World Organisation for Animal Health
Founded as OIE

WORLD ORGANISATION FOR ANIMAL HEALTH

MANUAL OF DIAGNOSTIC TESTS AND VACCINES FOR TERRESTRIAL ANIMALS

(mammals, birds and bees)

Volume 1, 2, 3,

2023

FOREWORD

The *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*) aims to prevent and control animal diseases, including zoonoses, to contribute to the improvement of animal health services world-wide and to allow safe international trade in animals and animal products. The principal target readership is laboratories carrying out veterinary diagnostic tests and surveillance, along with vaccine manufacturers and users, and regulatory authorities in Members. The main objective is to provide internationally agreed diagnostic laboratory methods and requirements for the production and control of relevant vaccines and other biological products.

This ambitious task has required the cooperation of highly renowned animal health specialists from many WOAH Members. The World Organisation for Animal Health (WOAH, founded as OIE), received the mandate from its Members to undertake this task on a global level. The main activities of the organisation, which was established in 1924, and in 2023 comprised 183 Members, are as follows:

1. To ensure transparency in the global animal disease and zoonosis situation.
2. To collect, analyse and disseminate scientific veterinary information on animal disease control methods.
3. To provide expertise and encourage international solidarity in the control of animal diseases.
4. Within its mandate under the WTO (World Trade Organization) Agreement on Sanitary and Phytosanitary Measures (SPS Agreement), to safeguard world trade by publishing health standards for international trade in animals and animal products.
5. To improve the legal framework and resources of national Veterinary Services.
6. To provide a better guarantee of the safety of food of animal origin and to promote animal welfare through a science-based approach.

The *Terrestrial Manual*, covering infectious and parasitic diseases of mammals, birds and bees, was first published in 1989. Each successive edition has extended and updated the information provided. This twelfth edition includes over 15 updated chapters. The structure of the edition remains unchanged: Part 1 contains ten introductory chapters that set general standards for the management of veterinary diagnostic laboratories and vaccine production facilities; Part 2 comprises specific recommendations and includes eight chapters of recommendations for validation of diagnostic tests and three chapters of recommendations for the manufacture of vaccines; Part 3 comprises chapters on WOAH listed diseases and other diseases of importance.

As a companion volume to the *Terrestrial Animal Health Code*, the *Terrestrial Manual* sets laboratory standards for all WOAH listed diseases as well as several other diseases of global importance. It describes the diagnostic tests available and fit for purpose, including those that are suitable for certifying individual animals prior to movement. The *Terrestrial Manual* has become widely adopted as a key reference book for veterinary laboratories around the world. Aquatic animal diseases are included in a separate *Aquatic Manual*.

The task of commissioning chapters and compiling the *Terrestrial Manual* was assigned to the WOAH Biological Standards Commission by the World Assembly of national Delegates. Manuscripts were requested from specialists (the WOAH designated experts at WOAH Reference Laboratories when relevant) in each of the diseases or the other topics covered. When needed, an *ad hoc* Group of experts was convened tasked with updating or developing a chapter. The chapters were reviewed and approved by the Biological Standards Commission for circulation to all WOAH Member Countries for review and comment. The Commission, elected every 3 years by the Assembly, took the resulting comments into consideration, often referring back to the contributors for further clarification, before

finalising the chapters and approving them for circulation a second time to all WOAH Members. The final texts were then presented for adoption by the Assembly at the General Session held in May each year.

The Standards presented in each chapter of the *Terrestrial Manual* are International Standards in accordance with the mandate of the WOAH as recognised by the WTO under the SPS Agreement, and considering their approval by the WOAH World Assembly.

A procedure for the official recognition of commercialised diagnostic tests, under the authority of the Assembly, was finalised in September 2004. Data are submitted using a validation template that was developed by the Biological Standards Commission. Submissions regarding terrestrial animal diseases are evaluated by appointed experts, who advise the Biological Standards Commission before the final opinion of the WOAH World Assembly is sought. All information on the submission of applications can be found on the WOAH website.

The *Terrestrial Manual* continues to expand and to extend its range of topics covered. In 2022, it was decided to no longer publish a paper edition of the *Terrestrial Manual*. The online version of the *Terrestrial Manual* is therefore the only valid version of the *Terrestrial Manual*. The *Terrestrial Manual* is available in English and Spanish on the WOAH website.

At the 89th General Session in May 2022, the World Assembly of Delegates adopted Resolution No. 10, recognising that "OIE" will be replaced by "WOAH" as part of a rebranding of the Organisation, which will be applied in future editions of the *Terrestrial Manual*.

Dr Monique Eloit
Director General
World Organisation for Animal Health

June 2023

Professor Emmanuel Couacy-Hymann
President
WOAH Biological Standards Commission

June 2023

INTRODUCTION
(How to use this *Terrestrial Manual*)

- **Arrangement of the *Terrestrial Manual***

Part 1, the beginning of this *Terrestrial Manual,* contains ten introductory chapters that set general standards for the management of veterinary diagnostic laboratories and vaccine facilities.

Part 2 elaborates specific recommendations on topics, many of which are introduced in Part 1 such as validation of diagnostic tests and for the manufacture of vaccines. The Section also includes chapters that have been developed on topics such as biotechnology and antimicrobial susceptibility testing.

The main part of the *Terrestrial Manual* (Part 3) covers standards for diagnostic tests and vaccines for specific diseases listed in the WOAH *Terrestrial Animal Health Code* and other important diseases. The diseases are in alphabetical order, subdivided by animal host group. WOAH listed diseases are diseases that have the potential for very serious and rapid spread, irrespective of national borders. They have particularly serious socio-economic or public health consequences and are of major importance in the international trade of animals and animal products.

Three of the diseases in Section 3.10 are included in some individual species sections, but these chapters cover several host species and thus give a broader description. Some additional diseases that may also be of importance but that do not have a chapter in the *Terrestrial Code* are also included in Section 3.10. This section also includes some important zoonotic infections.

Although there is a list of contributors at the beginning of the *Terrestrial Manual*, the final responsibility for the content of the *Terrestrial Manual* lies with the World Assembly of WOAH.

- **Part 3: Format of the disease-specific chapters**

Each disease chapter includes a summary intended to provide information for veterinary officials and other readers who need a general overview of the tests and vaccines available for the disease. This is followed by a text giving greater detail for laboratory workers. In each disease chapter, Part A gives a general introduction to the disease, Part B deals with laboratory diagnosis of the disease, and Part C (where appropriate) with the requirements for vaccines or *in vivo* diagnostic biologicals. The information concerning production and control of vaccines or diagnostics is given as an example; it is not always necessary to follow these when there are scientifically justifiable reasons for using alternative approaches. Bibliographic references that provide further information are listed at the end of each chapter.

- **Table of tests available for each disease and their purpose**

It has been agreed to include in each disease-specific chapter a table of the diagnostic tests available and in use for the disease graded against six purposes. The purposes relate to Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*, which identifies six main purposes for which diagnostic tests may be carried out. The aim of the table is to give a concise guide (elaborated in the text of the chapter) as to which tests are appropriate for which purpose.

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COMMON ABBREVIATIONS USED IN THIS *TERRESTRIAL MANUAL*

ABTS	2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulphonic acid	EMTM	Evans' modified Tobie's medium
AGID	Agar gel immunodiffusion	EYL	Earle's yeast lactalbumin (balanced salt solution)
ATCC[1]	American type culture collection	FAT	Fluorescent antibody test
BBAT	Buffered *Brucella* antigen test	FAVN	Fluorescent antibody virus neutralisation
BCIP	5-bromo-4-chloro-3-indolyl-phosphate	FBS	Fetal bovine serum
BFK	Bovine fetal kidney (cells)	FITC	Fluorescein isothiocyanate
BGPS	Beef extract-glucose-peptone-serum (medium)	FLK	Fetal lamb kidney (cells)
BHK	Baby hamster kidney (cell line)	FPA	Fluorescence polarisation assay
BLP	Buffered lactose peptone	***g***	Relative centrifugal force
BPAT	Buffered plate antigen test	GIT	Growth inhibition test
BSA	Bovine serum albumin	HA	Haemagglutination
BSF	Bovine serum factors	HAD	Haemadsorption
CAM	Chorioallantoic membrane	HBSS	Hanks' balanced salt solution
CAT	Card agglutination test	H&E	Haematoxylin & eosin (stain)
CEF	Chicken embryo fibroblast	HEP	High-egg-passage (virus)
CF	Complement fixation (test)	HEPA	High-efficiency particulate air (filter)
CFU	Colony-forming unit	HEPES	N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid (buffer)
CIEP	Counter immunoelectrophoresis	HI	Haemagglutination inhibition
CK	Calf kidney (cells)	HRPO	Horseradish peroxidase
CNS	Central nervous system	IB	Immunoblot test
CPE	Cytopathic effect	ICFTU	International complement fixation test unit
CPLM	Cysteine-peptone-liver infusion maltose (medium)	ICPI	Intracerebral pathogenicity index
CSY	Casein-sucrose-yeast (agar)	ID_{50}	Median infectious dose
Ct	cycle threshold (PCR tests)	IFA	Indirect fluorescent antibody (test)
DEAE	Diethylaminoethyl	IGRA	Interferon gamma release assay
DEPC	Diethylpyrocarbonate	IHA	Indirect haemagglutination
DIVA	Detection of infection in vaccinated animals	IPMA	Immunoperoxidase monolayer assay
DMEM	Dulbecco's modified Eagle's medium	IU	International units
DMSO	Dimethyl sulfoxide	IVPI	Intravenous pathogenicity index
DTH	Delayed-type hypersensitivity	LA	Latex agglutination
EDTA	Ethylene diamine tetra-acetic acid	LD	Lethal dose
EGTA	Ethylene glycol tetra-acetic acid	LEP	Low egg passage (virus)
EID	Egg-infective dose	LPS	Lipopolysaccharide
ELISA	Enzyme-linked immunosorbent assay	MAb	Monoclonal antibody

1 American Type Culture Collection, P.O. Box 1549, Manassas, Virginia 20108, United States of America.

MALDI TOF MS	Matrix assisted laser desorption ionisation time of flight mass spectrometry	PPD	Purified protein derivative
MCS	Master cell stock	MAT	Microscopic agglutination test
MDBK	Madin–Darby bovine kidney (cell line)	PHA	Passive haemagglutination (test)
MDT	Mean death time	PPLO	Pleuropneumonia-like organisms
MEM	Minimal essential medium	PRN	Plaque reduction neutralisation
MHC	Major histocompatibility complex	PSG	Phosphate-buffered saline glucose
MLST	Multi-locus sequence typing	RBC	Red blood cell
MLV	Modified live virus (vaccine)	RFLP	Restriction fragment length polymorphism
m.o.i.	multiplicity of infection	RK	Rabbit kidney
MSV	Master seed virus	RPM	Revolutions per minute
NI	Neutralisation index	RSA	Rapid serum agglutination
NBT	Nitro blue tetrazolium	RT-PCR	Reverse-transcriptase polymerase chain reaction
NPLA	Neutralising peroxidase-linked assay	SAN	Specific antibody negative
OD	Optical density	SAT	Serum agglutination test
OGP	1-octyl-beta-D-glucopyranoside (buffer)	SDS	Sodium dodecyl sulphate
OPD	Orthophenyldiamine (chromogen)	SOP	Standard operating procedure
OPG	Oxalase-phenol-glycerin (preservative solution)	SPF	Specific pathogen free
ORF	Open reading frame	SPG	Sucrose phosphate glutamic acid
PAGE	Polyacrylamide gel electrophoresis	SRBC	Sheep red blood cells
PAP	Peroxidase–antiperoxidase (staining procedure)	$TCID_{50}$	Median tissue culture infective dose
PAS	Periodic acid-Schiff (reaction)	TMB	Tetramethyl benzidine
PBS	Phosphate-buffered saline	TSI	Triple sugar iron (medium)
PCR	Polymerase chain reaction	VB	Veronal buffer
PD	Protective dose	VBS	Veronal buffered saline
PFGE	Pulsed field gel electrophoresis	Vero	African green monkey kidney (cells)
PFU	Plaque-forming unit	VN	Virus neutralisation

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GLOSSARY OF TERMS

The definitions given below have been selected and restricted to those that are likely to be useful to users of this WOAH Terrestrial Manual*.*

- **Absorbance or optical density**

Absorbance, also termed optical density (OD), describes the amount of light transmitted through a medium. Many assays are designed so that the absorbance is proportional to the amount of analyte. OD at a specific wavelength is determined with a spectrophotometer. It is calculated as OD = $\log_{10}$(incident light/transmitted light)

- **Accuracy**

Nearness of a test value to the expected value for a reference standard reagent of known activity or titre.

- **Anthroponosis**

Anthroponosis is an infection or disease that is transmissible from humans to animals under natural conditions.

- **Assay**

Synonymous with test or test method, e.g. enzyme immunoassay, complement fixation test or polymerase chain reaction tests.

- **Batch**

All vaccine or other reagent, such as antigen or antisera, derived from the same homogeneous bulk and identified by a unique code number.

- **Biohazard (CWA[1] 15793:2011)**

Potential source of harm caused by biological agents or toxins.

- **Biological agent (adapted from CWA 15793:2011)**

Any microorganism including those which have been genetically modified, cell cultures, and parasites, which may be able to provoke any infection, allergy, or toxicity in humans, animals or plants. *Note:* for the purpose of Biorisk Analysis, prions are regarded as biological agents.

- **Biosafety**

Laboratory biosafety describes the principles and practices for the prevention of unintentional exposure to biological materials, or their accidental release.

1 CWA: CEN Workshop Agreement (2011). CEN: European Committee for Standardization

- **Biosecurity**

Laboratory biosecurity describes the controls on biological materials within laboratories, in order to prevent their loss, theft, misuse, unauthorised access, or intentional unauthorised release.

- **Biorisk (CWA 15793:2011)**

Combination of the probability of occurrence of harm and the severity of harm where the source of harm is a biological agent or toxin. Note: the source of harm may be an unintentional exposure, accidental release or loss, theft, misuse, diversion, unauthorised access or intentional unauthorised release.

- **Biorisk analysis (adapted from WOAH *Terrestrial Animal Health Code*)**

The process composed of biohazard identification, biorisk assessment, biorisk management and biorisk communication.

- **Biorisk assessment (CWA 15793:2011)**

Process of evaluating the biorisks arising from biohazards, taking into account the adequacy of any existing controls, and deciding whether or not the biorisk(s) is acceptable.

- **Biorisk Managment Advisor (CWA 15793:2011)**

Individual who has expertise in the biohazards encountered in the organisation and is competent to advise top management and staff on biorisk management issues.

- **Biorisk Management (adapted from WOAH *Terrestrial Animal Health Code*)**

Process of identifying, selecting and implementing measures that can be applied to reduce the level of biorisk.

- **Biorisk Management System (CWA 15793:2011)**

Part of an organisation's management system used to develop and implement its biorisk policy and manage its biorisks.

- **Cell line**

A stably transformed line of cells that has a high capacity for multiplication *in vitro*.

- **Centrifugation**

Throughout the text, the rate of centrifugation has been expressed as the Relative Centrifugal Force, denoted by '***g***'. The formula is:

$$\frac{(\text{RPM} \times 0.10472)^2 \times \text{Radius (cm)}}{980} = g$$

where RPM is the rotor speed in revolutions per minute, and where Radius (cm) is the radius of the rotor arm, to the bottom of the tube, in centimetres.

It may be necessary to calculate the RPM required to achieve a given value of ***g***, with a particular rotor. The formula is:

$$\frac{\text{RPM} = \sqrt{g \times 980}\,/\text{Radius (cm)}}{0.10472}$$

- **Comparability**

The preferred term when performance characteristics of a new test, which has undergone a minor change, are as good as those of a validated test within statistically defined limits.

- **Cross-reaction**

See False-positive reaction.

- **Ct value**

The number of amplification cycles in a real-time polymerase chain reaction (PCR) required for fluorescent signal to exceed the background.

- **Cut-off/threshold**

In immunoassays, cut-off or threshold values are those selected for distinguishing between negative and positive test results, and may include an indeterminate or suspicious zone.

- **Dilutions**

Where dilutions are given for making up liquid reagents, they are expressed as, for example, 1 in 4 or 1/4, meaning one part added to three parts, i.e. a 25% solution of A in B.

a) v/v – This is volume to volume (two liquids).

b) w/v – This is weight to volume (solid added to a liquid).

- **Dilutions used in virus neutralisation tests**

There are two different conventions used in expressing the dilution used in virus neutralisation (VN) tests. In Europe, it is customary to express the dilution before the addition of the antigen, but in the United States of America and elsewhere, it is usual to express dilutions after the addition of antigen.

These alternative conventions are expressed in the *Terrestrial Manual* as 'initial dilution' or 'final dilution', respectively.

- **Efficacy**

Specific ability of the biological product to produce the result for which it is offered when used under the conditions recommended by the manufacturer.

- **False-negative reaction**

Negative reactivity in an assay of a test sample obtained from an animal exposed to or infected with the organism in question, may be due to lack of analytical sensitivity, restricted analytical specificity or analyte degradation, decreases diagnostic sensitivity.

- **False-positive reaction**

Positive reactivity in an assay that is not attributable to exposure to or infection with the organism in question, maybe due to immunological cross-reactivity, cross-contamination of the test sample or non-specific reactions, decreases diagnostic specificity.

- **Final product (lot)**

All sealed final containers that have been filled from the same homogenous batch of vaccine in one working session, freeze-dried together in one continuous operation (if applicable), sealed in one working session, and identified by a unique code number.

- **Harmonisation**

The result of an agreement between laboratories to calibrate similar test methods, adjust diagnostic thresholds and express test data in such a manner as to allow uniform interpretation of results between laboratories.

- **Incidence**

Estimate of the rate of new infections in a susceptible population over a defined period of time; not to be confused with prevalence.

- **In-house checks**

All quality assurance activities within a laboratory directly related to the monitoring, validation, and maintenance of assay performance and technical proficiency.

- **In-process control**

Test procedures carried out during manufacture of a biological product to ensure that the product will comply with the agreed quality standards.

- **Inter-laboratory comparison (ring test)**

Any evaluation of assay performance and/or laboratory competence in the testing of defined samples by two or more laboratories; one laboratory may act as the reference in defining test sample attributes.

- **Laboratory biosafety**

See Biosafety.

- **Laboratory biosecurity**

See Biosecurity.

- **Limit of detection (LOD)**

The LOD is the estimated amount of analyte in a specified matrix that would produce a positive result at least a specified per cent of the time and is a measure of the analytical sensitivity.

- **Master cell (line, seed, stock)**

Collection of aliquots of cells of defined passage level, for use in the preparation or testing of a biological product, distributed into containers in a single operation, processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination.

- **Master seed (agent, strain)**

Collection of aliquots of an organism at a specific passage level, from which all other seed passages are derived, which are obtained from a single bulk, distributed into containers in a single operation and processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination.

- **Methods comparison (equivalency testing)**

Determination of certain assay performance characteristics of new or different test methods by means of an inter-laboratory comparison to a standard test method; implied in this definition is that participating laboratories are using their own test methods, reagents and controls and that results are expressed qualitatively.

- **Optical density**

See absorbance.

- **Performance characteristic**

An attribute of a test method that may include analytical sensitivity and specificity, accuracy and precision, diagnostic sensitivity and specificity and/or repeatability and reproducibility.

- **Phylogeography**

Phylogeography is the study of the genetic and geographic structure of populations and species.

- **Potency**

The potency of a biological product is the concentration of the immunologically active component. For a vaccine it is the concentration of the specific immunogen, and for an antiserum it is the concentration of the specific antibody.

- **Precision**

The degree of dispersion (variance, standard deviation or coefficient of variation) within a series of measurements of the same sample tested under specified conditions.

- **Predictive value (negative)**

The probability that an animal is free from infection given that it tests negative; predictive values are a function of the DSe (diagnostic sensitivity) and DSp (diagnostic specificity) of the diagnostic assay and the prevalence of infection.

- **Predictive value (positive)**

The probability that an animal has been infected given that it tests positive; predictive values are a function of the DSe and DSp of the diagnostic assay and the prevalence of infection.

- **Prevalence**

Estimate of the proportion of infected animals in a population at one given point in time; not to be confused with incidence.

- **Primary cells**

A pool of original cells derived from normal tissue up to and including the tenth subculture.

- **Production seed**

An organism at a specified passage level that is used without further propagation for initiating preparation of a production bulk.

- **Proficiency testing**

One measure of laboratory competence derived by means of an inter-laboratory comparison; implied in this definition is that participating laboratories are using the same test methods, reagents and controls and that results are expressed qualitatively.

- **Purity**

Quality of a biological product prepared to a final form and:

a) Relatively free from any extraneous microorganisms and extraneous material (organic or inorganic) as determined by test methods appropriate to the product; and

b) Free from extraneous microorganisms or material which could adversely affect the safety, potency or efficacy of the product.

- **Qualitative Risk Assessment (*Terrestrial Animal Health Code*)**

An assessment where the outputs of the likelihood of the outcome or the magnitude of the consequences are expressed in qualitative terms such as high, medium, low or negligible.

- **Quantitative Risk Assessment (*Terrestrial Animal Health Code*)**

An assessment where the outputs of the of the risk assessment are expressed numerically.

- **Receiver operator characteristic (ROC)**

ROC analysis provides a cut-off-independent approach for evaluation of the global accuracy of a test where results are measured as ordinal or continuous values. The area under the ROC curve provides a single numerical estimate of overall accuracy ranging from 0.5 (useless test) to 1 (perfect test).

- **Reference Laboratory**

Laboratory of recognised scientific and diagnostic expertise for a particular animal disease or testing methodology; includes capability for characterising and assigning values to reference reagents and samples.

- **Repeatability**

Level of agreement between replicates of a sample both within and between runs of the same test method in a given laboratory.

- **Reproducibility**

Ability of a test method to provide consistent results when applied to aliquots of the same sample tested by the same method in different laboratories.

- **Risk (*Terrestrial Animal Health Code*)**

The likelihood of the occurrence and the likely magnitude of the biological and economic consequences of an adverse event or effect to animal or human health.

- **Risk communication (*Terrestrial Animal Health Code*)**

The interactive transmission and exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors and risk perceptions among risk assessors, risk managers, risk communicators, the general public, and other interested parties.

- **Robustness**

Robustness refers to an assay's capacity to remain unaffected by minor variations in test situations that may occur over the course of testing.

- **Room temperature**

The term 'room temperature' is intended to imply the temperature of a comfortable working environment. Precise limits for this cannot be set, but guiding figures are 18–25°C. Where a test specifies room temperature, this should be achieved, with air conditioning if necessary; otherwise the test parameters may be affected.

- **Safety**

Freedom from properties causing undue local or systemic reactions when used as recommended or suggested by the manufacturer and without known hazard to in-contact animals, humans and the environment.

- **Sample**

Material that is derived from a specimen and used for testing purposes.

- **Sensitivity (analytical)**

Synonymous with 'Limit of Detection', smallest detectable amount of analyte that can be measured with a defined certainty; analyte may include antibodies, antigens, nucleic acids or live organisms.

- **Sensitivity (diagnostic)**

Proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false-negative results.

- **Sensitivity (relative)**

Proportion of reference animals defined as positive by one or a combination of test methods that also test positive in the assay being compared.

- **Seroconversion**

i) Demonstration of a change from a seronegative to a seropositive condition using a serological assay specific for the antigen

OR

ii) Demonstration of a four-fold or more rise in antibody titre between an acute and convalescent serum using an ordinal test specific for the antigen.

- **Specific antibody negative (SAN)**

Used in relation to animals that have been shown by the use of appropriate tests to be free from antibodies to specific pathogenic microorganisms, and also eggs derived from SAN birds.

- **Specific pathogen free (SPF)**

Animals that have been shown by the use of appropriate tests to be free from specified pathogenic microorganisms, and also refers to eggs derived from SPF birds.

- **Specificity (analytical)**

Degree to which the assay distinguishes between the target analyte and other components in the sample matrix; the higher the analytical specificity, the lower the level of false-positives.

- **Specificity (diagnostic)**

Proportion of known uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results.

- **Specificity (relative)**

Proportion of reference animals defined as negative by one or a combination of test methods that also test negative in the assay being compared.

- **Specimen**

Material submitted for testing.

- **Standard Reagents**

  - **International Standard Reagents**

    Standard reagents by which all other reagents and assays are calibrated; prepared and distributed by an International Reference Laboratory.

  - **National Standard Reagents**

    Standard reagents calibrated by comparison with International Standard Reagents; prepared and distributed by a National Reference Laboratory.

- **Working Standards (reagents)**

  Standard reagents calibrated by comparison with the National Standard Reagent, or, in the absence of a National Standard Reagent, calibrated against a well-characterised in-house standard reagent; included in routine diagnostic tests as a control and/or for normalisation of test results.

- **Sterility**

Freedom from viable contaminating microorganisms, as demonstrated by approved and appropriate tests.

- **Test method**

Specified technical procedure for detection of an analyte (synonymous with assay).

- **Tests**

  - **Screening**

    Tests of high diagnostic sensitivity suitable for large-scale application.

  - **Confirmatory**

    Test methods of high diagnostic specificity that are used to confirm results, usually positive results, derived from other test methods

- **Thermotolerant**

For vaccines, the term is used to describe the ability to retain protective immunogenicity after exposure to temperatures above the storage temperature required according to the manufacturer's recommendations. Claims of thermotolerance must be supported by data.

- **Vaccine**

Includes all products designed to stimulate active immunisation of animals against disease, without regard to the type of microorganism or microbial component or toxin from which they may be derived or that they contain.

- **Validation**

Is a process that determines the fitness for purpose of an assay, which has been properly developed, optimised and standardised, for an intended use.

- **Working seed**

Organism at a passage level between master seed and production seed.

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NB: FIRST ADOPTED IN 1996. MOST RECENT UPDATES ADOPTED IN 2023.

CONTRIBUTORS

CONTRIBUTORS AND PROFESSIONAL ADDRESS AT TIME OF WRITING

The chapters in the Terrestrial Manual *are prepared by invited contributors* (*WOAH Reference Experts, where possible*). *In accordance with WOAH standard procedure, all chapters are circulated to WOAH Members for comment. The WOAH Biological Standards Commission and the Consultant Editor then modify the text to take account of comments received, and the text is circulated a second time as the final version that will be presented for adoption by the World Assembly of Delegates to WOAH at the General Session in May of each year. The* Terrestrial Manual *is thus deemed to be a WOAH Standard that has come into being by international agreement. For this reason, the names of the contributors are not shown on individual chapters but are listed below. The Biological Standards Commission greatly appreciates the work of the following contributors* (*address at the time of writing*):

1.1.1. Management of veterinary diagnostic laboratories	**Dr T. Drew (retired)** Australia.
1.1.2. Collection, submission and storage of diagnostic specimens	**WOAH *ad hoc* Group on Biosafety and Biosecurity in Veterinary Laboratories**
1.1.3. Transport of biological materials	**WOAH *ad hoc* Group on Transport of Biological Materials**
1.1.4. Biosafety and biosecurity: standard for managing biological risk in the veterinary laboratory and animal facilities	**WOAH *ad hoc* Group on Biosafety and Biosecurity in Veterinary Laboratories**
1.1.5. Quality management in veterinary testing laboratories	**Dr A. Colling** Australian Centre for Disease Preparedness, CSIRO, Geelong, Victoria, Australia.
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1 This chapter was updated by consensus of the WOAH *ad hoc* Group on High Throughput Sequencing, Bioinformatics and Computational Genomics.

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2.1.2. Biotechnology advances in the diagnosis of infectious diseases	**Dr S. Belak (retired)**[2] Sweden.
2.1.3. Managing biorisk: examples of aligning risk management strategies with assessed biorisks	**WOAH *ad hoc* Group on Biosafety and Biosecurity in Veterinary Laboratories**
2.2.1. Development and optimisation of antibody detection assays *2.2.2. Development and optimisation of antigen detection assays* *2.2.3.Development and optimisation of nucleic acid detection assays* *2.2.4. Measurement uncertainty* *2.2.5. Statistical approaches to validation* *2.2.6. Selection and use of reference samples and panels* *2.2.7*. Validation of diagnostic tests for infectious diseases applicable to wildlife* *2.2.8. Comparability of assays after changes in a validated test method*	**WOAH *ad hoc* Group on Validation of Diagnostic Assays** ***WOAH *ad hoc* Group on Validation of Diagnostic Tests for Wildlife**
2.3.1. The application of biotechnology to the development of veterinary vaccines	**Dr A.A. Potter, Dr V. Gerdts, Dr G. Mutwiri, Dr S. Tikoo & De S. van Drunen Littel-van den Hurk** Vaccine and Infectious Disease Organization, Saskatoon, Canada.
2.3.2. The role of official bodies in the international regulation of veterinary biologicals	**Dr J.-P. Orand (retired) and Dr C. Lambert** Agence Nationale du Médicament Vétérinaire, Anses Fougères, France. **Dr B. Rippke (retired)** USA. **Dr T. Tsutsui** National Institute of Animal Health, Division of Viral Disease and Epidemiology, National Institute of Animal Health, Ibaraki, Japan.

2 This chapter was updated by consensus of an Expert Consultation

2.3.3. Minimum requirements for the organisation and management of a vaccine manufacturing facility	
2.3.4. Minimum requirements for the production and quality control of vaccines	**WOAH Collaborating Centre for Veterinary Medicinal Products, Anses Fougères, France**
2.3.5. Minimum requirements for aseptic production in vaccine manufacture	
3.1.1. Anthrax	**Dr K. Amoako** Canadian Food Inspection Agency, National Centre for Animal Disease (NCAD), Lethbridge Laboratory, Alberta, Canada. **Dr G. Harvey** USDA, APHIS, National Veterinary Services Laboratories, Ames, Iowa, USA.
3.1.2. Aujeszky's disease (*infection with Aujeszky's disease virus*)	**Dr A. Jestin & Dr M.F. Le Potier** Anses-Ploufragan, Laboratoire d'études et de recherches avicoles et porcines, Ploufragan, France. **Dr W. Loeffen** Wageningen Bioveterinary Research, Lelystad, The Netherlands. **Dr S.L. Swenson (formerly)** USDA, APHIS, National Veterinary Services Laboratories, Ames, Iowa, USA.
3.1.3. Bluetongue (*infection with bluetongue virus*)[3]	**Dr Debbie Eagles** Australian Centre for Disease Preparedness, CSIRO, Geelong, Victoria, Australia.
3.1.4. Brucellosis (*infection with* Brucella abortus, B. melitensis *and* B. suis)[4]	**Dr A. Whatmore** APHA Weybridge, New Haw, Addlestone, Surrey, Weybridge, UK.
3.1.5. Crimean–Congo haemorrhagic fever	**Dr J.C. Manuguerra** Institut Pasteur, Paris, France.
3.1.6. Echinococcosis (*infection with* Echinococcus granulosus and *with* E. multilocularis *virus*)	**Dr G. Masala** Istituto Zooprofilattico Sperimentale (IZS) of Sardinia, Sassari, Italy. **Dr M. Donadeu & Dr M. Lightowlers** Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Werribee, Australia.
3.1.7. Epizootic haemorrhagic disease (*infection with epizootic hemorrhagic disease virus*)	**Dr S. Zientara & Dr C. Sailleau** Laboratoire de santé animale de Maisons-Alfort, Maisons-Alfort, France.
3.1.8. Foot and mouth disease (*infection with foot and mouth disease virus*)[5]	**Dr D.J. King** The Pirbright Institute, Ash Road, Woking, Surrey, UK.

3 This chapter was updated by consensus of all WOAH Reference Laboratories for bluetongue.
4 This chapter was updated by consensus of all WOAH Reference Laboratories for brucellosis.
5 This chapter was updated by consensus of all WOAH Reference Laboratories for foot and mouth disease.

3.1.9. Heartwater	**Dr N. Vachiéry & Dr I. Marcelino** UMR CIRAD-INRA 117 ASTRE, Campus International de Baillarguet, Montpellier, France.
3.1.10. Japanese encephalitis	**Dr D.-K. Yang** Animal and Plant Quarantine Agency, Gyeongsangbuk-do, Korea (Rep. of).
3.1.11. Leishmaniosis	**Dr F. Vitale** Istituto Zooprofilattico Sperimentale della Sicilia, National Reference Centre for Leishmaniasis, Palermo, Italy.
3.1.12. Leptospirosis[6]	**Dr J. Petrakovsky** Laboratorio de Leptospirosis, Dirección General de Laboratorios y Control Técnico, Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA), Martínez, Pcia de Buenos Aires, Argentina.
3.1.13. Mammalian tuberculosis (*infection with* Mycobacterium tuberculosis *complex*)	**WOAH *Ad hoc* Group on Replacement of the International Standard Bovine Tuberculin**
3.1.14. New World screwworm (Cochliomyia hominivorax) *and* Old World *screwworm* (Chrysomya bezziana)	**Dr J. Welch** COPEG (Panama–US Commission for the Eradication and Prevention of NWS), Panama, Panama. **Dr M.J.R. Hall** Department of Entomology, The Natural History Museum, Cromwell Road, London, UK.
3.1.15. Nipah and Hendra virus diseases	**Dr K. Halpin** Australian Centre for Disease Preparedness, CSIRO, Geelong, Victoria, Australia.
3.1.16. Paratuberculosis (*Johne's disease*)[7]	**Dr Bernardo Alonso** DILAB (Dirección de Laboratorios y Control Técnico), Servicio Nacional de Sanidad y Calidad, Agroalimentaria (SENASA), Martínez, Prov. de Buenos Aires, Argentina.
3.1.17. Q fever	**Dr E. Rousset & Dr K. Sidi-Boumedine** Anses Sophia Antipolis, Laboratoire d'Études et de Recherches sur les Petits Ruminants et les Abeilles, Sophia Antipolis Cedex, France. **Dr B. Kadra & Dr B. Kupcsulik** Ceva-Phylaxia Co. Ltd, Budapest, Hungary.
3.1.18. Rabies (*infection with rabies virus and other lyssaviruses*)[8]	**Dr T. Müller** Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler Institut, Federal Research Institute for Animal Health, Insel Riems, Germany.

6 This chapter was updated by consensus of all WOAH Reference Laboratories for leptospirosis.
7 This chapter was updated by consensus of all WOAH Reference Laboratories for paratuberculosis.
8 This chapter was updated by consensus of all WOAH Reference Laboratories for rabies.

3.1.19. Rift Valley fever (*infection with Rift Valley fever virus*)[9]	**Dr C. Cetre-Sossah** Campus international de Baillarguet, Montpellier, France. **Dr B.A. Lubisi** Onderstepoort Veterinary Institute, Agricultural Research Council, Onderstepoort, South Africa.
3.1.20. Rinderpest (*infection with rinderpest virus*)	**Dr G. Libeau (retired)** France. **Dr M. Baron (retired)** UK. **Dr K. Yoshida** National Institute of Animal Health (NIAH), National Agriculture and Food Research Organization, Tokyo, Japan.
3.1.21. Surra in all species (Trypanosoma evansi *infection*)[10]	**Dr M. Desquesnes** UMR177-Intertryp (CIRAD-IRD), CIRAD-bios, Campus international de Baillarguet, Montpellier, France.
3.1.22. Trichinellosis (*infection with* Trichinella *spp.*)	**Dr B. Scandrett** Canadian Food Inspection Agency, Centre for Foodborne & Animal Parasitology, Saskatoon, Saskatchewan, Canada. **Dr M.A. Gomez Morales** Istituto Superiore di Sanita, Laboratorio di Parasitoligia, Roma, Italy
3.1.23. Tularemia	**Dr T.E. Rocke** USGS National Wildlife Health Center, Wisconsin, United States of America. **Dr M. Gyuranecz** Laboratory of Zoonotic Bacteriology and Mycoplasmology, Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest, Hungary.
3.1.24. Vesicular stomatitis	**Dr E.M. Pituco** PANAFTOSA, Rio de Janeiro, Brazil. **Dr M.K. Torchetti** USDA, APHIS, National Veterinary Services Laboratories, Ames, Iowa, USA.
3.1.25. West Nile fever	**Dr F. Monaco** Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy. **Dr T. Sturgill** USDA, APHIS, National Veterinary Services Laboratories, Ames, Iowa, USA.

9 This chapter was updated by consensus of the WOAH *ad hoc* Group on Rift Valley fever.
10 This chapter was updated by consensus of the WOAH *ad hoc* Group on Diagnostic Tests for Trypanosomoses.

Introductory note on bee diseases	**Dr M.-P. Chauzat** Anses Sophia Antipolis, Bee Pathology Unit, Sophia Antipolis, France.
3.2.1. Acarapisosis of honey bees (*infestation of honey bees with* Acarapis woodi)	**Dr R. Hall** Diagnostic and Surveillance Services, Biosecurity New Zealand, Ministry for Primary Industries, Upper Hutt, New Zealand.
3.2.2. American foulbrood of honey bees (*infection of honey bees with* Paenibacillus larvae) *3.2.3. European foulbrood of honey bees* (*infection of honey bees with* Melissococcus plutonius)	**Dr K. Sidi-Boumedine** Anses Sophia Antipolis, Bee Pathology Unit, Sophia Antipolis, France.
3.2.4. Nosemosis of honey bees	**Dr I. Fries** Honey Bee Research Group, Department of Ecology, Swedish University of Agricultural Sciences, Uppsala, Sweden.
3.2.5. Infestation with Aethina tumida (*small hive beetle*) *3.2.6. Infestation with* Tropilaelaps *spp.*	**Dr M.-P. Chauzat, Dr S. Franco, Dr V. Duquesne & Dr M.-P. Rivière** Anses Sophia Antipolis, Bee Pathology Unit, Sophia Antipolis, France.
3.2.7. Varroosis of honey bees (*infestation of honey bees with* Varroa *spp.*)	**Dr M.O. Schäfer** National Reference Laboratory for Bee Diseases, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Insel Riems, Germany.
3.3.1. Avian chlamydiosis	**Dr C. Schnee** Institute of Molecular Pathogenesis, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Jena, Germany. **Prof. D. Vanrompay** Laboratory for Immunology and Animal Biotechnology, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University, Coupure Links, Ghent, Belgium. **Dr K. Laroucau** Anses Maisons-Alfort, Animal Health Laboratory Bacterial Zoonoses Unit, Maisons-Alfort, France.
3.3.2. Avian infectious bronchitis	**Dr J.J. (Sjaak) de Wit** Department R&D, GD Animal Health, Deventer, The Netherlands. **Dr P. Britton** The Pirbright Institute, Compton Laboratory, Newbury, Berkshire, UK.
3.3.3. Avian infectious laryngotracheitis	**Dr A.H. Noormohammadi & Dr J. Devlin** Faculty of Veterinary Science, The University of Melbourne, Werribee, Victoria, Australia.

3.3.4. Avian influenza (*including infection with high pathogenicity avian influenza viruses*)[11]

Dr D. Swayne(retired)
Southeast Poultry Research Laboratory, Athens, Georgia, USA.

Prof. I. Brown
APHA Weybridge, New Haw, Addlestone, Surrey, Weybridge, UK.

3.3.5. Avian mycoplasmosis (Mycoplasma gallisepticum, M. synoviae)

Dr S. Catania
Mycoplasma Unit, Istituto Zooprofilattico Sperimentale delle Venezie, Verona, Italy

Dr Evelin Lobo Riveroi (formerly)
MYCOLAB Laboratorio para diagnóstico de micoplasmas, Centro Nacional de Sanidad Agropecuaria, San José de las Lajas, Provincia Mayabeque, Cuba.

3.6. Avian tuberculosis

Dr I. Pavlik (formerly) & Dr I. Slaná (formerly)
Veterinary Research Institute, Brno, Czech Republic.

3.3.7. Duck virus enteritis

3.3.8. Duck virus hepatitis

Dr S. Stoute
California Animal Health and Food Safety Laboratory System, University of California, Davis, California, USA.

3.3.9. Fowl cholera

Dr P. Blackall
Poultry Hub Australia, Queensland Alliance for Agriculture and Food Innovation, University of Queensland, EcoSciences Precinct, Brisbane, Queensland, Australia.

3.3.10. Fowlpox

Dr H.S. Sellers
Poultry Diagnostic and Research Center, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA.

3.3.11. Fowl typhoid and Pullorum disease

Dr R. Davies
APHA Weybridge, New Haw, Addlestone, Surrey, UK.

3.3.12. Infectious bursal disease (*Gumboro disease*)

Dr N. Eterradossi
Anses, Laboratoire de Ploufragan-Plouzané, Laboratoire d'études et de recherches avicoles, porcines et piscicoles, Ploufragan-Plouzané, France.

Dr Y. Saif
Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, Ohio, USA.

11 This chapter was updated by consensus of all WOAH Reference Laboratories for avian influenza.

3.3.13. Marek's disease

Dr Y. Yao & Dr V. Nair (retired)
The Pirbright Institute, Ash Road, Woking, Surrey, UK.

Dr J.R. Dunn
US National Poultry Research Center, USDA-ARS Southeast Poultry Research Laboratory, Athens, Georgia, USA.

3.3.14. Newcastle disease (infection with Newcastle disease virus)[12]

Dr D. Swayne (retired)
USA.

Prof. I. Brown
APHA Weybridge, New Haw, Addlestone, Surrey, Weybridge, UK.

3.3.15. Turkey rhinotracheitis (avian metapneumovirus)

Dr N. Eterradossi & Dr P. Brown
Anses, Laboratoire de Ploufragan-Plouzané, Laboratoire d'études et de recherches avicoles, porcines et piscicoles, Ploufragan-Plouzané, France.

3.4.1. Bovine anaplasmosis

Dr F. Parrodi (formerly) & Dr J.J. Mosqueda Gualito
Centro Nacional de Servicios de Constatación en Salud Animal (CENAPA), Morelos, Mexico.

3.4.2. Bovine babesiosis

Prof. N. Yokoyama
National Research Center for Protozoan Disease Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

Dr J.J. Mosqueda Gualito
Centro Nacional de Servicios de Constatación en Salud Animal (CENAPA), Morelos, Mexico.

3.4.3. Bovine cysticercosis

See chapter 3.10.3.

3.4.4. Bovine genital campylobacteriosis

Prof. J.A. Wagenaar & Dr L. van der Graaf-van Bloois
Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

3.4.5. Bovine spongiform encephalopathy[13]

Prof. T. Seuberlich
NeuroCentre, Department of Clinical Research and Veterinary Public Health, Division of Experimental Clinical Research, University of Bern, Bern, Switzerland.

3.4.6. Bovine tuberculosis

Dr D.V. Cousins (retired)
Australia.

3.4.7. Bovine viral diarrhoea[14]

Dr P. Kirkland
Elizabeth Macarthur Agriculture Institute (EMAI), Virology Laboratory, Menangle, Camden, New South Wales, Australia.

12 This chapter was updated by consensus of all WOAH Reference Laboratories for Newcastle disease.
13 This chapter was updated by consensus of all WOAH Reference Laboratories for bovine spongiform encephalopathy.
14 This chapter was updated by consensus of all WOAH Reference Laboratories for bovine viral diarrhoea.

3.4.8. Contagious bovine pleuropneumonia (*infection with* Mycoplasma mycoides *subsp.* mycoides)[15]	**Dr F. Thiaucourt (retired)** France.
3.4.9. Enzootic bovine leukosis	**Prof. T.W. Vahlenkamp** Institute of Virology, Centre for Infectious Diseases, Faculty of Veterinary Medicine, Leipzig University, Leipzig, Germany. **Dr B. Choudhury** APHA Weybridge, New Haw, Addlestone, Surrey, UK. **Dr J. Kuzmak** National Veterinary Research Institute, Pulawy, Poland.
3.4.10. Haemorrhagic septicaemia (Pasteurella multocida *serotypes 6:b and 6:e*)	**Dr S.B. Shivachandra** ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Karnataka, India.
3.4.11. Infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis	**Dr M. Beer** Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Insel Riems, Germany. **Dr A. Dastjerdi** APHA Weybridge, New Haw, Addlestone, Surrey, UK.
3.4.12. Lumpy skin disease	**Dr P. Beard (formerly)** The Pirbright Institute, Ash Road, Woking, Surrey, UK. **Dr D. Wallace** Onderstepoort Veterinary Institute, Agricultural Research Council, Onderstepoort, South Africa.
3.4.13. Malignant catarrhal fever	**Dr G. Russell** Moredun Research Institute, International Research Centre, Pentlands Science Park, Penicuik, Scotland, UK.
3.4.14. Nagana: infections with salivarian trypanosomoses (*excluding* Trypanosoma evansi *and* T. equiperdum)[16]	**Dr M. Desquesnes** UMR177-Intertryp (CIRAD-IRD), CIRAD-bios, Campus international de Baillarguet, Montpellier, France.
3.4.15. Theileriosis in cattle (*infection with* Theileria annulata, T. orientalis *and* T. parva)	**Dr P. Toye** Animal Health and Genetics, International Livestock Research Institute, Nairobi, Kenya. **Dr D. Geysen** Institute of Tropical Medicine, Department of Animal Health, Antwerp, Belgium.

15 This chapter was updated by consensus of all WOAH Reference Laboratories for contagious bovine pleuropneumonia.

16 This chapter was updated by consensus of the following WOAH experts on trypanosomes: Dr L. Touratier (deceased), Prof. N. Inoue, Prof. Ph. Büscher, Dr K. Suganuma, Dr M. Gonzatti.

3.4.16. Trichomonosis	**Dr E. Authie (formerly)** Laboratoire National de Contrôle des Reproducteurs, Maisons-Alfort, France. **Prof. A. Lew-Tabor** The University of Queensland, St. Lucia, Brisbane Queensland, Australia. **Prof. I. Diallo** Biosecurity Sciences Laboratory, Health and Food Sciences Precinct, Brisbane, Queensland, Australia.
3.5.1. Camelpox	**Dr U. Wernery, Dr K. Kamal-Aldin, Mrs S. Joseph & Mrs A. Riya Thomas** Central Veterinary Research Laboratory, Dubai, United Arab Emirates.
2.5.2. Middle East respiratory syndrome (*infection of dromedary camels with MERS-CoV*)	**WOAH *ad hoc* Group on Middle East Respiratory Syndrome Coronavirus (MERS-Cov)**
3.6.1. African horse sickness (*infection with African horse sickness virus*)	**Prof. J.M. Sánchez-Vizcaíno** Centro de Vigilancia Sanitaria Veterinaria, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain. **Dr M. Agüero Garcia** Laboratorio Central de Veterinaria, Algete (Madrid), Spain. **Dr J. Baron Castillo-Olivares** The Pirbright Institute, Ash Road, Woking, Surrey, UK.
3.6.2. Contagious equine metritis	**Dr I. Mawhinney** APHA Bury St Edmunds, Suffolk, UK **Dr M.M. Erdman** USDA, APHIS, National Veterinary Services Laboratories, Ames, Iowa, USA.
3.6.3. Dourine in horses (Trypanosoma equiperdum *infection*)[17]	**Prof. Ph. Büscher (retired)** Belgium.
3.6.4. Epizootic lymphangitis	**Dr C. Scantlebury** Department of Functional and Comparative Genomics, Institute of Integrative Biology, Biosciences Building, University of Liverpool, UK.
3.6.5. Equine encephalomyelitis (*Eastern, Western and Venezuelan*)	**Dr T. Sturgill** USDA, APHIS, National Veterinary Services Laboratories, Ames, Iowa, USA.

17 This chapter was updated by consensus of the following WOAH experts on trypanosomes: Dr M.I. Gonzatti, Dr I. Pascucci, Dr L. Touratier (deceased), Dr M. Desquesnes, Dr A. Schnaufer, Dr K. Suganuma, Dr N. Inoue, Dr N. Van Reet, Dr N. Ledesma, Dr L. Hébert.

3.6.6. Equine infectious anaemia	**Dr E.N. Ostlund (retired)** USA. **Dr J. Zhou** Laboratory of Equine Infectious Anemia Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences Harbin, China (People's Rep. of). **Dr K. Murakami** National Institute of Animal Health, Viral Disease Section, Ibaraki, Japan.
3.6.7. Equine influenza (*infection with equine influenza virus*)[18]	**Prof. A. Cullinane** Irish Equine Centre, Johnstown, Naas, Co. Kildare, Ireland.
3.6.8. Equine piroplasmosis	**Prof. N. Yokoyama** National Research Center for Protozoan Disease Obihiro University of Agriculture and Veterinary Medicine, Hokkaido Japan.
3.6.9. Equine rhinopneumonitis (*equine herpesvirus-1 and -4*)[19]	**Dr D. Elton & Dr N. Bryant** Animal Health Trust, Centre for Preventive Medicine, Kentford, Suffolk, UK.
3.6.10. Equine viral arteritis (*infection with equine arteritis virus*)	**Dr P.J. Timoney (retired)** USA. **Dr T. Drew & Prof. F. Steinbach** APHA Weybridge, New Haw, Addlestone, Surrey, UK.
3.611. Glanders and melioidosis	**Dr H. Neubauer** Institute of Bacterial Infections and Zoonoses, Friedrich-Loeffler Institut, Federal Research Institute for Animal Health, Jena, Germany. **Prof. U. Wernery** Central Veterinary Research Laboratory, Dubai, United Arab Emirates.
3.7.1. Myxomatosis *3.7.2. Rabbit haemorrhagic disease*	**Dr A. Lavazza, Dr L. Capucci & Dr P. Cavadini** Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy.
3.8.1. Border disease	**Dr P. Kirkland** Elizabeth Macarthur Agriculture Institute (EMAI), Virology Laboratory, Camden, New South Wales, Australia.
3.8.2. Caprine arthritis/encephalitis & Maedi-visna	**Dr D. Knowles (retired) & Dr L.M. Herrmann** USDA-ARS, Animal Disease Research Unit, Washington State University, Pullman, Washington, USA.

18 This chapter was updated by consensus of all WOAH Reference Laboratories for equine influenza.
19 This chapter was updated by consensus of all WOAH Reference Laboratories for equine rhinopneumonitis.

3.8.3. Contagious agalactia	**Dr R. Ayling** APHA Weybridge, New Haw, Addlestone, Surrey, UK. **Dr G. Loria** Istituto Zooprofilattico Sperimentale della Sicilia (IZSSi), Palermo, Italy.
3.8.4. Contagious caprine pleuropneumonia	**Dr F. Thiaucourt (retired)** France.
3.8.5. Enzootic abortion of ewes (*ovine chlamydiosis*) (*infection with* Chlamydophila abortus)	**Dr C. Schnee** Institute of Molecular Pathogenesis, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Jena, Germany. **Dr N. Borel** Institute for Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland. **Dr K. Laroucau** Anses Maisons-Alfort, Animal Health Laboratory Bacterial Zoonoses Unit, Maisons-Alfort, France.
3.8.6. Nairobi sheep disease	*See chapter 3.10.1.*
3.8.7. Ovine epididymitis (Brucella ovis)[20]	**Dr B. Garin-Bastuji (retired)** France. **Dr J.M. Blasco** Centro de Investigación y Tecnología Agroalimentaria de Aragón, Zaragoza, Spain.
3.8.8. Ovine pulmonary adenocarcinoma (*adenomatosis*)	**Dr M.J. Sharp (formerly)** APHA, Lasswade Laboratory, Pentlands Science Park, Bush Loan, Penicuik, Scotland, UK.
3.8.9. Peste des petits ruminants (*infection with small ruminant morbillivirus*)[21]	**Dr M. Baron (retired)** UK.
3.8.10. Salmonellosis (*S. abortusovis*)	*See chapter 3.10.7*
3.8.11. Scrapie[22]	**Dr J. Spiropoulos** APHA Weybridge, New Haw, Addlestone, Surrey, UK.
3.8.12. Sheep pox and goat pox	**Dr P. Beard (formerly)** The Pirbright Institute, Ash Road, Woking, Surrey, UK. **Dr B.A. Lubisi** Onderstepoort Veterinary Institute, Agricultural Research Council, Onderstepoort, South Africa. **Dr H. Reza Varshovi (retired)** Iran.

20 This chapter was updated by consensus of all WOAH Reference Laboratories for brucellosis and other experts.
21 This chapter was updated by consensus of all WOAH Reference Laboratories for peste des petits ruminants.
22 This chapter was updated by consensus of all WOAH Reference Laboratories for scrapie.

3.8.13 Theileriosis in sheep and goats (*infection with* Theileria lestoquardi, T. luwenshuni *and* T. uilenbergi)	**Dr A. Torina** Istituto Zooprofilattico Sperimentale della Sicilia (IZSSi), Palermo, Italy.
3.9.1. African swine fever	**Dr C.A.L. Oura (formerly)** The Pirbright Institute, Ash Road, Woking, Surrey, UK. **Dr M. Arias** Centro de Investigación en Sanidad Animal (CISA-INIA), Madrid, Spain.
3.9.2. Atrophic rhinitis of swine	**Dr K.B. Register** USDA, ARS, National Animal Disease Center, Ames, Iowa, USA.
3.9.3. Classical swine fever (*infection with classical swine fever virus*)[23]	**Prof. P. Becher** Department of Infectious Diseases, Institute of Virology, University of Veterinary Medicine of Hannover, Hannover, Germany.
3.9.4. Nipah virus encephalitis	*See chapter 3.1.14.*
3.9.5. Porcine cysticercosis (*infection with* Taenia solium)	*See chapter 3.10.3.*
3.9.6. Porcine reproductive and respiratory syndrome[24]	**Prof. Z. Pejsak & Dr K. Podgórska** National Veterinary Research Institute, Pulawy, Poland. **Dr K. Tian** Veterinary Diagnostic Laboratory, China Animal Disease Control Center, Beijing, China (People's Rep. of).
3.9.7. Influenza A viruses of swine[25]	**Prof. I. Brown** APHA Weybridge, New Haw, Addlestone, Surrey, Weybridge, UK.
3.9.8. Swine vesicular disease	**Dr D. King** The Pirbright Institute, Ash Road, Woking, Surrey, UK. **Dr E. Brocchi,** Istituto Zooprofilattico Sperimentale della e dell'Emilia Romagna (IZSLER), Brescia, Italy.
3.9.9. Teschovirus encephalomyelitis	**Mr N. Knowles** The Pirbright Institute, Ash Road, Woking, Surrey, UK.

23 This chapter was updated by consensus of the WOAH *ad hoc* Group on Classical Swine Fever (vaccine section) and of all WOAH Reference Laboratories for classical swine fever (diagnostic section).

24 This chapter was updated with help from: Nicolas Ruggli (The Institute of Virology and Immunology, Mittelhäusern, Switzerland); Tomasz Stadejek (Warsaw University of Life Sciences, Warsaw, Poland).

25 This chapter was updated by consensus of all WOAH Reference Laboratories for swine influenza.

3.9.10. Transmissible gastroenteritis	**Dr L.J. Saif** The Ohio State Universtiy, Ohio Agricultural Research and Development Center, Food Animal Health Research Program, Wooster, Ohio, USA.
3.10.1. Bunyaviral diseases of animals (excluding Rift Valley fever and Crimean–Congo haemorrhagic fever)	**Dr B.A. Lubisi** Onderstepoort Veterinary Institute, Agricultural Research Council, Onderstepoort, South Africa. **Dr M. Beer & K. Wernike** Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Insel Riems, Germany. **Dr M. Baron (retired)** UK. **Dr P. Kirkland** Elizabeth Macarthur Agriculture Institute (EMAI), Virology Laboratory, Menangle, Camden, New South Wales, Australia.
3.10.2. Cryptosporidiosis	**Dr R. Chalmers** Cryptosporidium Reference Unit, Public Health Wales Microbiology, Singleton Hospital, Swansea, UK.
3.10.3. Cysticercosis	**Prof. P. Dorny & Prof. S. Gabriël** Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
3.10.4. Infection with Campylobacter jejuni *and* C. coli	**Prof. J.A. Wagenaar & Dr L. van der Graaf-van Bloois** Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.
3.10.5. Listeria monocytogenes[26]	**Dr A. Leclercq** Institut Pasteur, CNR & CCOMS Listeria, Unité de Biologie des Infections, Paris, France.
3.10.6. Mange	**Dr J.L. Schlater & Dr J.W. Mertins** Parasitology and Clinical Pathology Section, Pathobiology Laboratory, National Veterinary Services Laboratories, USDA, APHIS, VS, Ames, Iowa, USA.
3.10.7. Salmonellosis[27]	**Dr R. Davies (retired)** UK.
3.10.8. Toxoplasmosis	**Dr J.P. Dubey** Animal Parasitic Diseases Laboratory, USDA, Agricultural Research Service, Beltsville, Maryland, USA.

26 This chapter was updated with help from: Dr R. Rathbone (AOAC, USA); Dr G. Riegler (AOAC, USA); Dr K. Jinneman (FDA, USA); Dr Y. Chen (FDA, USA); Dr T. Hammack (FDA, USA); Dr S. Granier (Anses Maisons-Alfort, France); Dr R. Danguy-des-Deserts (Laboratoire départementale de développement et d'analyses, France); Dr A. Oevermann (University of Bern, Switzerland).

27 This chapter was updated by consensus of all WOAH Reference Laboratories for salmonellosis.

3.10.9. Verocytotoxigenic Escherichia coli

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PART 1

GENERAL STANDARDS

SECTION 1.1.

INTRODUCTORY CHAPTERS

CHAPTER 1.1.1.

MANAGEMENT OF VETERINARY DIAGNOSTIC LABORATORIES

INTRODUCTION

Reliable laboratory services can be delivered only by specialised facilities that are appropriately constructed and managed to provide the operating environment where the complex interaction of qualified staff, infrastructure and scientific methods can be coordinated to deliver specialised outputs consistently and safely. This chapter describes components of governance and management of veterinary laboratories that are necessary for the effective delivery of a diagnostic service, highlighting the critical elements that should be established as minimum requirements. Subsequent chapters set more specific standards for managing biological risks associated with laboratory facilities and for the range of aspects to be addressed to ensure confidence in laboratory test results.

The essential prerequisite for effective laboratory management is a clear understanding of the outputs required by the managing jurisdiction. National governments should support laboratory systems by developing a national laboratory policy based on the definition of the categories of laboratory test results required for effective implementation of the national animal health policy, including tests in support of international trade. Such clarity regarding national animal health requirements for laboratory services will guide the formation of national strategic planning for the delivery of these services. A clear statement of expectations of the laboratory service will guide governance and resourcing arrangements.

Further to these considerations, this chapter specifies components of diagnostic service management and delivery including the key support services that are considered essential. In addition to making provision for the scientific and technical aspects of the laboratory activities, the laboratory management system must address biorisk management and quality assurance. Laboratory management must also understand and meet the national and international regulatory requirements governing diagnostic laboratory operations. The outputs from a veterinary laboratory must be based on sound science, and mechanisms must be in place to prevent corrupt practices and inappropriate political influences.

A. GENERAL CONSIDERATIONS

1. Introduction

Laboratories fulfil an essential role in the delivery of veterinary services. Without the data and information supplied by veterinary laboratories, animal disease detection, control and prevention would be significantly weakened (Edwards & Jeggo, 2012). Veterinary laboratories are also a valuable resource to increase national laboratory capacity during major human health events such as pandemics.

Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* lists the usual purposes for which laboratory testing is conducted, which include demonstration of freedom from infection in defined animal populations, certification of freedom from infection in individual animals or products for trade/movement purposes,

contributions to the elimination of infection from defined populations, confirmation of diagnosis of suspect or clinical cases, estimation of prevalence of infection or exposure to facilitate risk analysis, and determination of the immune status of individual animals or populations.

These roles can be provided by governments (public sector laboratories), by industry (private sector laboratories), by universities (university laboratories) or by other organisations. Combinations of such providers in a complex matrix of services create challenges in the management and expectations of service delivery.

The governance of public sector veterinary laboratories will vary from country to country according to their public sector processes. This chapter sets out the general principles of governance and management for all types of veterinary laboratories that should ensure that Veterinary Services have access to reliable, trustworthy laboratory services, data and advice. The governance framework should ensure strong and effective delivery of services in a manner that is politically accountable, transparent, ethical, forward-looking and fair to staff and customers.

2. Accountability and oversight

A veterinary laboratory is held accountable for a range of issues apart from the delivery of basic diagnostic services. These may include health and safety, biosecurity, animal welfare and ethics, environmental contamination, genetic manipulations and quality assurance. It is essential that processes are established for the management and reporting of these issues and that individual staff are held accountable for their formally delegated responsibilities. As part of the process, it is critical to recognise and manage the resource implications, as failure to deliver to these accountabilities can bring the laboratory service into disrepute, detracting from the credibility of national animal health services.

There must be a clearly communicated and effective process by which the laboratory management is assessed and held accountable for delivery of all aspects of service delivery and accountability. This may be through a formally constituted governing body or through line management by the veterinary services or other qualified arm of government. Where a governing board is appointed, an independent chairman should be selected who understands both the political and the scientific environments in which the laboratory operates. The governing board should advise the laboratory director[1] on how to meet the expectations of the customers and owners of the laboratory, but should also represent the laboratory's interests by ensuring that these customers and owners have realistic expectations of the laboratory's capability and capacity, both in normal day-to-day operation and during outbreaks.

Whatever the governance structure, those responsible must ensure that the managers and scientific staff of the laboratory can operate in a scientifically sound environment and are free from inappropriate political influences. This includes the publication of the results of scientific research. A zero-tolerance approach to corruption at all levels must be in place.

A laboratory should have a medium-term strategic plan and a more detailed business plan for the year ahead, including financial and resource management. The director of the laboratory is ultimately responsible for ensuring the management of the laboratory according to these plans and obtaining appropriate governance agreement. The laboratory should also prepare an annual report for approval through the established oversight processes.

The governing body must not become involved with the operational management of the laboratory, which must remain firmly in the hands of the director and the management team.

It is important to review regularly the overall laboratory objectives and agreed deliverables with government to ensure transparency in meeting of expectations. Staff should be kept informed on such deliverables, understand priorities and not feel unduly threatened by the need to ensure financial security for the laboratory. There may be competing pressures with regards to the activities that need to be undertaken, and the director should continually provide leadership and guidance to staff on these issues.

3. Executive management

It is essential that operational activities in the laboratory are conducted under the authority of a single individual who is given an appropriate title, e.g. director or chief executive. The director (or equivalent) should be fully

1 The term "director" is used generically in this chapter to refer to the senior responsible person in the laboratory. Local terminology may vary, and this role is further discussed in Section A.3 of this chapter.

accountable for the delivery of outputs from the laboratory and for the deployment of resources within the institution. As the core role of the laboratory is to participate in the diagnosis of animal disease and disease control programmes, the director should ideally be a qualified veterinarian and also have personal experience of working in a laboratory environment. Where the director does not have a veterinary qualification, a senior deputy should be appointed in the role of veterinary director. The key attributes of the director are to have an understanding of the operating environment of laboratory work, to be fully aware of the end-user requirements so that the outputs are relevant, trustworthy and timely and to demonstrate leadership qualities that will motivate the laboratory staff to deliver their best and to the required quality standard, both during normal operations and in outbreaks.

The director should be supported by a senior management team whose members will lead specific aspects of the work of the laboratory. The size of this team, and the scope of their individual responsibilities, will depend on the size of the laboratory, but it will typically involve leaders of different scientific disciplines (e.g. pathology, bacteriology, virology) as well as business leaders with expertise in human resources (HR), finance, procurement, engineering, information technology (IT) and communication. At least one of the senior team should be designated deputy director; the deputy will work closely with and in support of the director and fulfil the director's responsibilities in his or her absence.

4. Infrastructure

Laboratories are highly specialised facilities with very particular requirements in terms of buildings, services and operational environments. Although some smaller laboratories can operate within an adapted general-purpose building, it is highly recommended that veterinary laboratories are housed in purpose-built units, designed with considerable input from scientific staff, along with architects, environmental experts, safety advisers and others in the design team. The structure and functions of the laboratory must comply with all relevant national regulations and international standards, such as for biocontainment, biosafety and environmental impact. Local issues must also be taken into account, such as the likelihood of extreme conditions (high or low temperatures, earthquakes, hurricanes, floods) and the reliability of water and electricity supplies.

National authorities must recognise that laboratories, whilst very expensive to build, are equally expensive to operate and maintain. It is absolutely essential that an adequate, ongoing budget be allocated for annual operating costs (see section on finance below). Factors to support include the IT data support requirements (including future-proofing), utility costs and waste management. Likewise, as technologies and legislative requirements change, budgetary commitments to equipment and laboratory upgrades should be planned well in advance.

5. Human resources

A veterinary laboratory, like any organisation employing staff, must have a clear, transparent HR policy that is seen to treat all individuals fairly. Appropriate procedures should be in place to determine remuneration, performance management, appraisal and promotion, leading to incentivisation and reduced risk of departure of well-trained staff. A robust mechanism for addressing poor performance is also essential; it should provide clear and fair procedures for dismissal, in extreme circumstances. Veterinary laboratories employ an unusually high proportion of specialised staff, and this can cause difficulties where work patterns change as new technologies are introduced. HR policies should include training and retraining programmes to ensure that all staff are developed to their full potential and contribute to a flexible work force. It is prudent to encourage staff, particularly new entrants, to move around different sections of the laboratory as part of their development. This breadth of experience ensures increased resilience in outbreaks, and also builds tolerance, as staff develop an appreciation of the challenges of different disciplines – and they also make better leaders.

6. Compliance

6.1. Health and safety

Veterinary laboratories are hazardous environments. Most hazards fall into three main categories: biological, chemical and physical. There are biological risks from handling dangerous pathogens, including infectious zoonotic agents (i.e. those that may infect humans), recombinant forms of infectious agents, viral vectors, biologicals introduced into experimental animals and allergens from handling animals. Hazardous chemicals can result in exposure during use, if misused or mishandled, or through inappropriate storage. Appropriate personal protective equipment must be used to protect personnel from exposure to toxic, carcinogenic or otherwise hazardous chemicals. Risks from physical hazards can include ergonomic issues associated with manual tasks, handling sharps, poor housekeeping, ionising

radiation, ultraviolet radiation, fire, high-pressure steam, liquid nitrogen, solid CO_2 vessels and animals (bites, kicks and other trauma to staff). Health and safety (H&S) must comply with the applicable national H&S legislation where such exists and be managed in a transparent and documented manner. The laboratory must have policies and procedures in place to assess all risks to staff (and visitors) and to mitigate those risks to acceptable levels. Risk assessments should be performed by the individuals most familiar with the specific characteristics of the pathogens being considered for use, the equipment and procedures to be employed, animal models that may be used and the containment equipment and facilities available.

It is the responsibility of the laboratory senior management to ensure the development and adoption of H&S policies and procedures. Training of new and existing personnel is key to avoiding accidents. Appointment of an H&S professional should be a serious consideration for larger laboratories, and this should be supported by an appropriate, dedicated H&S budget. The role of the H&S professional must be clearly defined and documented, and all personnel (including visitors) should understand that the presence of an H&S professional does not mean that they are any less responsible for carrying out their work in a safe and responsible manner, in compliance with agreed protocols. The H&S professional must have the full support of laboratory senior management. Personnel should be advised of special hazards, required to read and have easy access to the laboratory safety or operations manual, follow standard practices and procedures and participate in regular training.

A H&S committee should be established consisting of representatives from both staff and management of the laboratory. A requirement for such committee structures and operations is usually included in national legislation, and the laboratory managers must be fully conversant with these defined processes, including the appointment of H&S representatives, actions and reporting procedures for all H&S incidents, H&S training requirements and the minimum laboratory infrastructures and processes to meet these requirements. For larger facilities that may also carry out additional activities, including research, a biological safety officer, a chemical safety officer and a more formal institutional biosafety committee, with external representation, may also be of benefit in assessing and managing additional biosafety risks. Such a committee should also apply oversight and guidance on dual use research of concern[2].

6.2. Biosafety and biosecurity

In addition to health and safety issues, veterinary laboratories have a responsibility to contain pathogens and to prevent unauthorised access to reduce the risk of their accidental or deliberate release that might threaten neighbouring human or animal populations, or the environment. Standards on biorisk management are given in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities* and in the WHO[3] Laboratory Biosafety Manual (WHO, 2020). All veterinary laboratories should comply with the relevant standards in these documents and also adhere to national standards and regulations. In many countries there is a national compliance monitoring authority for biosecurity or biocontainment. This authority will inspect the laboratory on a regular basis. The laboratory managers must understand the regulations and ensure that sufficient resources are available to ensure compliance. For laboratories undertaking investigations of new and emerging diseases or pathogens, the potential zoonotic risk to staff and the general population should also be subject to a risk assessment and appropriate mitigation measures put in place where potential risks are identified. Brass *et al.* 2017 provide a useful summary of methodologies used to assess risks posed by novel pathogens, as well as covering the fundamentals of laboratory biosafety and biosecurity.

Whilst minimum legal requirements exist, individual laboratories should examine their processes and procedures to determine where elements of biosecurity risk may arise and how best these should be managed on a local basis. A biorisk manual that contains standard operating procedures (SOPs) for all activities should be maintained, with version control. Such SOPs should highlight biosecurity controls, training should be provided, and it is recommended that local procedures are put in place to manage non-compliance. This is a matter of good laboratory practice, regardless of the legislative background.

2 WOAH Guidelines for responsible conduct in veterinary research: https://www.woah.org/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/BTR/A_GUIDELINES_VETERINARY_RESEARCH.pdf

3 WHO: World Health Organization

Laboratory biorisk management should specifically recognise the potential for bioterrorist threats including the concept of the insider threat (e.g. the bioterrorist threat posed by a staff member or visiting scientist). When working with high consequence pathogens, a process should be developed by which this threat can be managed. Physical security measures and an annual staff threat appraisal would be a minimum requirement in such circumstances and security screening of staff may also be considered. In addition, measures must be in place to control access by visiting scientists and to ensure their supervision when working with this class of pathogens.

Where required by regulation or organisational policy, written confidentiality agreements should be in place for appropriate personnel including staff, visiting scientists, contractors and others, to prevent any confidential information being released inappropriately.

6.3. Animal welfare

Veterinary laboratories must ensure their activities comply with animal welfare standards (Section 7 of the *Terrestrial Animal Health Code*, particularly Chapter 7.8 *Use of animals in research and education*). It is also essential to understand fully the national legislation governing the ethical use of animals and put in place processes to ensure compliance. An institutional animal ethics committee, with external representation, is recommended.

6.4. Gene regulation

Many laboratories now use modified genes or gene products in their activities. Compliance with national regulations governing their use must be ensured including establishment of systems in the laboratory to monitor and ensure such compliance. International obligations concerning ethical behaviours in genetic manipulation in the context of Dual Use Research of Concern (DURC)[4] also apply (WOAH, 2019). Particular attention should be given to experiments that might fall into the category or "Gain of Function", where the host specificity or virulence of a pathogen might be altered.

6.5. Environment

Laboratory waste may create concerns of environmental pollution. The risk of environmental damage from carcass disposal and disposal of other biological material is an issue that requires specific attention. Understanding and managing, as far as possible, any potential negative impacts of the laboratory on the surrounding environment is important and may be subject to national and local regulations. Certification of compliance with standard ISO[5] 14001:2015 *Environmental Management Systems* (ISO, 2015) should be a target for laboratory managers. The laboratory should not be established in an inappropriate environment such as close to other facilities that may impact on the safety of the laboratory.

B. SCIENTIFIC SERVICES

1. Diagnostic service delivery

The national Veterinary Services must be very clear in specifying the purposes for which laboratory capability is required, and hence the test methods and technologies to be supported. The defined purposes will include the list of diseases or infectious agent groups in scope, the nature of the government programmes to be supported in terms of the purposes of testing outlined in chapter 1.1.6, the likely scheduling and volume of submissions, and the required turnaround time for test reports. The cost implications must be identified and agreed. These discussions should be recorded in a service level agreement or similar documentation.

A key component of the delivery of scientific services is the routine monitoring, calibration and maintenance of scientific equipment. This is a real challenge in terms of both the resources to maintain the process and the availability of trained engineers and calibration equipment. Managing these processes should be a priority for

4 DURC: https://www.who.int/csr/durc/en/
5 ISO: International Organization for Standardization

resource allocation as test results generated on unmaintained and uncalibrated equipment cannot be trusted to be accurate.

Provision should also be made for the laboratory services that will be required in a disease emergency. The laboratory maximum (surge) capacity for processing samples should be defined as well as a plan for scale-up of operations. This may include a diversion of resources from lower priority tasks. Capacity may be disease-specific and test turnaround times are also an important element in this specification.

All countries should support WOAH designated Reference Centres through submission of specimens, isolates of infectious agents and other information of potential regional or international significance. It is only through receipt of such submissions that the Reference Centres can fulfil their WOAH mandated role on behalf of the international community. Involvement with the designated Reference Centres is necessary for international public good.

In turn, national veterinary laboratories with special expertise in particular areas may seek recognition from international bodies such as WOAH, FAO[6] or WHO as reference laboratories or collaborating centres. This is encouraged, as it facilitates the harmonisation of laboratory procedures worldwide, and strongly supports the work of WOAH and other international organisations. Funding for reference laboratory status needs to be allocated from national sources, and this should be part of the national planning with the Veterinary Services.

Many veterinary laboratories carry out work for a range of different customers. As well as meeting the needs of Veterinary Services, the laboratory may conduct contract work for national or international parties, provide diagnostic and surveillance procedures for private veterinarians, veterinary organisations or livestock industries, test food or environmental samples for food safety or other public health reasons, perform regulatory testing of veterinary medicinal products, and carry out contract testing for the private sector, e.g. for pharmaceutical companies. It is the responsibility of the laboratory director and management team to ensure that a balanced approach is taken in the allocation of resources in order to deliver this complex array of services. There should be clear recognition of priorities to facilitate dealing with unexpected events such as disease emergencies.

In some countries, laboratory services are delivered through a number of laboratories, rather than through a single laboratory. In such cases, it is essential that the laboratories form a network, sharing best practice and information on testing, such that the Competent Authority has assurance that the same result is obtained, wherever testing is performed.

2. Quality assurance

Veterinary laboratories must be managed under a quality assurance system as specified in Chapter 1.1.5 *Quality management in veterinary testing laboratories* and should preferably be accredited to an international standard such as ISO/IEC 17025 *General requirements for the competence of testing and calibration laboratories* (ISO, 2017). The laboratory should ensure that all of its procedures, not just those concerning the laboratory bench but also those for supporting documentation and computer records, are robust, reliable and repeatable.

The quality standards require that diagnostic tests used in the laboratory should be validated as fit for purpose. The international standard for validation of diagnostic tests is established by WOAH and is set out in chapter 1.1.6. Validation is not a once-for-all-time procedure but requires continual monitoring and refinement as the test is used. Laboratories should strive at all times to use tests that have reached at least stage 3 on the WOAH validation pathway (chapter 1.1.6) and to continue refining the validation data as explained in the text.

3. Research

Laboratories are likely to engage in research, such as development or adoption of new tests or test methods, or pathogenesis or epidemiological studies of infections important in the particular country. It is essential to manage effectively the balance between research and diagnostic service delivery and the potential for competition for resources, including staff time. Many laboratories undertake research aimed at disease awareness, preparedness and mitigation, and to inform policy in “peacetime”, with the research space configured and scientists trained and deployed to disease investigation and surge capacity diagnostic functions during outbreaks. Furthermore, such capability can provide for investigation of new and emerging diseases, or variants of known disease. Publication of

6 FAO: Food and Agriculture Organization of the United Nations

emerging disease threats, novel methods of detection and sharing of protocols with Reference Laboratories is encouraged.

4. Information and data for disease surveillance

Diagnostic laboratories may play a critical role in providing laboratory information and data to support routine disease surveillance activities and hence the claim of disease status in the country, which is essential to maintaining domestic and international trade. Apart from using fit-for-purpose surveillance tests as detailed in the relevant disease and quality management chapters of this *Terrestrial Manual*, laboratories should endeavour to ensure the compatibility of relevant information and data with that used in their central or national animal health systems if available or applicable.

C. SUPPORT SERVICES

1. Internal governance: policies and procedures

To ensure adequate standards of laboratory management across the spectrum of roles and responsibilities as identified in this chapter the responsible authority for the laboratory must ensure that laboratory management has adequate arrangements in place to deliver the required outcomes. These arrangements will include clearly defined policies and procedures supported by a management structure that is adequately resourced for implementation, audit and review. In many areas of activity, national legislation may provide the overarching standards to which laboratories must work and it is essential that compliance is seen to be mandatory, for the welfare of staff, the protection of the environment and the reputation of the laboratory.

Laboratory management should agree and document its policies for all aspects of operational activities. The processes by which such policies are implemented should also be documented in the form of clear procedures that are communicated to all staff who are involved in the particular activity. This approach has been introduced in some laboratories through the development of a quality assurance system, and is also applicable to other aspects of laboratory activity. The responsibilities of designated staff for oversight and implementation of policies and procedures should be included in the documentation and communicated clearly to all staff on the laboratory site.

2. Information management

Modern laboratories are increasingly dependent on computerised systems to manage their data. This can include an all-encompassing laboratory information management system (LIMS), bespoke systems for controlling individual laboratory equipment, and sophisticated analytical systems for use by specialised information scientists in disciplines such as molecular biology, informatics, epidemiology, risk analysis and statistics. A system that monitors location and quantities of high consequence pathogens is also common in high containment laboratories. There will also be office support systems for word processing, finance, HR and bibliographic databases. Systems for internal and external communications such as websites and email services will be needed. As with other elements of the laboratory's activities, it is essential that the computer systems are managed by competent professionals and that the scientific staff are consulted in specifying the services they require. Measures must be in place to protect the integrity of the data, for archiving and retrieval, and for privacy protection of personal or sensitive items. It is important that the laboratory clearly determines its needs and procures the necessary resources, either through a service contract with an IT support company or through the direct employment of IT professionals, so as to provide adequate support in this essential area.

3. Finance

The budget is an integral part of the annual laboratory business plan and will set a basis for negotiation with customers and funders. The director should be personally accountable for delivering the work programme of the laboratory within budget, while individual managers of projects or activities should be set delegated delivery and financial targets. For any but the smallest of laboratories, the director should be supported in this area by one or more finance professionals.

Laboratory management should identify all costs and their allocation to the appropriate area of activity, so that the total cost of delivering any particular service can be identified. The operating costs should include directly attributable items (such as reagents and equipment), staff time per procedure, administration (booking in samples,

generating reports), capital equipment (the cost of which may need to be spread across multiple activities or projects) and an appropriate proportion of overhead costs (covering such items as management, buildings, utilities, IT services, safety and quality procedures, and storage and archiving of samples and records).

Cost control is an essential part of laboratory management. Continual efforts should be made to improve efficiency without compromising on quality. It is to be expected that customers will seek to minimise the costs to them of the services received; however; it is also important that the Veterinary Services or other laboratory customers recognise the complexity of the expenses in running a laboratory.

For many laboratories revenue generation through the sale of services and products is an important component of their finances. There may be political or regulatory constraints that determine whether such activities can make a profit, break even or be subsidised from the government allocated funds, but in all cases where the laboratory recovers costs from submitters, the laboratory should have a transparent pricing policy.

An important aspect of financial management is procurement of equipment, laboratory supplies and services. It is likely that there will be government regulations with which the actual procurement processes must comply. However it is important that the scientific staff of the laboratory should prepare detailed specifications of their requirements, whether for reagent supplies, equipment, or external provision of services. If the specification is well prepared, then the procurement process should be able to secure appropriate supplies of the product at the required quality. Clear rules must be in place to prevent undue pressure or bribery being applied to procurement officers by suppliers. This risk must be monitored closely by the senior management of the laboratory and, if necessary, by the governing body.

4. Engineering and maintenance

A modern veterinary laboratory requires substantial and adequate engineering maintenance and support. It is possible to outsource many of these maintenance requirements, but in many cases an in-house capability may better serve the need. Familiarity of engineering staff with the biological impacts and risks associated with the systems is also important to ensure maintenance and repairs can be conducted safely. Most laboratories have site-specific needs and requirements that are best met with a reasonable complement of engineering and trade skills on site, with staff who are familiar with local needs and issues. A robust reliability programme helps to identify critical points of failure and ensure planned, preventive maintenance of these elements. Sufficient stocks of spare parts and supply lines should also be in place, supported by a stock management system, to ensure continuity. Maintenance schedules affecting laboratories should be carefully programmed in consultation with laboratory users to ensure continuity of operations as far as practicable. Laboratory management should regularly review how best to supply these support services.

5. Communications

Good communications that result in transparency of decision making and operations are vital to the success of a laboratory enterprise. This includes internal communications within the laboratory, ensuring that all staff are aware of the current priorities and how these impact on their work individually, as well as the wider activities of the laboratory and how their efforts contribute to the whole. It is essential that senior management is visible and has a system for communicating with staff throughout the laboratory and that this process genuinely works both ways. Senior managers must make efforts to be aware of the concerns and aspirations of their staff.

Externally, the director and management team must be effective advocates for the laboratory and represent it in meetings with Veterinary Services and other government officials, with scientists from other institutions, nationally and abroad, or with the wider public, including the media. It follows that the director and senior managers should be trained to interact with the media. This is a major priority particularly during a disease emergency, when effective communications with laboratory stakeholders is essential.

The key outputs from a veterinary laboratory are the scientific results and interpretation stemming from its analytical and investigational activities. These must be communicated to the customers or end-users in a clear, unambiguous and meaningful manner. Laboratory reports should include, where appropriate, indications of the level of uncertainty in the results, whether further results are still pending, and how to raise queries or clarifications or request further work. For their part, customers should be encouraged to provide as much additional relevant information as possible, when submitting samples, as this can greatly aid laboratory investigation and the interpretation of results.

A public information policy and procedures should provide a mechanism for individuals and outside bodies to ask about specific activities in the laboratory. Communications support staff should be involved in ensuring that the laboratory's customers are kept informed about the work of the laboratory, its successes and any constraints on future work. Laboratories may provide an internet website or other IT-based strategies to assist with such communications. The management team should also ensure that procedures are in place to ensure compliance with obligatory reporting and notification requirements.

Scientific staff should be encouraged and supported to attend conferences and present papers, while the production of a steady stream of good-quality written papers in refereed journals is vital to the success of a laboratory institution. Importantly, this does not apply only to the research scientists; those working in diagnostic and surveillance work can also play an important role.

D. CONCLUSIONS

Good governance and management of a veterinary diagnostic laboratory are essential for the safe, sustainable and effective delivery of a diagnostic service. This chapter identifies the range of issues to be addressed if laboratories are to meet international standards. Many aspects of the delivery of laboratory services are now highly regulated by national authorities, and laboratory managers must be familiar with these regulations and have compliance processes in place. Key elements of staff safety, biocontainment, biosecurity, quality assurance, animal welfare and environmental management are vital components of operating such facilities. The governance and management of these aspects are as important as the delivery of the actual diagnostic service.

A well managed laboratory will further ensure that the general provisions specified in the remaining chapters of Part 1 of this *Terrestrial Manual* are met as well as the specific standards for the diagnostic testing for specific disease agents as outlined in Part 2. A key component in providing customer assurance is conforming with the WOAH quality standard (chapter 1.1.5) supported by accreditation to quality standards such as ISO 17025. Accreditation is an important achievement of which laboratory staff can be proud, and implies that underlying compliance issues have been addressed.

Fundamental to the effective delivery of diagnostic services is the operation and maintenance of the facility and the scientific equipment. Allocation of adequate ongoing resources to this area is vital.

A successful veterinary diagnostic laboratory will have a highly trained, motivated workforce, with respect and support given to all individuals including both the frontline scientific staff and the important support teams providing vital services in areas such as finance, HR, safety, quality, procurement, engineering, IT and communications.

The achievement of all the above, and delivery of a respected and reliable service, requires a management system with checks and balances and effective review. This will include mechanisms to ensure political accountability, transparency, responsiveness, and coherent planning to ensure sustainability. A structure that includes an oversight process is strongly advocated to assist both financial management and strategic approaches to the delivery of all aspects of the laboratory's activities.

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NB: FIRST ADOPTED IN 2015. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 1.1.2.

COLLECTION, SUBMISSION AND STORAGE OF DIAGNOSTIC SPECIMENS

INTRODUCTION

Laboratory investigation of animal disease is critically dependent on the quality and appropriateness of the specimens collected for analysis. This chapter sets out the general standards involved in specimen collection, submission, and storage. The individual disease chapters in this Terrestrial Manual provide specific information on appropriate specimens needed to test for particular pathogens or toxins. Sampling may be from individual animals, from animal populations, or from the environment for a variety of purposes, such as disease diagnosis, disease surveillance, health certification, and monitoring of treatment or vaccination responses. To provide scientifically and statistically valid results the specimens collected must be appropriate for the intended purpose, and adequate in quality, volume, and number for the proposed testing. Additionally, the animals and tissues sampled must be appropriately representative of the condition being investigated.

Specimens must be collected using appropriate biosafety and containment measures in order to prevent contamination of the environment, animal handlers, and individuals doing the sampling as well as to prevent cross-contamination of the specimens themselves. Care should additionally be taken to avoid undue stress or injury to the animal and physical danger to those handling the animal. Biological materials should be packaged to rigorously control for leakage, and then labelled with strict adherence to the applicable regulations guiding their transport as outlined in Chapter 1.1.3 Transport of biological materials.

A. COLLECTION OF SAMPLES

1. General considerations

Careful consideration must be given to the collection, containment, and storage of the specimens, including biosafety measures that must be in place to prevent contamination of the environment or exposure of other animals and humans to potentially infectious materials (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). For information on transport of specimens see Chapter 1.1.3 *Transport of biological materials.*

The reliability of the diagnostic testing is critically dependent on the specimen(s) being appropriate, of high quality, and representative of the disease process being investigated. Prior to sampling, consideration must be given to the type of specimen(s) needed including the purpose of the testing and the test technologies to be used. The volume or quantity of specimen must be sufficient to perform initial testing, to perform any subsequent confirmatory testing and to provide sufficient residual specimen for referral or archival purposes.

The purposes of testing will be aligned with the purposes for which tests are validated, as listed in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*, namely:

i) Demonstration of freedom from infection in a defined population.

ii) Certification of freedom from infection or presence of the agent in individual animals or their products for trade/movement purposes.

iii) Eradication of disease or elimination of infection from defined populations.

iv) Confirmatory diagnosis of suspect or clinical cases.

v) Estimation of prevalence of infection or exposure to facilitate risk analysis.

vi) Determination of immune status of individual animals or populations (post-vaccination).

Epidemiologically appropriate sampling plans should be developed prior to collection of specimens, as described in Section B and Appendix 1.1.2.1. These will specify the number of animals or other sampling units to be sampled.

Specimens must be collected according to a sound knowledge of the epidemiology and pathogenesis of the disease under investigation, or the disease syndrome to be diagnosed. This will lead to the sampling of tissues or fluids most likely to contain the infectious agent or evidence of the infection. Considerations will include the tissue predilection(s) or target organ, the duration and site of infection in each tissue type and the duration and route of shedding, or the time frame in which evidence of past infection, such as an antibody response, can be detected reliably by the tests to be deployed. These considerations will also indicate the method(s) of collection to be used. In many herd or flock-based disease investigations it is beneficial to collect specimens from a healthy cohort for comparative epidemiological or baseline testing (e.g. case-control and cohort approaches for diagnostic testing) and for validation purposes.

Where chemical euthanasia or anaesthesia is required for animal restraint, the impact of the chemical on the test result (e.g. toxicology testing) must be considered. Some laboratory tests are not compatible with specific blood anticoagulants and tissue preservatives, such as heparin, formalin, dry ice (exposure of the test sample to elevated levels of CO_2), or even freezing. While it is critical to collect specimens as aseptically as possible, equal care must be taken to avoid contamination with detergents and antiseptic treatments used to clean the collection site on the animal, as these agents may interfere with the laboratory test procedures. Procedures requiring tissue culture of pathogens, as well as many molecular-based tests, can be negatively affected by chemicals or detergents commonly used in the manufacture or preparation of collection tools (e.g. chemicals used in manufacture of some types of swabs and detergents used in cleaning glassware).

Specific information on diagnostic test methodologies and the recommended specimens, preservatives, and specimen handling procedures can be found in the individual *Terrestrial Manual* disease chapters or through direct consultation with the laboratory that will be performing the required testing. Procedures for collection and submission of specimens are available from most diagnostic laboratories, including national and international authorities, where the information is frequently accessible via the specific laboratory's web page. The WOAH web page provides contact information for all WOAH reference laboratories[1].

It is critical not only to collect the most diagnostically-appropriate specimens, but to also inform the laboratory of the associated disease epidemiology in order for the laboratory scientists to assign the most appropriate tests or panels of tests. Epidemiological information to be submitted with specimens is outlined in Section C of this chapter.

Where investigating diseases of unknown cause multiple different specimens that represent the different stages of the disease progression in an animal or the population of animals (e.g. the pre-clinical, early clinical, active clinical, chronically affected and convalescent phases) should be collected. Epidemiological considerations for sampling are particularly critical for diagnosing population-related diseases, as would occur with beehives, flocks, and herds. Epidemiological principles used for sampling are further introduced in Section B of this chapter.

Specimens can be collected ante-mortem or post-mortem. Specific considerations regarding different specimen types are as outlined below.

2. Blood

Whole blood samples may be collected for haematology, clinical chemistry, toxicology, direct examination for bacteria or parasites, PCR testing, immunological testing, or for culture for bacteria or viruses. Dependent on testing needs, whole blood, blood cells, and/or plasma samples can be obtained from whole blood collected into appropriate anticoagulants. In selecting the anticoagulant to be used the collector must be aware of the laboratory tests, including PCR-based diagnostics, clinical chemistry, and toxicology, which may be negatively affected by the presence of specific anticoagulants or preservatives. Specific disease chapters in this *Terrestrial Manual* provide guidance for individual tests and sample requirements. To be effective anticoagulants require that the collected

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

blood be thoroughly mixed with the chosen anticoagulant during or immediately following its sampling from the animal.

To obtain serum, whole blood is collected without anticoagulants and the clot is allowed to contract at ambient temperature protected from extremes of heat and cold for periods that may range from a few hours to overnight. Clear serum can be decanted or collected by pipette following physical removal of the clot, ideally following gentle centrifugation to separate cell components from the serum. In the absence of a centrifuge, separation of the clot can be facilitated by tilting the freshly collected blood tube at an approximate 45 degree angle until the clot has retracted, "ringing" the clot with a sterile rod or pipette to separate the clot from the tube surface, and then removing the clot with forceps. The results of serological testing can be compromised by the quality of the sample. Bacterial contamination and red blood cell debris in serum samples can produce false positive reactions in agglutination-type assays. Serological assays can be negatively impacted by haemolysis in the serum sample. Microbial contamination and haemolysis are significant concerns especially when obtaining blood and serum samples from post-mortem animals. Frequent causes of haemolysed serum and plasma include exposure to excessive temperatures or time delays prior to separating sera from the red blood cells, blood collection using a needle of too small gauge, or failure to remove the needle when transferring the blood sample from the collection syringe.

Whole blood should be collected aseptically, typically by venipuncture of the live animal. Depending on the animal and sampling situation jugular, caudal, brachial, cephalic, mammary veins or the vena cava may be used. Specific techniques for sampling small laboratory animals have been reviewed (Anon, 1993; Hem *et al.,* 1998). Care should be taken to collect and dispense blood samples as gently as possible to prevent damage to red blood cells, which causes haemolysis. Blood and sera are typically shipped and stored cool (or frozen in the case of sera) in non-breakable vials, tubes, or bottles; however for some laboratory tests that require viable peripheral blood mononuclear cells, the blood must be packaged, transported and stored so as to prevent exposure to temperature extremes. For some tests, aliquots of specimens can be dried onto a piece of untreated, or specifically-treated commercial filter paper designed for stabilised sample transport and storage.

3. Faeces

Faeces can be collected freshly voided or preferably directly from the rectum/cloaca for tests such as culture for microorganisms, parasite examination, or faecal occult blood determination; or can be collected for culture and molecular-based diagnostics from the rectum/cloaca using cotton, dacron, or gauze-tipped swabs, dependent on the volume of sample required by the specific test methodology. Samples collected on swabs should be kept moist by placing them in the transport media recommended for use with the specific test to be performed, which may range from sterile saline to culture media containing antimicrobials or stabilisers. Faecal specimens should be kept chilled (e.g. refrigerated at 4°C or on ice) and tested as soon after collection as possible to minimise the negative impacts on test results caused by death of the targeted microorganism, bacterial overgrowth or hatching of parasite eggs. Double-packaging of faecal samples in screw cap or sealable containers that are subsequently contained within sealed plastic bags will help prevent cross-contamination of samples and associated packaging materials. Faeces contained only in rectal exam gloves, plastic bags, or rubber-stoppered tubes are unsuitable as they are very frequently comprised by bacterial growth with gas production that can rupture plastic bags, displace stoppers, and allow leakage of the specimen.

4. Epithelium

Epithelial tissue in the form of biopsies or skin-scrapings; swabs of oral, nasal, pharyngeal, and gastrointestinal surfaces, as well as plucked hair or wool can be used variously for direct examinations or laboratory tests to identify surface parasites such as mites and lice, fungal, bacterial or viral infections, allergic reactions, and neoplasia. The specimens should be collected aseptically and preserved as specified for the intended test(s). Deep skin-scrapings obtained using the edge of a scalpel blade are useful for burrowing mites. Feather tips have been validated for use in the detection of viral antigen for Marek's disease, and used as a sample for molecular detection of additional avian diseases. Epithelial tissues, particularly those associated with vesicular lesions and collected into viral transport media, can be critical in the laboratory diagnosis of specific viral infections such as foot and mouth disease.

5. Ocular sampling

The surface of the eye can be sampled by swabbing or ocular scraping, ensuring that cells rather than mucopurulent discharge or lacrimal fluids are collected for testing. Specimens from the conjunctiva are typically collected by holding the palpebra apart and gently swabbing the surface of the eye with a cotton, dacron, or gauze swab that has been pre-moistened with sterile saline or equivalent media. Such swabs should be kept moist in saline or transport

media specifically recommended for use with the testing to be performed. Biopsies from the third eyelid of sheep have been used as a lymphoid-rich tissue for prion detection.

6. Sampling the reproductive tract

Preputial and vaginal wash fluids and swabs of the cervix and urethra can be used as specimens for investigation of reproductive disease. The swabs should be kept moist following collection by placing in the recommended volume of transport media required by the laboratory test, typically sterile saline or specified culture media. Semen specimens are typically obtained using an artificial vagina or by extrusion of the penis and artificial stimulation. Avoid contamination of the specimen with antiseptic or detergent solutions used to prepare the animal/site for sampling.

7. Nasal discharge, saliva, and vesicular fluids

Secretions can be collected directly into a vial or tube, or can be collected using swabs. Vesicular fluids provide a highly concentrated source of pathogen for diagnostic testing, and can be collected from unruptured vesicles using a sterile needle and syringe, and immediately transferred to a securely sealed vial or tube. Specifically developed sampling tools, such as probang cups, can be used for collecting cellular material and mucus from the pharynx of livestock. Cotton ropes that animals are allowed to mouth and chew have been validated for use in collecting saliva specimens from domestic swine.

8. Milk

Milk can be collected from individual animals or from bulk milk in tanks pooled from multiple animals in a herd. The teat(s) used for sample collection should be cleaned, and any detergent thoroughly rinsed off before collection of the specimen. In collecting milk from individual teats, the initial stream must be discarded and only the subsequent streams sampled. The method of preservation prior to testing varies with the requirements of the test; in some cases it will be critical to avoid freezing or addition of chemical preservatives. The individual disease chapters of this *Terrestrial Manual* or the advice of the testing laboratory should be consulted for appropriate specimen handling and preservation recommendations.

9. Tissues collected at necropsy

Necropsies should be conducted only by qualified veterinarians and pathologists. Paraveterinary staff may be trained by veterinarians to conduct post mortem examinations for specific purposes. Importantly, the purpose of the necropsy is not only to collect specimens but to make informed observations regarding the pathology of the condition. Such observations are an important adjunct to epidemiological and clinical observations in the comprehensive veterinary investigation of the case or outbreak. It is useful for veterinary authorities to retain specialist veterinary pathologists to lead post mortem investigations in important cases. Where this expertise is managed from a veterinary laboratory the methods employed should be formally described in the laboratory's Quality Assurance Manual and the capability should be recognised in the laboratory's scope of accreditation. Detailed procedures for conducting post-mortem examinations and tissue collection are available in most pathology text books, and are additionally provided in many of the web-page accessible national laboratory testing guidelines. Specimens that are critical for the laboratory investigation of listed diseases are included in the chapters of the *Terrestrial Manual* relating to each disease.

Whether the necropsy is performed in a designated laboratory facility or in the field, appropriate biosafety and containment procedures should be followed to ensure operator safety and to provide non-contaminated and useful tissues for testing as well as to protect the environment and other animals from potential exposure to pathogens. As a minimum requirement the collector(s) must wear personal protective equipment that protects the skin and mucous membranes and that can be discarded or decontaminated. All remaining tissues or carcass parts and fluids should be contained and treated with an appropriate disinfectant or destruction method, and the immediate environment should be thoroughly disinfected.

Dependent on the suspected disease, condition of the carcass and facilities available for necropsies post-mortem specimens can be collected from one or multiple organs and submitted to the laboratory as either fresh (no preservative) or preserved specimens for further laboratory testing. The process of carcass autolysis can destroy diagnostically relevant tissues and infectious agents and so should be considered prior to collecting and submitting post-mortem specimens.

For fresh specimens particular attention must be paid to their handling and storage to avoid autolysis and overgrowth by bacterial and fungal contaminants. Ideally, freshly collected specimens are kept at a constant cool temperature from collection until processing for testing. Where such a cold chain cannot be provided fresh specimens for some test procedures can be collected into fluids such as ethylene glycol that inhibit the growth of secondary organisms. Where such strategies are compatible with the subsequent test methods the option is mentioned in the relevant chapters of the *Terrestrial Manual* for each disease.

Preservation of post mortem specimens is most frequently achieved by collection into formalin solution. Where such chemical fixative is supplied to pathology staff by laboratories or competent authorities they must ensure adequate training in health and safety aspects of the use of such chemicals and training in compliance with regulations relating to the transport of such chemicals.

10. Environment and feed

Environmental sampling may be of litter, bedding, water from troughs and drinkers, or feed which has been exposed to urine, faeces, and/or saliva of affected animals, or swabbed surfaces of facilities, ventilation ducts, drains or feed containers. If specialised equipment is available circulating air may be sampled.

11. Honey bees

Adult moribund or recently dead bees can be collected in the vicinity of colonies. Live bees can be killed by freezing. Brood specimens are typically collected by removing a piece of brood comb showing abnormalities and including dead or discoloured brood followed by wrapping in a paper towel or newspaper rather than in foil or wax paper in order to help prevent microbial overgrowth. Alternately, diseased cells in a comb may be sampled using a toothpick or equivalent. A sticky board can be used to collect hive debris, including trapping of mobile parasites. More information on the specimens that need to be collected can be found in the disease-specific chapters of this *Terrestrial Manual* related to bees.

B. EPIDEMIOLOGICAL APPROACHES TO SAMPLING

To provide scientifically and statistically valid results specimens must be appropriate for the intended purpose for the proposed investigation, and adequate in quality, volume, and number. The range of purposes for which investigations supported by laboratory testing may be conducted have been outlined in Section A above.

For the purpose of laboratory testing to establish a diagnosis it is important to sample animals that are either clinically affected, or suspected on good evidence to be infected or, for serology, to have been infected. Specimens that are most likely to give highest sensitivity and specificity to the investigation should be collected. In general, the stage of infection, as well as the route and duration of shedding will determine the appropriate animal(s), stage of clinical disease, timeline for sampling, and tissue or anatomical site for sampling. These criteria will be addressed through an understanding of the pathogenesis of the disease for known conditions and on an hypothesis of the pathogenesis of the diseases for conditions of unknown aetiology.

To detect evidence of infection in line with the other five purposes of testing as listed in Section A above the sampling should be done within the context of a surveillance programme. The criteria for the design and implementation of effective surveillance are described in Chapter 1.4 *Animal health surveillance* of the *Terrestrial Animal Health Code* (*Terrestrial Code*). Identification of animals for sampling in surveillance programmes may be targeted (risk-based) or random. Risk-based sampling based on epidemiological knowledge of the infection under study or on epidemiological observations of the population under study is intended to result in the most likely detection of infected individuals.

Inferences on the status of a population such as estimation of prevalence, immune status or disease freedom should be based on random sampling. Random sampling ensures that the animals sampled are representative of the population, within the practical constraints imposed by different environments and production systems. Additionally, random sampling allows extrapolation of the findings of the study to the overall population (with an appropriate confidence interval). The epidemiological principles for sample size estimation are addressed in Appendix 1.1.2.1.

The specific requirements for surveillance to demonstrate freedom from disease/infection, and the associated sampling requirements, are addressed in detail in Article 1.4.6 of Chapter 1.4 of the *Terrestrial Code*.

Sampling and laboratory testing may also be used in support of epidemiologically based diagnostics and studies such as case control, structured longitudinal, and cohort studies (Fosgate & Cohen, 2008; Mann, 2003; Pfeiffer, 2010). The number and selection of the animals to be sampled and the nature of the specimens will be part of the study design.

C. INFORMATION TO BE SENT WITH SPECIMENS

Individual specimens must be clearly identified using appropriate methods. Marking instruments should be able to withstand the condition of use, i.e. being wet or frozen. Use of an indelible marking pen is required. Pencil may rub off containers. Labels attached to plastic may fall off when stored at –70°C.

Information regarding the location and contact information of the submitter and the premises sampled, the case information and associated epidemiological information, as detailed below, should always accompany the specimens to the laboratory. Such documentation should be placed in a plastic envelope on the outside of the shipping container so as to be available for reference during transport and should also be duplicated inside the shipping container between the secondary and the outer packaging (see also Chapter 1.1.3 *Transport of biological materials*). It would be advisable to contact the receiving laboratory to obtain an appropriate submission form and other relevant shipping and handling information.

Necessary information includes:

1. Location and contact information

i) Name and address of owner/occupier of the animal owner and/or the sampled premises and the geolocation (latitude and longitude, if available) where disease occurred, with appropriate contact information (telephone number, e-mail address).

ii) Name, postal and e-mail address, telephone and fax numbers of the sender.

2. Case information

i) Disease agents suspected and tests requested.

ii) Species, breed, sex, age and identity of the animals sampled, and trackability number when available.

iii) Date samples were collected and submitted.

iv) List and type of samples submitted with transport media used.

v) Case history:

   a) The clinical signs and their duration including the temperature of sick animals, condition of mouth, eyes and feet, and milk or egg production data as relevant.

   b) A list and description of the animals examined and the findings of the ante- and post-mortem examinations.

   c) The length of time sick animals have been on the premise; if they are recent arrivals, from where did they originate.

   d) The date of the first cases and of subsequent cases or losses, with, for tracking, any appropriate previous submission reference numbers.

3. Epidemiological information

i) A description of the spread of infection in the herd or flock.

ii) The number of animals on the premise by species, the number of animals dead, the number showing clinical signs, and their age, sex and breed.

iii) The type and standard of husbandry, including biosecurity measures and other relevant factors potentially associated with the occurrence of cases.

iv) History of foreign travel by owner or of introduction of animals from other countries or regions.

v) Any medication given to the animals, and when given.

vi) Vaccination history describing the type of vaccines used and dates of application.

vii) Other observations about the disease, husbandry practices and other disease conditions present.

D. RECEIPT, STORAGE AND ARCHIVES OF LABORATORY SUBMISSIONS

1. Reception of samples

Receiving, unpacking and aliquoting specimens must be done in a way to avoid cross-contamination in order to guarantee reliable testing of samples and prevent exposure of personnel.

A risk assessment (RA) as outlined in chapter 1.1.4 should be performed before systems for handling biological agents and toxins are established in order to define the appropriate biosafety and laboratory biosecurity measures. The RA should lead to the development of stated policy and procedures for the operation of the whole process of receiving submissions to the laboratory.

Submissions delivered to the laboratory should be received in accordance with specified standard operating procedures by staff who are appropriately trained, and when possible are made aware of potential arrivals so that parcels are treated correctly upon reception. To enable appropriate specimen tracking the following information should be logged: a) the time of arrival, b) the sender, c) the person receiving the samples, and d) the shipper with the tracking number. Where a specified chain of custody is required for the purposes of legal action or investigation the consignment should remain unopened and secured in a cool, dry place away from direct sunlight until authorised laboratory personnel are notified and available to receive the package and continue chain of custody. Laboratories should have a written operating procedure detailing the requirements to meet national legal requirements for such submissions.

1.1. Specimen reception area

Specimen reception areas should be equipped to facilitate the safe handling and processing of diagnostic submissions to avoid contamination of the work area, the personnel, cross-contamination among specimens and to allow easy disinfection in situations where specimen containers may have leaked. The specimen reception room should be clean with adequate bench space for organising submissions and paperwork. Depending on the number of submissions expected and depending on the RA the specimen reception area may be either a dedicated part of the diagnostic laboratory or a separate space outside the diagnostic laboratory.

The receiving room should contain an area dedicated to the unpacking of the specimens, with easily cleanable surfaces and trays and/or a biosafety cabinet, depending on the RA. There should be adequate and appropriate space to store specimens, either refrigerators or freezers, taking into consideration the time the specimens are to be stored. Specimen registration equipment such as computers, printers or logbooks should be available. A bar code system can be used to identify and track the specimens.

1.2. Submission unpacking, registration and preparation for further processing

Consignments should remain unopened until transferred to the specimen reception area for further processing. The submission should be unpacked and opened according to defined standard operation procedures. Surface decontamination should be considered to avoid cross-contamination and be part of the designated procedures arising from the RA.

Information to be recorded at specimen log-in includes the delivery source, the date the submission was consigned, the condition of the outer package, and the condition of inner packages (noting the presence of leaks or breakage), the condition of the specimen material, the inner package temperature, and any specific requests from the sender.

Further activities may include labelling of specimen containers, transfer of specimens to the laboratory and storage of specimens.

Packaging material should be disposed of appropriately according to national regulations which may include autoclave destruction of all packaging materials, depending on the RA.

Personal protective equipment (PPE) should be provided to protect the personnel. Minimal PPE is a laboratory coat and gloves. Depending on the relevant RA respiratory protection or effective splash protection (e.g. protective glasses) may be required as well.

After it has been determined that the submission contains the appropriate paperwork matching the specimens and that the specimens are in good condition appropriately trained laboratory personnel become responsible for transfer to the appropriate laboratory area, including maintenance of the chain of custody of the specimens as required. Only properly contained registered (identified) specimens should be transferred into the diagnostic laboratory. It is good practice, and at times a requirement for biosafety and laboratory biosecurity dependent on the RA, to enclose the submitted specimen containers in a secondary container to transfer the specimens safely within the laboratory.

1.3. Emergencies

A comprehensive RA will identify credible and foreseeable emergency scenarios, and be the basis for preparing a response plan. In particular, leaky samples represent a biohazard for the laboratory personnel and could contaminate the environment and other samples. Written instructions on how to deal with broken or leaky tubes should be available in the sample reception area. Personnel should be trained and regular emergency exercises and simulations should take place.

Samples that are degraded or in a condition unacceptable for testing should be decontaminated and appropriately disposed of according to the laboratory's response plan as noted in the prior paragraph. Contaminated laboratory surfaces should be decontaminated with the appropriate disinfectant. Sample rejection and discrepancies between the sample and accompanying paperwork should be resolved by contacting the sender immediately to resend a duplicate sample or to clarify paperwork.

2. Storage and archives

Collections of well characterised specimens including infectious agents, infected tissues and fluids, as well as negative control tissues and fluids, are critical for future research and development efforts, for retrospective studies, epidemiological investigations, and for providing critical reference materials used in assay standardisation, validation, and proficiency testing programmes. In addition, specimens being investigated for legal purposes should be banked.

Materials routinely needed as reference standards and for assay validation are described in Chapter 1.1.5 *Quality management in veterinary testing laboratories*. The materials maintained in laboratory archives should be representative of the agents handled and the types of samples used in the different testing methods performed, which would variously include fresh and fixed tissues and fluids, paraffin-embedded tissues, and stabilised or otherwise preserved cultures. The World Federation for Culture Collections (WFCC: *www.wfcc.info/collections*) is a useful source of information and reference documentation for developing, maintaining, and sharing culture collections, and has published a comprehensive guide for establishing and operating microbial culture collections (WFCC, 2010). It is part of the remit of WOAH Reference Laboratories to supply reference materials.

The principal components of any laboratory archive include the appropriate means of stabilisation and storage, a complete system of documentation and inventory of the material stored, and implementation of biosafety and laboratory biosecurity measures needed to manage the collection.

2.1. Stabilisation and storage

The method of preserving tissues, fluids, and cultures will depend on their anticipated use(s). Samples stored for periodic access such as assay reference materials should be aliquoted to avoid potential problems associated with repeated retrieval and return to storage. They may be stored separately from specimens or samples stored for historical, long-term preservation. Storage conditions should be managed to maintain viability, biochemical, and immunological properties of the samples to the

maximum extent possible. Considerations for preserving the integrity of the samples must include protection from desiccation (e.g. as can happen in certain freezers), frequent or extreme temperature fluctuations, UV degradation, humidity, contamination, and the potential for loss of identification and associated archive documentation. Unique or valuable isolates and materials should be stabilised and stored using at least two different procedures and storage locations.

Storage at ultra-low temperatures (e.g. liquid nitrogen, cryopreservation in freezers at –140°C or lower) is considered the optimum method for long-term storage of biological materials. Storage at low-freezer temperatures of –80°C and –20°C is common for periods that may range from months to 5–10 years. Ultra-low freezing may not be a practical choice as it is expensive to maintain, but cost must be balanced with the fact that biological degradation of the sample over time is an increasing risk at warmer freezer temperatures. Reference materials that are to be accessed with any regularity should be stored in appropriately-sized aliquots to allow access while minimising the number of times the "master stock" is handled. Repeated freezing and thawing of samples should be avoided as it can denature antigens, result in loss of viability of fastidious agents, and can precipitate the over-growth of contaminants or unwanted microorganisms in the sample.

Methods for stabilisation and storage of samples at room temperature range from commercially available technologies that largely target nucleic acid stabilisation, lyophilisation processes, to the relatively low-tech versions of drying fluids on filter paper disks or storage of biological samples in the presence of desiccating agents such as silica gel or grains of rice to absorb moisture.

Considerations such as speed at which a sample is frozen or chemically preserved, size and density of the material to be preserved, storage container and media, and also protocols for reconstitution, thawing, and reviving agents will vary with different tissues and agents. Whether the plan is to store samples frozen or at ambient temperature, for most tissues and groups of infectious agents there are specific preservatives, stabilisation protocols, and storage conditions that are considered optimal; the current published literature should be consulted.

2.2. Documentation and Inventory

Agents and tissues maintained in an archive must be correctly identified and sufficient supporting data that characterises the sample or agent must be recorded. For reference materials, further documentation that authenticates the agent or tissue is required. The unique identity of the tissue, fluid, or agent and the storage location are best maintained in an electronic or paper inventory record which also documents the date the material was obtained, date and method of preservation, volume of material stored, source of the material including associated species, geographical location, and the clinical history of the donor animal and the disease situation of the flock or herd. Additional information is extremely useful and generally includes the original method of isolation/recovery, characterisation (e.g. available data on biochemical properties, antibody or antigen titre, and genetic sequence) as well as additional history on handling of the material (e.g. number of passages for infectious agents and cell lines, dates the archived material was frozen-thawed or rehydrated and the dates it was transferred to different storage conditions or containers). Inventories are most often organised by assigning a unique identifying number or alphanumeric code to each sample (sample container) that is cross-referenced to a database or inventory log. Inventory records can be manual data logs, computerised spreadsheets, or specialised computer programs. However the records are managed, they must be kept current and the information entered must be traceable to its source. The identification of the individual making an entry or modification to the sample inventory should be recorded.

2.3. Biosafety and laboratory biosecurity

As a first step in establishing an archive, a laboratory biorisk assessment addressing biosafety and laboratory biosecurity issues, including any control or mitigation measures to be implemented, must be completed. As further defined in chapter 1.1.4, an appropriate risk assessment for archived samples should address the technical competency needed of staff handling the tissues, fluids, and agents, with particular emphasis on those materials that are potentially infectious or toxic to workers, animals, and the environment in and around the laboratory. The laboratory should consider all biorisk management measures needed to protect the integrity of the sample, as well as the health of the workers and environment, from the time the original sample is received through the long-term storage and ultimate use or destruction of the material(s).

The appropriate level of laboratory biosecurity, including controlled access to the archived samples and inventory records, is an important consideration for laboratories maintaining biological inventories and archives. The laboratory should also have a back-up plan for the transfer or destruction of potentially dangerous archived materials in the event of power failures or other compromises to the storage environment. National and international regulations and legislation, including requirements for permits and licenses to receive, maintain, work with, and distribute specific agents and tissues must be followed for all laboratory archives. Current regulatory information in regards to receiving and storage of biological materials can be found on the European Biological Resource Centre Network website (www.ebrcn.net/), in the WFCC guidelines (2010) and from relevant national government agencies.

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NB: FIRST ADOPTED IN 2008 AS COLLECTION AND SHIPMENT OF DIAGNOSTIC SPECIMENS.
MOST RECENT UPDATES ADOPTED IN 2013.

APPENDIX 1.1.2.1.

EPIDEMIOLOGICAL APPROACHES TO SAMPLING: SAMPLE SIZE CALCULATIONS

The type and number of samples needed depends on the desired purpose. Sample size calculation for each of the main purposes of testing, where random sampling has been used, may be approached as follows:

1. Demonstrate freedom from infection in a defined population (country/zone/ compartment/herd where the prevalence is apparently zero)

Frequently, the objective of sampling is to determine if a disease is present or absent in a population at a specific threshold (design prevalence). These sampling methods are needed to perform the scientifically based surveys specified in the WOAH *Terrestrial Animal Health Code* in order to determine freedom with and without vaccination as well as to re-establish freedom after outbreaks.

It is possible to calculate how many animals should be sampled from a herd/flock of a certain size, to achieve a 95% probability of detecting infection or previous exposure assumed to be present in a certain percentage of the animals. The following formula can be applied:

$$n \cong \frac{(1-(1-CL)^{1/D})(N-{}^{1}/_{2}\,(SeD-1))}{Se}$$

Where
n: is the required sample size
CL: is the confidence level (generally 0.95)
N: is the population size
D: is the number of diseased animals expected in the population
Se: is the diagnostic sensitivity of the test used

For example, to determine the sample size required to detect with 95% confidence at least one infected animal in a herd of 500 animals at a design prevalence of 10%, the formula above would be used as follows (assuming perfect diagnostic sensitivity):

$$n \cong (1-(1-0.95)^{1/50})(500-{}^{1}/_{2}\,(50-1)) \cong 28$$

If the laboratory results are negative for all samples the epidemiologist can conclude, with 95% confidence, that the prevalence is lower than 10%. If the disease in question is highly infectious and it is unlikely that only 10% of the animals would be infected, the herd could be considered free. If, however, one or more samples are positive the epidemiologist may conclude, with 95% confidence, that the disease prevalence is at least 10%.

2. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes

The *Terrestrial Code* provides specific recommendations for trade purposes. Some are based on demonstration of disease freedom at a herd or flock level and others on testing of individual animals for export. When certification of a disease free herd or flock is recommended, the approach described in point 1 above can be followed to calculate the number of samples required.

In the case of testing individual animals, it is generally expected that all animals will be tested. The critical question in this case is related to the negative predictive value (NPV) of the test and the probability of having at least one false negative individual in a group. The negative predictive value of a test is defined as the probability that an animal

is not infected given that it tested negative. The NPV is a function of the diagnostic sensitivity and specificity of the test(s) used and the prevalence of the infection in the population where the animals come from. In general, the probability of having at least one false negative in a group is calculated as:

$$P(x \geq 1) = 1 - (1 - NPV)^n$$

The negative predictive value is calculated as follows:

$$NPV = \frac{TN}{TN + FN} = \frac{(1-p)Sp}{(1-p)Sp + p(1-Se)}$$

Where
TN: is the true negative
FN: is the false negative
Se: is the diagnostic sensitivity
Sp: is the diagnostic specificity
p: is the prevalence
n: is the number of animals in the group

Additional information on quantifying these types of probabilities can be found in the WOAH *Handbook on Import Risk Analysis for Animals and Animal Products, Quantitative Risk Analysis*.

3. Eradication of disease or elimination of infection from defined populations

The objective of surveillance in the event of an outbreak is to try to find any remaining pockets of infection. Sampling should be directed at populations having higher risk of exposure and where the agent is most likely to be found, such as animals exhibiting clinical signs or suspected to have been in contact with infected animals. If animals within a selected herd or flock are not exhibiting clinical signs, a representative sample, based on the formula presented under point 1 above for presence or absence of disease, should be collected.

4. Confirmatory diagnosis of suspect or clinical cases (includes confirmation of positive screening test)

Suspect or clinical cases with a positive screening test result should be retested with a confirmatory test. Usually, a test with high diagnostic sensitivity is used for screening purposes and one with high diagnostic specificity for confirmation. If the status of the herd or flock is of interest, animals exhibiting clinical signs compatible with the disease of interest should be sampled. This will increase the probability of confirming the infection. If required, the formula for presence or absence in point 1 above can be used to calculate the number of samples required. Given that sample collection is directed to animals exhibiting clinical signs, the design prevalence used can be relatively high, yielding a lower sample size.

5. Estimate prevalence of infection

Disease control programmes may need to periodically assess the impact of control measures. One of the key indicators of success is a reduction in the prevalence of the disease. To determine the prevalence of disease within a group of animals, the following formula can be used to determine the number of samples required.

$$n = \frac{Z^2 pq}{L^2}$$

Where
n: is the required simple size
Z: is the value of the Z distribution for the desired confidence level (usually 95%)
p: is the expected prevalence in the population
q: is 1-p
L: is the level of precision (or acceptable error)

There are no fixed rules to determine the level of precision (sometimes called also margin of error), the choice of it is left to the epidemiologist conducting the survey. However, finer levels of precision require larger sample sizes.

The corresponding value of the Z distribution for 95% confidence is 1.96. To determine the prevalence of a disease with 95% confidence in a herd of 500 animals with an expected disease prevalence of 20%, at a level of precision of ±3%, the required sample size can be calculated:

$$n = \frac{1.96^2 \times 0.2 \times 0.8}{0.03^2} = \frac{0.6146}{0.0009} \cong 683$$

Note that in this case the required sample size is larger than the population, so the sample size will need to be adjusted to take account the population size (N):

$$n_{adj} = \frac{1}{1/n + 1/N} = \frac{1}{1/683 + 1/500} \cong 289$$

Therefore, 289 animals would have to be randomly sampled.

6. Determine immune status of individual animals or populations (post-vaccination).

Disease control programmes often rely on vaccination as a tool, in such cases it is important to assess immunity coverage and not merely count the number of animals or herds that have been vaccinated. The proportion of animals that need to be immunised to stop the spread of disease in a population is a function of the number of secondary infections arising from a single infected case (the reproductive number, R_0). For many infectious diseases the proportion needing immunity in order to control, disease spread is around 80%. Two approaches can be applied. If the objective is finding the proportion of immune animals, the formula for determining prevalence in point 5 above, can be applied. If, however, programme managers want to assess if herds have an immunity level at or above a certain threshold, the formula for presence or absence, in point 1 above, should be used. The difference in sample size varies greatly depending on the objective.

If the immune status of a herd of 500 animals that have all been vaccinated wants to be estimated, the following approaches can be followed.

6.1. Estimating the proportion of immune animals in a group

- Expected prevalence (of immune animals) 80%
- Precision, for this example assume 3%
- Confidence level 95%

$$n = \frac{1.96^2 \times 0.8 \times 0.2}{0.03^2} = \frac{0.6146}{0.0009} \cong 683$$

$$n_{adj} = \frac{1}{1/n + 1/N} = \frac{1}{1/683 + 1/500} \cong 289$$

6.2. Estimating immunity at a defined threshold

- Expected prevalence (of immune animals) 80%, i.e. 400 out of 500 animals
- Confidence level 95%
- Perfect diagnostic sensitivity

$$n \cong (1 - (1 - 0.95)^{1/400})(500 - 1/2\,(400 - 1)) \cong 3$$

If at least one of the three samples is positive to the test, the interpretation is, with 95% confidence, that the proportion of immune animals in the herd is at least 80%. If none of the samples test positive, the herd cannot be considered adequately immunised. Such an approach can be used to determine geographical locations or types of production systems where immune coverage is low and might need to be re-vaccinated.

On-line resources

Open Epi: http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm

Free Calc: http://epitools.ausvet.com.au/content.php?page=FreeCalc2

Win Episcope: http://www.winepi.net/

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CHAPTER 1.1.3.

TRANSPORT OF BIOLOGICAL MATERIALS

INTRODUCTION

The transport of biological materials, including infectious substances, is covered by international, regional or national regulations that are updated on a regular basis and are widely accessible via the internet, or through commercial and regulatory transportation affiliates. The transport of biological materials within a country and between countries will be explained in this chapter.

The international regulations for the transport of infectious substances by any mode of transport are based upon the Recommendations on the Transport of Dangerous Goods made by the Subcommittee of Experts on the Transport of Dangerous Goods (UN SCETDG), a subcommittee of the United Nations Economic and Social Council. The Recommendations are presented in the form of Model Regulations covering air, rail, road, sea and also include international mail. The World Health Organization (WHO) guidance document on "Transport of Infectious Substances" summarising the different transport regulations is regularly updated. Countries, other international organisations, international treaties and conventions such as the International Air Transport Association (IATA), the World Customs Organization (WCO), the Convention on International Trade in Endangered Species (CITES), and the Convention on Biodiversity (CBD), especially the Nagoya Protocol, provide additional guidance and regulations that should be considered in planning the transportation of biological materials.

In the interest of animal and human health, biological materials collected from animals must be transported safely, efficiently and legally from the place where they are collected to the place where they are analysed, studied or used. The collection of specimens from animals is covered in Chapter 1.1.2 Collection, submission and storage of diagnostic specimens*.*

For the purpose of this chapter, animals are defined as all members of the Kingdom Animalia except humans, and biological materials include specimens or samples from animals, cell cultures, zoonotic and animal microorganisms and genetically modified or synthetic organisms, and biological products such as vaccines and reagents.

A. RESPONSIBILITIES

All personnel involved in the packaging, labelling and shipping of biological materials must be appropriately trained, certified, competent and knowledgeable of the relevant national, regional and international regulations.

Biological materials should be transported to ensure a rapid and reliable system for delivery to the recipient using individuals such as professional logistics service providers that are trained and competent in the shipping and transportation process.

The efficient transport and transfer of biological materials requires co-ordination between the sender (shipper, consignor), the logistic providers, the carrier and the recipient (consignee) to ensure safe transport and arrival on time and in proper condition.

The sender (shipper, consignor) is responsible for providing the applicable documentation (e.g. certifications, permits) required by the national authorities of the countries of export, transhipment and import as well as ensuring that the shipment also complies with all other applicable regulations, such as:

i) Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity (CBD): Biological material containing genetic

resources as defined under the CBD may be subject to Access and Benefit-Sharing legislation in both the country where it is sourced and the country where it is sent.

ii) CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora): All import, export, re-export and introduction from the sea of species covered by the Convention has to be authorised through a licensing system. Resolution Conf. 12.3. (Rev.CoP17) on Permits and Certificates, contains a section XII, regarding the use of simplified procedures to issue permits and certificates (https://cites.org/sites/default/files/document/E-Res-12-03-R17.pdf).

Procedures for incidents such as spills or theft of materials during transportation and any other realistic and foreseeable emergencies should be part of a risk management system in order to respond adequately to emergencies (for basic principles see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

1. The sender (shipper, consignor)

i) Before any shipment of biological materials, the sender must be able to:

   a) Identify and classify, pack (including temperature control), ensure quantity limits, mark and label the package of biological materials,

   b) Ensure the correct documentation of all biological materials intended for transport,

   c) Complete and produce a Shipper's Declaration for Dangerous Goods (DGD), when required,

   d) Ensure biological materials are not forbidden for transport;

ii) Prepares necessary documentation, including permits, dispatch and shipping documents if necessary;

iii) Notifies the recipient of transportation arrangements once these have been made, well in advance of the expected arrival time;

iv) The air way bill (AWB) is the standard shipping document for shipping goods by air. While it is common practice for the air carrier or freight forwarder to complete the air waybill, the sender may be required to provide it;

v) Makes advance arrangements with the recipient including investigating the need for import/export permits;

vi) Makes advance arrangements with the carrier to ensure:

   a) that the shipment will be accepted for appropriate transport;

   b) that the shipment is undertaken by the most direct routing, as appropriate.

2. The carrier/courier

i) The following measures must be taken by the carrier:

   a) Routing: appropriate routing must be ensured, such as by the shortest or most secure route.

   b) Transhipment: when transfers are necessary, precautions must be taken to assure special care, expeditious handling and monitoring of the substances in transit for both safety and security purposes.

ii) For air transport, the carrier is required by the regulations to use, when applicable, an acceptance checklist to verify that the shipment complies with:

   a) marking and labelling requirements; and

   b) documentation requirements.

iii) Provide advice to the sender and assistance regarding the necessary shipping documents and instructions for their completion as well as correct packaging

iv) Assists the sender in arranging the most appropriate routing and then confirms the routing and provides, if possible, ways to track the shipment;

v) Maintains and archives documentation for shipment and transport.

3. The recipient (consignee)

i) Obtains the necessary authorisation(s) from national authorities for the importation of the material;

ii) Provides the sender with the required import permit(s), letter(s) of authorisation, or other document(s) required by the national authorities;

iii) Arranges for the most timely and efficient collection on arrival;

iv) Should acknowledge receipt to the sender.

Shipments should not be dispatched until all the necessary arrangements between the sender, carrier and recipient have been made.

The transportation chain involves many more stakeholders with specific roles and responsibilities. These are explained in more details in the framework of aviation security in a joint ICAO (International Civil Aviation Organization) and WCO (World Customs Organization) brochure that can be accessed using the following link: http://www.wcoomd.org/en/topics/facilitation/instrument-and-tools/tools/~/media/4B167884A3064E78BCF5D29E29F4E57E.ashx.

In addition, Material Transfer Agreements (MTA) should be considered because they:

i) Protect the interests of all involved parties in relation to;
   a) Intellectual property
   b) Potential alternative uses
   c) Commercial aspects
   d) Liability to third parties
   e) Potential further transfers/uses

ii) Help to avoid misunderstandings around the use of materials

iii) Clarify ownership of property

With reference to Article 4 of the "Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (ABS) to the Convention on Biological Diversity", the MTA is intended to establish a platform for the open exchange of materials among laboratories and to constitute Prior Informed Consent (PIC) on Mutually Agreeable Terms, while avoiding as much as possible the potential adverse impacts of the Nagoya requirements.

Appendix 3 is provided as a generic template for use in transfer of materials. It is intended to be modified and adapted as required to meet individual circumstances.

Appendix 3 does not constitute legal advice and users are responsible to ensure it meets their objectives and fulfils requirements of local legislation. Users are encouraged to consult their own legal professionals for advice on use of this template.

B. CLASSIFICATION AND CATEGORISATION

When transporting biological materials, the sender must determine whether the material should be classified as dangerous goods or not. Dangerous goods (hazardous materials, HAZMAT) are materials that can harm humans, animals and other living organisms, property, or the environment, and their transport is regulated by United Nations (UN) regulations[1]. Dangerous goods are assigned a *UN number* and *proper shipping name* based on the classification of the dangerous goods. The transport regulations assign a packing instruction against the UN number and proper shipping name, to specify the packaging/packing method to ensure that the dangerous goods do not pose a hazard in transport. Of the biological materials that are discussed in this Chapter, infectious substances are classified as dangerous goods and are assigned to UN 2814, UN 2900, UN 3373, or UN 3291, as

1 http://www.unece.org/trans/danger/publi/unrec/rev13/13nature_e.html; http://www.unece.org/trans/danger/publi/unrec/rev20/20files_e.html

appropriate. In addition, Genetically Modified Microorganisms (GMMOs) and Genetically Modified Organisms (GMOs) are classified as Class 9 and assigned to UN 3245 if they are not classified as Category A or Category B.

Table 1. Summary of classification, categorisation, identification and packaging of infectious substances

Dangerous goods classifications	Categorisation	Proper shipping name[2]	UN number[2]	Packing instruction/ packaging requirements
Class 6, Division 6.2	Category A	Infectious substance, affecting humans	UN 2814	P620
		Infectious substance, affecting animals	UN 2900	
Class 6, Division 6.2	Category B	Biological substance, Category B	UN 3373	P650
Class 6, Division 6.2	Exempt human/animal specimens	Exempt human/animal specimens	N/A	Triple packaging
not subject to dangerous goods regulations	Biological materials not subject to dangerous goods regulations	N/A	N/A	N/A
Class 9	GMMOs and GMOs that are not classified as Category A or B infectious substances	Genetically modified microorganisms; Genetically modified organisms	UN 3245	P904 (ICAO/IATA PI 959), IBC99

If it is likely that microorganisms that are present in the biological materials can cause harm to humans or animals then they must be assigned either to Category A or B.

The proper shipping name (see Table 1) must be supplemented with the technical name (scientific name of the pathogen) in parenthesis on the transport document, but not on the outer packaging. When the identity of the infectious substances to be transported are unknown, but are suspected of meeting the criteria for inclusion in category A, the words “suspected category A infectious substance” must be shown, in parenthesis, following the proper shipping name on the transport document.

1. Category A

A Category A substance is an infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals. Assignment to UN 2814 or UN 2900 (see Table 1) must be based on the known medical history of the animal(s), signs and individual circumstances of the specimen source, and endemic local disease conditions, or professional judgement concerning individual circumstances of the source, human or animal.

Some organisms are considered Category A only when in culture form (e.g. *Bacillus anthracis*, foot and mouth disease virus). Indicative examples of substances that meet these criteria are given in the Table 3. The table is not exhaustive. Infectious substances, including new or emerging pathogens, which do not appear in the Table but which meet the same criteria must be assigned to Category A. In addition, if there is doubt as to whether or not a substance meets the criteria it must be assigned to Category A.

Some infectious substances may have a high economic or trade impact on specific countries should there be release to the environment. Therefore, other infectious substances may be added to the list by individual countries (e.g. cultures of Newcastle disease virus where the virus is exotic to the country or region).

2 Dangerous goods are assigned UN numbers and proper shipping names according to their hazard classification and condition under the Dangerous Goods Regulations. See the Dangerous Goods List at pages 191–304 of the UN Model Regulations http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol1.pdf

Medical or clinical waste containing Category A infectious substances shall be assigned to UN 2814 or UN 2900 as appropriate. **Solid** medical waste containing Category A infectious substances generated from the medical treatment of humans or veterinary treatment of animals may be assigned to UN 3549. It should be noted that Medical or clinical waste from bio-research or liquid waste must not be assigned to UN3549.

2. Category B

Biological materials containing pathogens which do not meet the criteria for Category A (i.e. do not cause life-threatening disease to humans or animals) shall be assigned to Category B (UN 3373).

Typically a specimen with a high likelihood to contain pathogenic organisms shipped for disease diagnosis (e.g. confirmatory diagnosis of suspected or clinical cases, specimens for differential diagnosis, such as blood samples for classical swine fever or sheep pox diagnostics or throat samples from chickens for avian influenza) can be assigned to Category B.

It is important to note that unlike cultures, *patient specimens* which may contain infectious microorganisms listed as 'cultures only' in Table 3 (Category A infectious substances) do not require Category A transport practices. For these specimens Category B transport practice should be applied. In this case, although directly collected specimens (e.g. serum) can be shipped as Category B, pure cultures of the same pathogens must follow the requirements of Category A due to the characteristics of the specific organism. Some examples are classical swine fever virus isolates or sheep pox virus isolates (see Table 3). Specimens from animals intentionally infected with Category A pathogens must be sent as Category A, even if they are assigned to Category A (cultures only).

Shipments of cultures of non-category A agents can be assigned to Category B.

Medical or clinical waste containing Category B infectious substances shall be assigned to UN 3291.

3. Exempt specimens

Animal specimens for which there is minimal likelihood that pathogens are present can be transported as Exempt Specimens. Examples of specimens in the veterinary field which may be transported as exempt include specimens from surveillance studies, export controls of healthy animals (e.g. certification of freedom from classical swine fever) or determination of immune status of individual animals or populations (post-vaccination).

These specimens are not subject to dangerous goods regulations if the specimen is transported in a packaging that will prevent any leakage and that is marked appropriately (triple packaging principle, see item C and Figure 3 of Appendix 1.1.3.2).

4. Biological materials not subject to Dangerous Goods Regulations

Based on the known medical history of the animal(s), signs and individual circumstances of the source of the biological materials, and endemic local disease conditions, the following *are not subject to dangerous goods regulations, unless they meet the criteria for inclusion in another class* (*such as Class 9*):

i) biological materials that do not contain infectious substances

ii) biological materials containing microorganisms that are non-pathogenic to humans or animals;

iii) biological materials in a form in which any pathogens present have been neutralised or inactivated such that they no longer pose a health risk;

iv) Environmental specimens (including food and water specimens) that are not considered to pose a significant risk of infection;

v) Dried blood spots, collected by applying a drop of blood onto absorbent material.

Note: There may be specific regulations in place in some countries for the shipment, export or import of nucleic acids.

5. Contaminated items

These listed below are also included in infectious substances in the international regulations on transport of dangerous goods, however the details are not discussed in this chapter. For more information see UN Model Regulations, paragraphs 2.6.3.2.3.3 and .9 respectively.

Table 2. Summary of classification, categorisation, identification and packaging of contaminated items with infectious substances

Dangerous goods classifications	Categorisation	Proper shipping name[3]	UN number[3]	Package
Class 6, Division 6.2	Category A	Medical* devices or equipment contaminated with or containing infectious substances in Category A	UN2814, UN2900 as appropriate	Must be marked "Used Medical Device" or "Used Medical Equipment"
Class 6, Division 6.2	Exemption when condition is met	Medical* devices, medical equipment	N/A	See UN Model Regulations 2.6.3.2.3.9 and IATA Dangerous Goods Regulations (DGR) 3.6.2.2.3.9
Class 6, Division 6.2	Category A	Medical* waste, Category A, affecting humans, **solid**; Medical waste, Category A, affecting animals only, **solid**	UN 3549	P622, LP622
Class 6, Division 6.2	Category B	Clinical waste, Unspecified, n.o.s.(not otherwise specified); (Bio) medical waste, n.o.s.; Regulated medical waste, n.o.s.	UN3291	P621 (PI622), IBC620, LP621

*including veterinary use

6. Infectious substances included in Category A

Table 3. Indicative examples of infectious substances included in Category A

UN number and proper shipping name	Microorganism
UN 2814 Infectious substance, affecting humans	*Bacillus anthracis* (cultures only)
	Brucella abortus (cultures only)
	Brucella melitensis (cultures only)
	Brucella suis (cultures only)
	Burkholderia mallei – glanders (cultures only)
	Burkholderia pseudomallei (cultures only)
	Chlamydia psittaci – avian strains (cultures only)

3 Dangerous goods are assigned UN numbers and proper shipping names according to their hazard classification and condition under the Dangerous Goods Regulations. See the Dangerous Goods List at pages 191–304 of the UN Model Regulations http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol1.pdf

UN number and proper shipping name	Microorganism
	Clostridium botulinum (cultures only)
	Coccidioides immitis (cultures only)
	Coxiella burnetii (cultures only)
	Crimean–Congo haemorrhagic fever virus
	Dengue virus (cultures only)
	Eastern equine encephalomyelitis virus (cultures only)
	Escherichia coli, verotoxigenic (cultures only)[4]
	Ebola virus
	Flexal virus
	Francisella tularensis (cultures only)
	Guanarito virus
	Hantaan virus
	Hantaviruses causing haemorrhagic fever with renal syndrome
	Hendra virus
	Hepatitis B virus (cultures only)
	Herpes B virus (cultures only)
	Human immunodeficiency virus (cultures only)
	Highly pathogenic avian influenza virus (cultures only)
	Japanese encephalitis virus (cultures only)
	Junin virus
	Kyasanur Forest disease virus
	Lassa virus
	Machupo virus
	Marburg virus
	Monkeypox virus
	Mycobacterium tuberculosis (cultures only)[1]
	Nipah virus
	Omsk haemorrhagic fever virus
	Poliovirus (cultures only)
	Rabies virus (cultures only)
	Rickettsia prowazekii (cultures only)
	Rickettsia rickettsii (cultures only)
	Rift Valley fever virus (cultures only)
	Russian spring–summer encephalitis virus (cultures only)
	Sabia virus

4 For surface transport (ADR) nevertheless, when the cultures are intended for diagnostic or clinical purposes, they may be classified as infectious substances of Category B.

UN number and proper shipping name	Microorganism
	Shigella dysenteriae type 1 (cultures only)
	Tick-borne encephalitis virus (cultures only)
	Variola virus
	Venezuelan equine encephalitis virus (cultures only)
	West Nile virus (cultures only)
	Yellow fever virus (cultures only)
	Yersinia pestis (cultures only)
UN 2900 Infectious substance, affecting animals only	African swine fever virus (cultures only)
	Avian paramyxovirus Type 1 – Velogenic Newcastle disease virus (cultures only)
	Classical swine fever virus (cultures only)
	Foot and mouth disease virus (cultures only)
	Lumpy skin disease virus (cultures only)
	Mycoplasma mycoides – contagious bovine pleuropneumonia (cultures only)
	Peste des petits ruminants virus (cultures only)
	Rinderpest virus (cultures only[5])
	Sheep-pox virus (cultures only)
	Goatpox virus (cultures only)
	Swine vesicular disease virus (cultures only)
	Vesicular stomatitis virus (cultures only)

C. PACKAGING

1. Principles

All biological materials should be packaged and transported in accordance with local, national and international regulations. The procedures should minimise the risk of exposure for those engaged in transportation and should protect the environment and susceptible animal populations from potential exposures. Additionally, ineffective packaging that does not protect specimens or preservatives (e.g. ice) from damage or prevent leakage will likely delay the delivery of the shipment to the laboratory, delaying or preventing critical laboratory analyses from being performed. Biological materials should always be packaged and transported to protect the integrity of the specimens, as well as to avoid cross-contaminating other specimens and environmental contamination. Minimum requirements for the transport of specimens follow the principle of triple packaging, consisting of three layers as described below:

i) a primary receptacle;

ii) a secondary packaging;

iii) an outer packaging;

of which either the secondary or the outer packaging must be rigid.

5 Subject to prior approval by the FAO-WOAH rinderpest secretariat

1.1. Primary receptacle

A primary receptacle, leak-proof for liquids or sift-proof for solids containing the specimen. Primary receptacle(s) must be packed into the secondary packaging with enough absorbent material (e.g. cellulose wadding, paper towels, house hold paper, cotton balls) to absorb all fluid in case of breakage. Even though the regulations do not prohibit glass, primary receptacles should preferably be non-breakable. In addition, they must not contain any sharps (e.g. vacutainer with needle), particularly when using soft secondary or outer containers. If screw cap vials are used, they shall be secured by e.g. tape. A flip-top vial must not be used.

1.2. Secondary packaging

A second durable, leak-proof packaging to enclose and protect the primary receptacle(s) (e.g. sealed plastic bag, plastic container, screw-cap can).

The primary receptacle or the secondary packaging shall be capable of withstanding, without leakage, an internal pressure of 95 kPa (0.95 bar) in the range of –40°C to +55°C (–40°F to +130°F).

1.3. Outer packaging

Secondary packaging is placed in outer shipping packaging (e.g. sturdy insulated fibre board box) with suitable cushioning material. Outer packaging protects the contents from outside influences, such as physical damage, while in transit.

2. Category A

Due to the highly hazardous nature of the Category A samples the packaging must meet special requirements. The principle of triple packaging applies here, and the transport containers and outer packaging must meet the criteria defined in the relevant regulations. Category A must only be transported in packaging that meets the United Nations class 6.2 specifications, complies with Packing Instruction P620 and have passed specific tests and with UN specification marking as P620. This ensures that strict performance criteria are met; tests for compliance with these criteria include a 9-metre (29.5 feet) drop test, a puncture test, a pressure test and a stacking test. The packages are labelled to provide information about the contents of the package, the nature of the hazard and the packaging standards applied.

Marking and labelling is as follows (see Fig. 3 of Appendix 1.1.3.2):

i) *The delivery address* (*consignee*) *and sender's details* (*shipper*), *as well as 24/7 emergency contact details including named persons with telephone numbers to guarantee safe delivery.*

ii) *The proper shipping name and the UN number.*

Proper shipping name	*UN number*
INFECTIOUS SUBSTANCE, AFFECTING HUMANS	*UN2814*
INFECTIOUS SUBSTANCE, AFFECTING ANIMALS only	*UN2900*

iii) *The Infectious Substance label* (*Figure 1*).

NB: This label is only for Category A. This label must not be used when shipping Category B.

Fig. 1. Infectious Substance label for the transport of Category A.

iv) *UN specification marking for P620 packaging (printed on the box).*

v) *Orientation label, Cargo only label, if required (depending on the Net Weight [kg] of the infectious substance in a P620 box).*

The exact details can be found in P620 Packing Instruction[6].

For air transport:

i) The primary receptacle or secondary packaging must be capable of withstanding, without leakage, an internal pressure of 95 kPa. The primary receptacle or secondary packaging must also be capable of withstanding temperatures in the range of –40°C to +55°C;

ii) For liquids: the net quantity of infectious substances per one P620 box shall not exceed 50 ml for transport in cargo space of a passenger aircraft; and must not contain more than 4 litres (contain multiple primary receptacles totalling more than 4 litres) for transport on a cargo only aircraft;

iii) For solids: the net quantity of infectious substances per one P620 box shall not exceed 50 g for transport in cargo space of a passenger aircraft, and must not contain more than 4 kg (even if containing multiple primary receptacles totalling more than 4 kg) for transport on a cargo only aircraft. This quantity limit doesn't apply for animal parts, organs and whole carcasses.

iv) The three triple packaging principle has to be adopted accordingly using appropriate packaging systems;

v) The entire package must have been tested and complies with Packing Instruction P620.

For further information on marking and labelling of the Category A shipment package, see P620 Packing Instruction for UN Nos 2814 and 2900[6]

3. Category B

Category B must be transported in a packaging that complies with the requirements of packing instruction P650. The approval of the box by the government is not required, thus UN specification marking is not required.

6 Page 81 at: http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol2.pdf

Marking is as follows:

i) Packages should be clearly labelled with the delivery address and sender's details to guarantee safe delivery in time at the correct destination.

ii) Label with the *proper shipping name* in letters at least 6 mm high: "BIOLOGICAL SUBSTANCE, CATEGORY B" (Figure 2)

iii) In addition to the proper shipping name, the mark shown below (UN3373 in diamond) is used for shipments of Category B substances. The UN3373 mark must always be visible on the outer packaging.

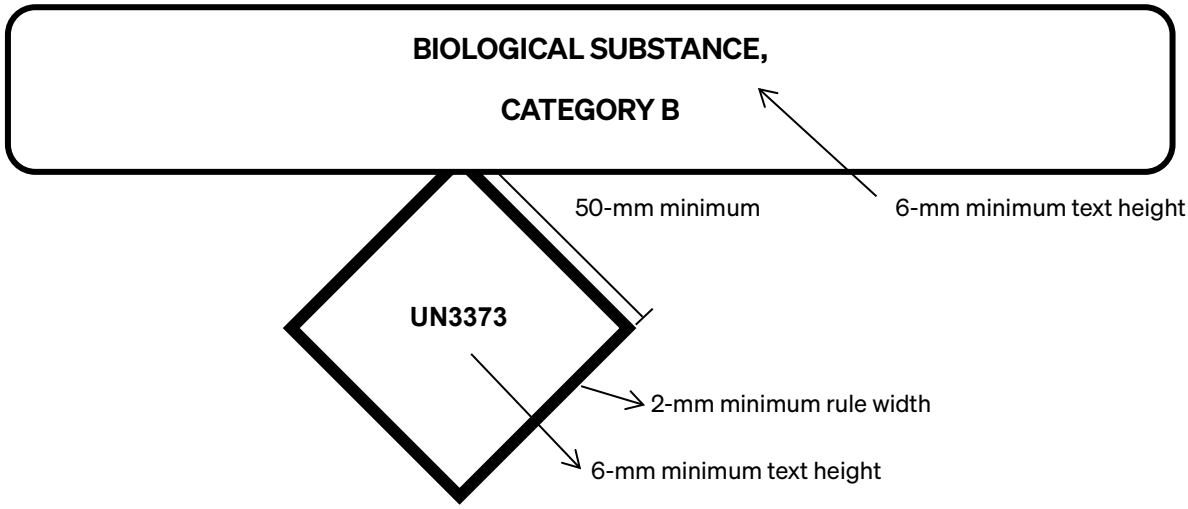


Fig. 2. UN3373 mark for the transport of Category B substances.

Additional requirements do apply as for category A for international shipment and air transport. One of the main differences between P650 and P620 is the reduced drop-test to 1.2 meters (4 feet).

For air transport:

i) The primary receptacle or secondary packaging must be capable of withstanding, without leakage, an internal pressure of 95 kPa. The primary receptacle or secondary packaging must also be capable of withstanding temperatures in the range of –40°C to +55°C;

ii) For liquids: no primary receptacle shall exceed 1 litre and the outer packaging must not contain more than 4 litres (contain multiple primary receptacles totalling more than 4 litres);

iii) For solids: the outer packaging must not contain more than 4 kg. This restriction doesn't apply for animal parts, organs and whole carcasses.

The exact details can be found in P650 Packing Instruction for UN No. 3373[7].

4. Exempt specimens

Biological materials for which there is a minimal likelihood that pathogens are present are not subject to regulation if the specimen is carried in a packaging which will prevent any leakage and which is marked with the words "Exempt animal specimens", as appropriate. The triple packaging system must still be applied.

5. Biological materials not subject to Dangerous Goods Regulations

This exemption refers to biological materials that do not contain infectious substances and are therefore not subject to dangerous goods regulations (such as class 6.2) and any packaging requirements*, unless they meet the criteria for inclusion in another class* (*such as class 9*).

7 Page 82 at: http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol2.pdf

Note: There may be specific regulations in place in some countries for the shipment, export or import of nucleic acids.

6. Overpack

"Overpack" is the term used when one or more packages are combined to form one unit and sent to the same destination by a single shipper. When refrigerants are used to protect contents, the overpacks may comprise insulated vessels or flasks. Whenever an overpack is used, the required marks and labels shown on the outer packaging must be repeated on the outermost layer of the overpack, except for the UN specification marking on P620. This requirement applies to all infectious substances including Categories A and B. Overpacks are also required to be marked with the word "overpack".

Combining different categories of infectious substance in a same overpack is permissible however in this case outer labelling should indicate the highest category included in the package.

7. Cold chain transportation

Refrigerants may be used to stabilise specimens during transport.

Ice, ice packs or dry ice shall be placed outside the secondary receptacle. Wet ice shall be placed in a leak-proof container; the outer packaging or overpack shall also be leak-proof.

Dry ice (solid carbon dioxide) must not be placed inside the primary or secondary receptacle because of the risk of explosion. A specially designed insulated packaging may be used to contain dry ice, typically a polystyrene or waxed-treated cardboard box to prevent leakage and maintain temperature. The packaging must permit the release of carbon dioxide gas if dry ice is used and the package (the outer packaging or the overpack) shall be marked "UN 1845" and "Carbon dioxide, solid as coolant" or "Dry ice as coolant" and the weight of the dry ice in Kilograms should also be indicated on the labelling. The package must also bear the Class 9 – Miscellaneous hazard label.

The secondary receptacle shall be secured within the outer package to maintain the original orientation of the inner packages after the refrigerant has melted or dissipated.

If liquid nitrogen is used as a refrigerant, additional requirements have to be followed according to the relevant regulations for dangerous goods (Division 2.2, UN 1977). Information on Dry Shipper is available in p19 of WHO Guidance on regulations for the transport of infectious substances 2017–2018[8].

D. ADDITIONAL CONSIDERATIONS

In addition to the transport regulations described above, other international agreements might be applicable.

1. CITES

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an international agreement between governments with the aim to ensure that international trade in specimens of wild animals and plants does not threaten their survival. The Convention is in effect in 183 Parties (including one economic integration organisation, the European Union).

Some specimens to be transported from one country to another may be derived from species covered by CITES (roughly 5,600 animal species and 30,000 plant species). Depending on the classification of the species in one of the three Appendices of the Convention and the movement involved, a CITES export permit, both an export and import permit, or re-export certificates may be required. The appropriate documents must be obtained from National CITES Management Authorities. Simplified procedures for the issuance of permits and certificates exist to facilitate and expedite trade in biological specimens from CITES-listed species.

8 http://www.who.int/ihr/publications/WHO-WHE-CPI-2017.8/en/

There may be some variation from one country to another in their CITES trade requirements (some countries take stricter domestic measures and some countries have added additional species requirements for permits in addition to the CITES lists), therefore It is always advisable to check the national legislation that applies.

Further information on CITES: https://cites.org

2. Nagoya protocol and access and benefit-sharing

"Fair and equitable sharing of benefits arising from the utilisation of genetic resources" (Access and Benefit Sharing, or ABS) is one of the three objectives of the Convention on Biodiveristy (CBD). The CBD confirms that States have sovereign rights over their genetic resources, including animals, plants, fungi and microorganisms. Consequently States may choose to regulate access to these, requiring researchers both within and outside their borders to seek permission. Permission (*Prior Informed Consent* or PIC) will be the responsibility of the State and maybe other stakeholders, and the conditions for sharing benefits (*Mutually Agreed Terms* or MAT) may be agreed with a range of actors, including laboratories.

In 2014 the *Nagoya Protocol on Access and Benefit Sharing* (NP) was agreed by the Parties of the CBD. Nagoya Protocol requirements have been implemented in a large number of countries. This obliges the Parties to take compliance measures and monitor researchers and others utilising genetic resources (accessed from other Parties) within their jurisdiction. Biological material containing genetic resources as defined under the CBD may be subject to national Access and Benefit-Sharing (ABS) legislation in both the country where it is sourced and the country where it is sent. This may include compliance with the NP if both countries are Party to the NP. To discover whether this is the case, consult the ABS Clearing House (https://absch.cbd.int/), and ask the National Focal Point of both countries (contact details on ABS Clearing House). If the source country has ABS legislation it may be necessary to seek a permit (Prior Informed Consent and Mutually Agreed Terms) prior to international transport of the material.

Benefits may be monetary or non-monetary. "Utilisation of genetic resources" is defined by the CBD as "means to conduct research and development of the genetic and/or biochemical composition of genetic resources, including through the application of biotechnology as defined in Article 2 of the Convention". Consequently many activities carried out by WOAH Members and their constituents may be classified as ABS.

Compliance will require researchers to produce documentary evidence that genetic resources were accessed with appropriate PIC and MAT, and declaring the type of utilisation being undertaken. This information will be transmitted to the provider country to check if the information accords with their records. There is no exemption for organisms of veterinary importance from ABS provisions in the NP, but countries where they are being accessed may choose to make this distinction and exemption.

Article 8 of the Nagoya Protocol states "*In the development and implementation of its access and benefit-sharing legislation or regulatory requirements, each Party shall: … (b) Pay due regard to cases of present or imminent emergencies that threaten or damage human, animal or plant health, as determined nationally or internationally. Parties may take into consideration the need for expeditious access to genetic resources and expeditious fair and equitable sharing of benefits arising out of the use of such genetic resources, including access to affordable treatments by those in need, especially in developing countries*;". However, this is not required and counties may choose to take no action. In addition, there is a 90-day grace period allowed to complete required documents after transport of the biological material.

If the research or diagnostic work on biological material of veterinary concern is considered 'utilisation' by the country (Party) in which it takes place the researcher will be required to provide information, including:

i) The Internationally Recognised Certificate of Compliance (number) from the ABS Clearing House, if available; or
   a) ABS Permit reference
   b) Evidence of Prior Informed Consent
   c) Evidence of Mutually Agreed Terms
   d) The entity to whom PIC and MAT was granted

ii) The Provider Country

iii) Date and place of access

iv) Description of the Genetic Resources

This information should be included in the documents with the transported biological material.

Although this documentation is only required if both providing country and recipient country are Party to the Nagoya Protocol the provider may have Access and Benefit-Sharing legislation even if not a Party. This should be respected and any documentation required, including permits, Prior Informed Consent and Mutually Agreed Terms, acquired and dispatched with the material.

Further information can be found on:

i) The ABS Clearing House - https://absch.cbd.int/

ii) The CBD Website - https://www.cbd.int/abs/default.shtml

E. REFERENCES AND FURTHER READING

WHO Guidance on regulations for the "Transport of Infectious Substances" 2017–2018, covering transport regulations on national and international and air transport by different means:
http://www.who.int/ihr/publications/WHO-WHE-CPI-2017.8/en/

Swiss Expert Committee on Biosafety: "Transport, import and export of substances consisting of or containing pathogenic or genetically modified (micro)organisms"; practical explanation on how to transport biological substances according to the specific dangerous goods transport regulations:

http://www.efbs.admin.ch/en/transport/index.html

IATA: http://www.iata.org/whatwedo/cargo/dgr/Documents/infectious-substance-classification-DGR56-en.pdf

F. ADDITIONAL INFORMATION ON THE UNITED NATIONS SYSTEM FOR THE TRANSPORT OF DANGEROUS GOODS

The United Nations dangerous goods web site provides comprehensive detail concerning the United Nations Recommendations on the Transport of Dangerous Goods. It also provides links to the modal agencies:
http://www.unece.org/trans/danger/danger.html

The site below provides the full text of the United Nations Recommendations on the Transport of Dangerous Goods – Model Regulations, which can be downloaded in PDF format. Readers wishing to see the text relating to the transport of infectious substances should download Part 2, Part 4 and Part 5 of the Recommendations:
http://www.unece.org/trans/danger/publi/unrec/rev20/20files_e.html

The site below provides the full text of the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) of 2017, which entered into force on 1 January 2017, which can be downloaded in PDF format. Readers wishing to study the text relating to the transport of infectious substances should download Part 2 (2.2.62), Part 4 (search P620, P650) and Part 5:
http://www.unece.org/trans/danger/publi/adr/adr2017/17contentse0.html and
http://www.unece.org/trans/danger/publi/adn/adn2017/17files_e0.html

Contracting parties to the various conventions for the transport of dangerous goods can be found on a number of web sites:

Air ICAO: http://www.icao.int/Pages/default.aspx and https://www.icao.int/safety/DangerousGoods/Pages/StateVariationPage.aspx

IATA: http://www.iata.org/whatwedo/cargo/dgr/Documents/infectious-substance-classification-DGR56-en.pdf

Rail RID (Intergovernmental Organisation for International Carriage by Rail): http://www.otif.org/. RID is primarily for the countries of Europe, North Africa and the Middle East. There are a number of

	countries (mainly Eastern Europe and Asia that apply RID through the Organization for Cooperation of Railways (OSJD); details of RID membership can be found at http://www.otif.org/en
Road	ADR: http://www.unece.org/trans/danger/publi/adr/country-info_e.htm (lists competent authorities)
Sea	IMO (International Maritime Organization): http://www.imo.org
Post	UPU (Universal Postal Union): http://www.upu.int/

NB: FIRST ADOPTED IN 1992 AS SAMPLING METHODS.
MOST RECENT UPDATES ADOPTED IN 2018.

APPENDIX 1.1.3.1.

DEFINITIONS

The following definitions are for the purposes of this chapter only. For general definitions please see the Glossary.

- **Biological products**

Biological products are those products derived from living organisms which are manufactured and distributed in accordance with the requirements of appropriate national authorities, which may have special licensing requirements, and are used either for prevention, treatment, or diagnosis of disease in humans or animals, or for development, experimental or investigational purposes related thereto. They include, but are not limited to, finished or unfinished products such as vaccines.

- **Cultures**

Cultures are the result of a process by which pathogens are intentionally propagated. This definition does not include human or animal patient specimens as defined below.

- **Infectious substances**

For the purposes of transport, infectious substances are defined as substances which are known or are reasonably expected to contain pathogens. Pathogens are defined as microorganisms (including bacteria, viruses, parasites, fungi) and other agents such as prions, which can cause disease in humans or animals. Infectious substances are further classified according to risk into two categories.

- **Genetically modified microorganisms (GMMOs) and organisms (GMOs)**

Genetically modified microorganisms not meeting the definition of infectious substance are classified in Class 9 (Miscellaneous dangerous substances and articles, including environmentally hazardous substances). GMMOs and GMOs are not subject to dangerous goods regulations when authorised for use by the competent authorities of the countries of origin, transit and destination. Genetically modified live animals shall be transported under terms and conditions of the competent authorities of the countries of origin and destination. DNA, RNA or plasmids are not considered as GMMO and not subject to dangerous goods regulations.

- **Medical or clinical wastes**

Medical or clinical wastes are wastes derived from the veterinary treatment of animals, the medical treatment of humans or from bio-research.

- **Patient specimens**

Patient specimens are those, collected directly from humans or animals, including, but not limited to, excreta, secreta, blood and its components, tissue and tissue fluid swabs, and body parts being transported for purposes such as research, diagnosis, investigational activities, disease treatment and prevention.

*
* *

APPENDIX 1.1.3.2.

EXAMPLE OF THE TRIPLE PACKAGING SYSTEM (IATA RECOMMENDATIONS) FOR THE PACKING AND LABELLING OF DIFFERENT TYPES OF BIOLOGICAL MATERIALS

Fig. 3. Example of triple packaging system for the packaging and labelling of Category A, UN2814 and UN2900 infectious substances (Figure kindly provided by IATA, Montreal, Canada).

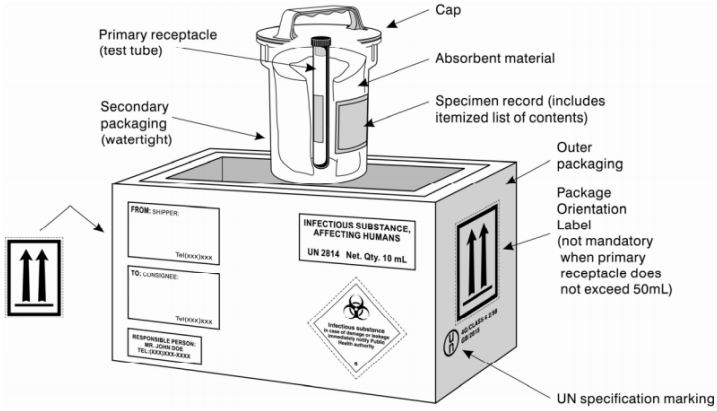


Fig. 4. Example of the triple packaging system for the packing and labelling of Category B, UN3373 infectious substances (Figure kindly provided by IATA, Montreal, Canada).

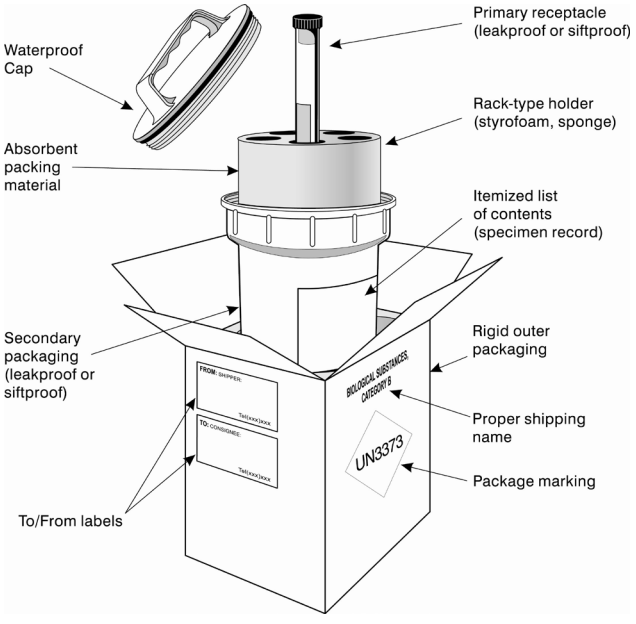


Fig. 5. Example of the triple packaging system for the packing and labelling of Category B, UN3373 infectious substances with non-rigid leakproof secondary packaging (Figure kindly provided by IATA, Montreal, Canada).

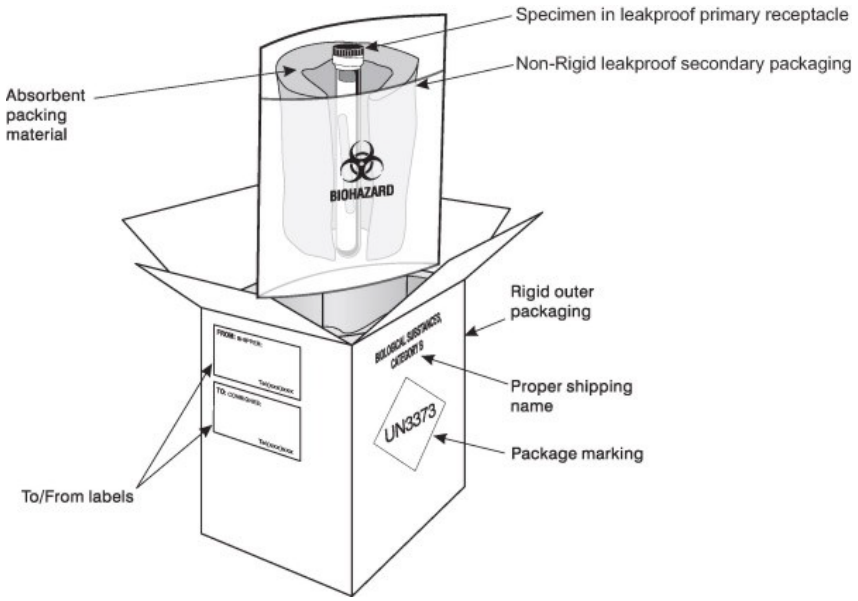
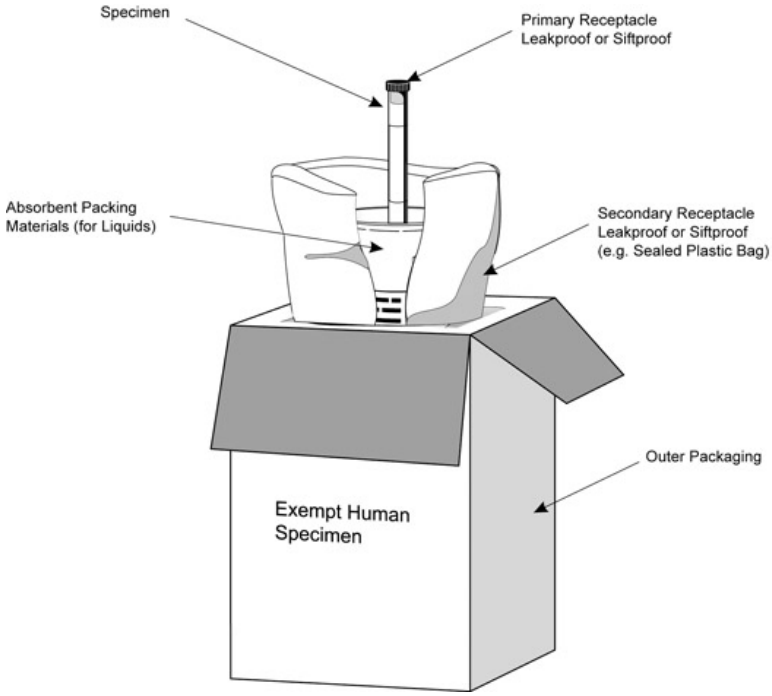


Fig. 6. Example of the triple packaging system for the packing and labelling of Exempt specimen (Figure kindly provided by IATA, Montreal, Canada).



APPENDIX 1.1.3.3.

MATERIAL TRANSFER AGREEMENT

MATERIAL TRANSFER AGREEMENT

BETWEEN

PROVIDER

Organisation:
Address:
Country:

PROVIDER SCIENTIST

Title and name:
Organisation:
Address:

AND

RECIPIENT

Organisation:
Address:
Country:

RECIPIENT SCIENTIST

Title and name:
Organisation:
Address:

ORIGINAL MATERIAL

Description of the material being transferred

SHIPPING ADDRESS

Title and name:
Address:

I. OBJECTIVE AND PURPOSE

[Insert a short statement about the objectives and intended purpose of the agreement and background to the parties to the agreement.]

II. DEFINITIONS

Provider
Organisation providing the *original material*. The name and address of this party will be specified in the first page of this MTA.

Provider scientist
The name and address of this party will be specified in an implementing letter.

Recipient
Organisation receiving the *original material*. The name and address of this party will be specified in an implementing letter.

Recipient scientist
The name and address of this party will be specified in an implementing letter.

Original material
The description of the material being transferred will be specified in an implementing letter.

Material
Original material, *progeny*, and *unmodified derivatives*. The *material* shall not include: (a) *modifications*, or (b) other substances created by the *recipient* through the use of the *material* which are not *modifications*, *progeny*, or *unmodified derivatives*.

Progeny
Unmodified descendant from the *material*, such as virus from virus, cell from cell, or organism from organism.

Unmodified derivatives
Substances created by the *recipient* which constitute an unmodified functional subunit or product expressed by the *original material*. Some examples include: subclones of unmodified cell lines, purified or fractionated subsets of the *original material*, proteins expressed by DNA/RNA supplied by the *provider*, or monoclonal antibodies secreted by a hybridoma cell line.

Modifications
Substances created by the *recipient* which contain/incorporate the *material*.

Commercial purposes
The sale, lease, license, or other transfer of the *material* or *modifications* to a for-profit organisation. *Commercial purposes* shall also include uses of the *material* or *modifications* by any organisation, including *recipient*, to perform contract research, to screen compound libraries, to produce or manufacture products for general sale, or to conduct research activities that result in any sale, lease, license, or transfer of the *material* or *modifications* to a for-profit organisation. However, industrially sponsored academic research shall not be considered a use of the *material* or *modifications* for *commercial purposes* per se, unless any of the above conditions of this definition are met.

Non-profit organisation(s)
A university or other institution of higher education or an organisation exempt from taxation or any nonprofit scientific or educational organisation qualified under a state nonprofit organisation statute. As used herein, the term also includes government agencies.

Agreement
Material Transfer Agreement (MTA)

III. TERMS AND CONDITIONS OF THIS AGREEMENT

Ownership
The *provider* retains ownership of the *material*, including any *material* contained or incorporated in *modifications*.

The *recipient* retains ownership of:

(a) *modifications* (except that, the *provider* retains ownership rights to the *material* included therein);

(b) those substances created through the use of the *material* or *modifications*, but which are not *progeny*, *unmodified derivatives* or *modifications* (i.e., do not contain the *original material*, *progeny*, *unmodified derivatives*). If either 2 (a) or 2 (b) results from the collaborative efforts of the *provider* and the *recipient*, joint ownership may be negotiated.

Non-commercial use
The *recipient* and the *recipient scientist* agree that the *material* is to be used solely for either teaching, non-commercial research or academic research purposes. In this Agreement, non-commercial research purpose and academic research purpose mean that the *material* cannot be used for *commercial purposes*, and the *recipient* may not exploit commercially the results, inventions, discoveries or know-how which incorporates the *materials* for its own benefit nor for a third party, without the consent of the *provider*.

Ownership of the results, inventions, discoveries or know-how generated by the *recipient* using the *material* shall rest with the *recipient*. Nevertheless, any results, inventions, discoveries or know-how which contain or incorporate the *material*, generated by the *recipient* using the *material* ("*modifications*") shall be jointly owned by the *provider* and the *recipient*. However, both the *provider* and the *recipient* agree that should the *recipient* having completed work under this MTA wish to use the *material* or *modifications* for commercial purposes it will be necessary for the *recipient* to negotiate the terms of a license Agreement with the *provider*, No right are given, implied or intended by this Agreement or the material transfer other than those explicitly stated in this Agreement.

Distribution to third parties
This *material* should be considered a property of the *provider*. The *recipient* therefore agrees to retain control over this material, and further agrees not to transfer the *material* to third parties or to personnel of the *recipient* not working under the supervision of the *recipient scientist*. The *recipient* agrees to refer to the *provider* any request for the *material* from anyone other than those persons working under the *recipient scientist*'s direct supervision. The *provider* reserves the right to distribute the *material* to others and to use it for its own purposes.

The *recipient* shall have the right, without restrictions, to distribute substances created by the *recipient* through the use of the *original material* only if those substances are not *progeny*, *unmodified derivatives*, or *modifications*.

Under a separate agreement at least as protective of the *provider*'s rights, the *recipient* may distribute *modifications* to *non-profit organisation(s)* for non-commercial research purposes and academic research purposes only, subject to prior written notice to the *provider*.

Confidentiality
The *recipient* agrees to treat the *materials* as it would treat its own confidential and proprietary information and at least no less than a reasonable degree of care, and to take all reasonable precautions to prevent unauthorised disclosure to any third party of the *material* which it receives hereunder. The *provider* agrees to keep confidential that the *recipient* is using the *material*.

Publications
This Agreement shall not be interpreted to prevent or delay publication of research findings resulting from the use of the *material* or the *modifications*. The *recipient scientist* agrees to provide appropriate acknowledgement of the source of the *material* in all publications.

Material use liability
The *material* is provided as a service to the research community at large. It is provided without warranty of merchantability or fitness for a particular purpose or any other warranty, express or implied. No indemnification for any damages is intended or provided under this Agreement. Each party should accept liability for their own actions. The parties make no express or implied warranty as to any matter whatsoever, including the conditions of the research or any invention or product, whether tangible or intangible, made, or developed under this Agreement, or the ownership, merchantability, or fitness for a particular purpose of the research or any invention or product. The

parties further make no warranty that the use of any invention or other intellectual property or product contributed, made or developed under this Agreement will not infringe any patent or other intellectual property right. In no event, will any party be liable to any other party for compensatory, punitive, exemplary or consequential damages.

Misuse, dual use and biosafety
The *recipient* accepts full responsibility for the safety of the research and warrants that the research will be performed in accordance with all local or national laws, rules and regulations. In particular, this *material* will only be used for research purposes by the *recipient* in its laboratory under suitable containment conditions.

Under the terms of this Agreement, the *material* may not be used in human beings.

Termination of the Agreement
The term of this Agreement is 2 years as of the effective date of termination, unless an extension is mutually agreed by the *provider* and the *recipient*. At the end of this term, the Agreement shall automatically terminate. Upon the effective date of termination, or if requested, the deferred effective date of termination, the *recipient* will discontinue its use of the *material* and will, upon direction of the *provider*, return or destroy any remaining *material*. The cost of return or destruction will be taken by the *recipient*.

Dispute
Any dispute arising under this Agreement instituted against the *recipient* by the *provider* shall be brought in the court of the *recipient*'s country of domicile. Any claims and proceedings against the *provider* by the *recipient* shall be brought in the courts of the *provider*'s country of domicile.

Modification of the Agreement and signatures
This agreement may not be modified, in whole or in part, except by the written consent of both parties. If any provision of this Agreement may be signed in counterpart, and by the parties hereto or separate counterparts, each of which shall be deemed an original.

This Agreement is effective when signed by all parties. The parties executing it certify that their respective organisations have accepted the terms of this Agreement, and further agree to be bound by the terms, for the transfer specified above.

Recipient responsibility
The Recipient undertakes to use the Material in full compliance with any national and international applicable law, including any disposition and guidelines regarding health and scientific research. In particular, the Material having intrinsic health risk shall be handled in full respect of the specific law and in compliance with all the necessary precautions.

The Recipient represents that within its laboratories:

- Access to the Material, Progeny and Modification will be restricted to personnel capable and qualified to safely handle those substances, using appropriate containment;
- Recipient shall use the utmost precaution to minimise any risk of harm to persons and property and to safeguard them from theft or misuse.

The Recipient also acknowledges that in no event the Material applies directly or indirectly to humans.

The Recipient assumes all liability for any and all third party damages and claims arising out of or relating to this Agreement, including the receipt, use, handling, storage, conservation of the Material. To the extent permitted by applicable law, the Recipient agrees to indemnify, defend and hold harmless the provider against all third party claims, losses, expenses and damages, including reasonable attorney's fees.

The provider shall have no liability towards the Recipient or its employees in the event that the Material and/or Derivatives infringe any intellectual property rights of third parties. The provider makes no warranties for the absence of any third party industrial property rights on the Material.

IV. PAGE OF SIGNATURES

AUTHORISED SIGNATURE OF THE PROVIDER SCIENTIST

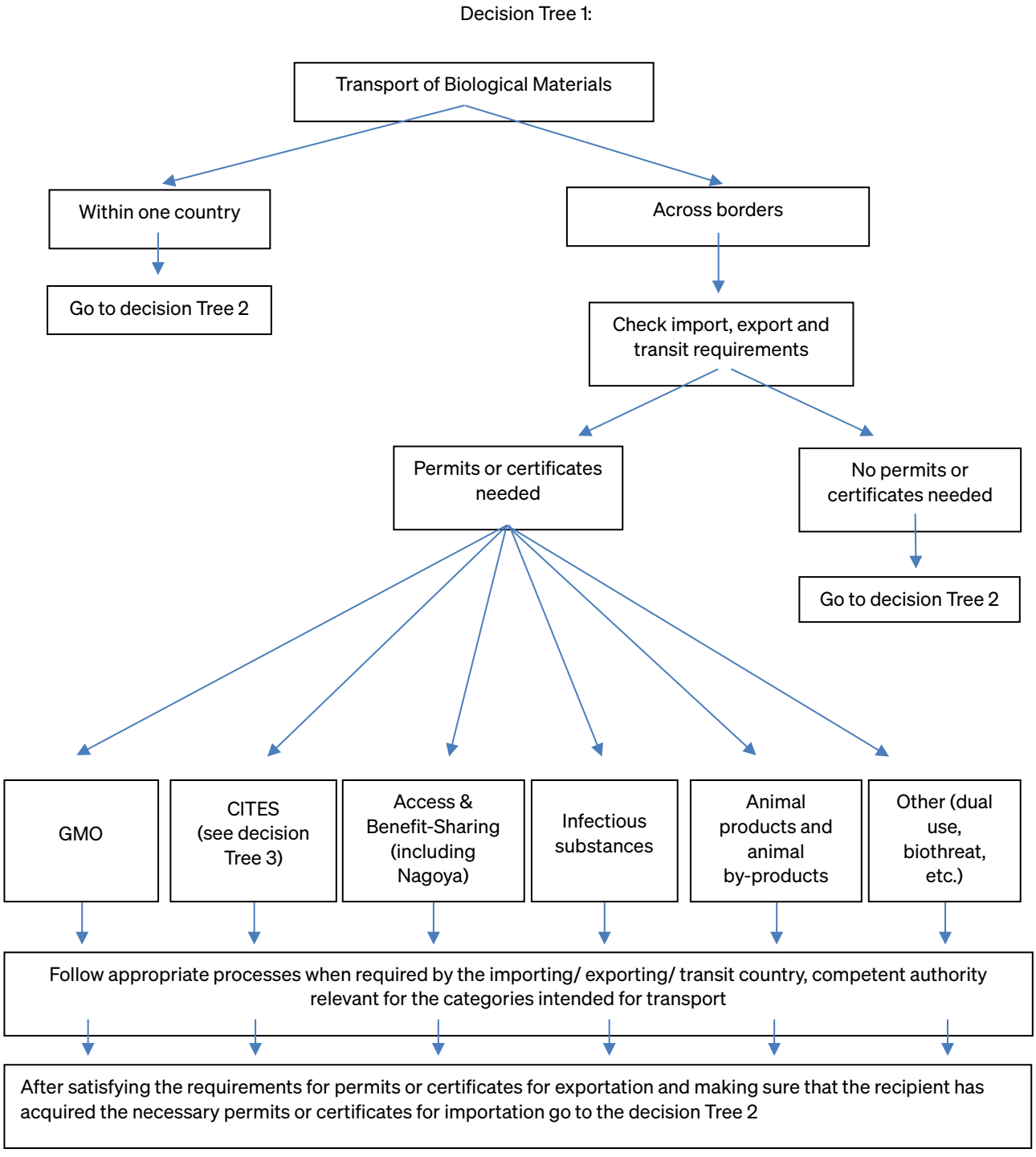
Signature:
Title and print name:
Date:

AUTHORISED SIGNATURE OF THE RECIPIENT SCIENTIST

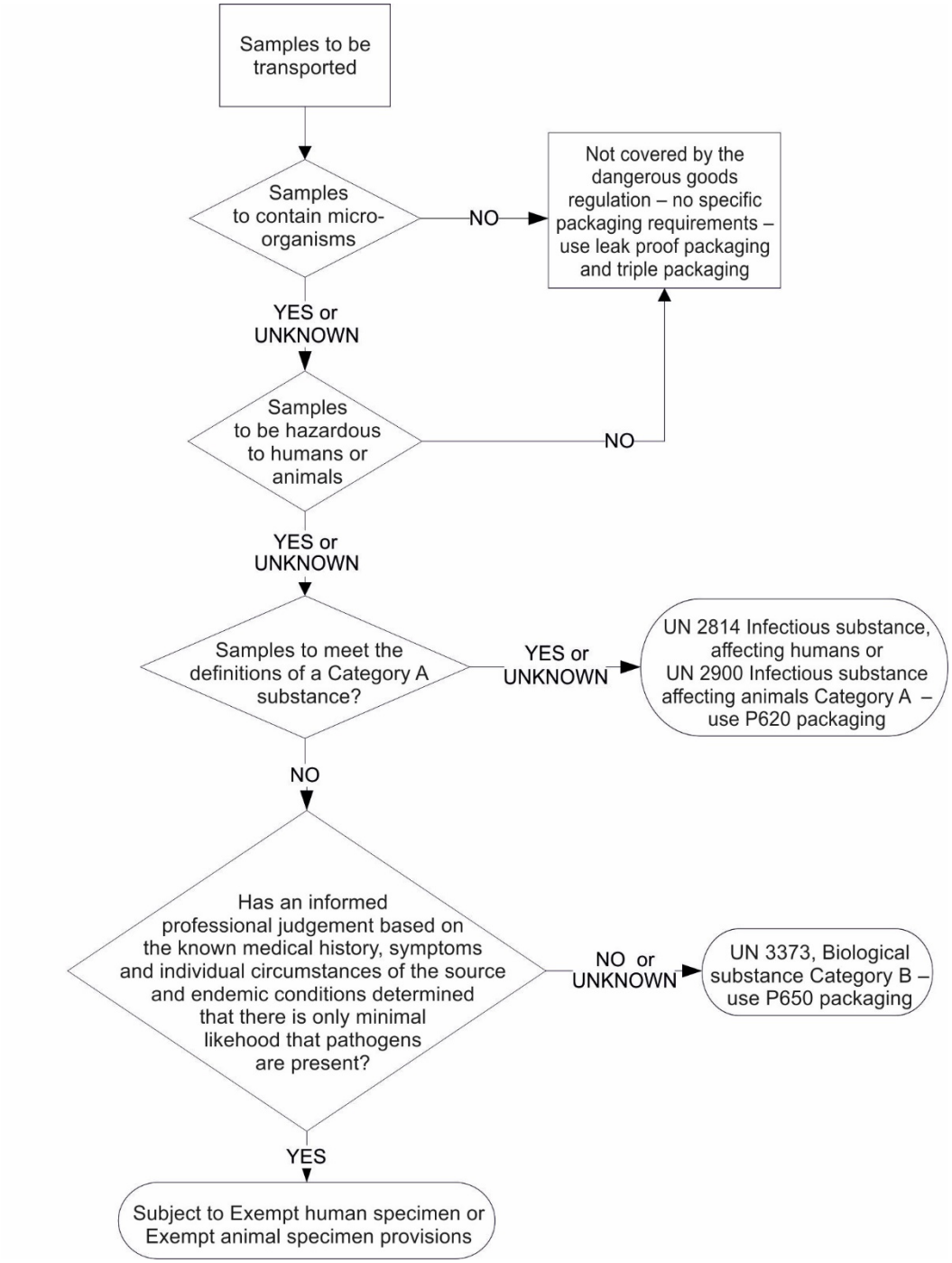
Signature:
Title and print name:
Date:

APPENDIX 1.1.3.4.

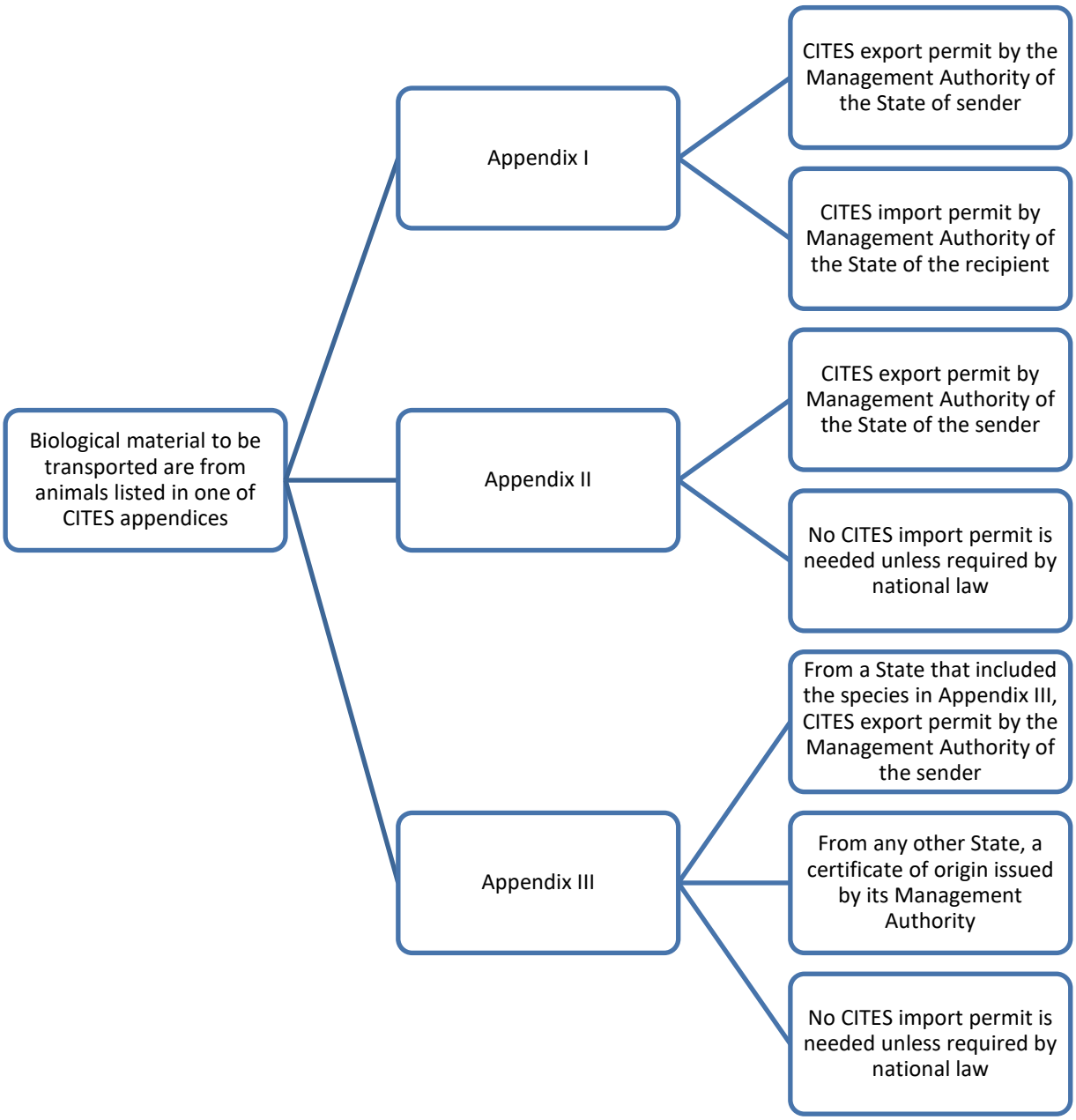
DECISION TREES FOR THE TRANSPORT REQUIREMENTS OF BIOLOGICAL MATERIALS



Decision Tree 2



Decision Tree 3



CHAPTER 1.1.4.

BIOSAFETY AND BIOSECURITY: STANDARD FOR MANAGING BIOLOGICAL RISK IN THE VETERINARY LABORATORY AND ANIMAL FACILITIES

INTRODUCTION

Chapter 1.1.1 Management of veterinary diagnostic laboratories *outlines the overall requirements and responsibilities to be addressed in the management of veterinary laboratories, of which management of the biological risks associated with the operation of a laboratory is an important aspect. This chapter outlines the principles on which the specific management of biological risks associated with veterinary laboratories and experimental animal handling facilities should be based. The terminology is aligned with the WOAH nomenclature for risk analysis, including the four components, namely hazard identification, risk assessment, risk management and risk communication, used in Chapter 2.1* Import Risk Analysis *of both the WOAH* Terrestrial Animal Health Code and *WOAH* Aquatic Animal Health Code*. In this way the process is consistent with and standardised against risk analysis processes already used by WOAH Members.*

Adoption of the risk analysis approach to management of biological risks for biosafety and biosecurity in veterinary laboratories and animal facilities provides Members with a means of tailoring their relevant national animal health policies and procedures regarding their laboratories to their particular circumstances and priorities. The biological risk management approach gives countries a mechanism to protect their human and animal populations from inadvertent or intentional release of or exposure to animal pathogens in an evidence-based, transparent, economically viable and sustainable manner. The approach is applicable in all countries from technologically advanced to in-transition or resource-limited countries.

The risk analysis approach moves towards a comprehensive biological risk management framework that is science-based and specific to an individual country and laboratory's circumstances. The process could accommodate the assigning of pathogens to risk groups relevant to the country and the subsequent restriction of the associated work to laboratory facilities defined by containment levels tailored to the types of risk identified if this suits an individual country's requirements as identified by its biological risk analysis. This chapter and the associated Chapter 2.1.3 Managing biorisk: examples of aligning risk management strategies with assessed biorisks *provide the framework for implementation of the risk management approach.*

Veterinary laboratories and animal facilities routinely handle biological materials that may constitute or contain infectious agents and toxins that may cause adverse animal or public health and economic effects due to uncontrolled release inside or outside the laboratory. Laboratory and animal facilities managers are responsible for providing a management system that ensures safe and secure handling, storage, and transport of these biological materials (a biological risk management system). This is needed not only to protect laboratory workers from inadvertent exposures and infection, but also to protect the local and regional animal populations, human populations, and environment from accidental or intentional release and spread of biological agents and toxins from laboratories. These considerations should also apply to animals and potential arthropod vectors that are handled in veterinary laboratories and animal facilities. The term "biological material" is used throughout this chapter to include all potential sources of biological risk for which laboratory management may be responsible. To classify the potential biological risk posed by the presence and handling of a particular biological material, laboratory managers should apply a systematic and evidence-based approach.

Biological risk analysis is the process of identifying and characterising health, safety, and security risks, followed by implementing, measuring the effectiveness of, and communicating the control measures used to reduce those risks to acceptable levels. Risk analysis has been used effectively by individuals in business and finance, engineering, energy, and health industries to characterise and control inherent risks associated with their business practices. This chapter focuses on biological-related risks, recognising that additional health and safety concerns exist, and should be controlled within the laboratory environment, such as radiation exposures, chemical burns, or liquid nitrogen hazards. A laboratory biological risk management system includes the policies, responsibilities, and operational procedures used to support biological risk analysis and the resulting biosafety and laboratory biosecurity measures implemented to manage laboratory biological risks.

Additional definitions and further explanation of the risk analysis principles and associated laboratory biological risk management system approach presented in this chapter can be found in the WOAH Handbook on Import Risk Analysis for Animals and Animal Products *and in the European Committee for Standardization* (*CEN*) *Workshop Agreement on Laboratory Biorisk Management. Following the overview presented in this chapter, a general guide for performing a risk analysis is included in Appendix 1.1.4.1. The types of biological hazards to be considered, the associated risks and the types of management strategies to be considered are provided in a table in Appendix 1.1.4.2. Chapter 2.1.3 contains worked examples based on hypothetical scenarios of how the checklist can be worked through for specific infectious hazards.*

A. LABORATORY BIOSAFETY AND BIOSECURITY BACKGROUND

As outlined in Chapter 1.1.1 *Management of Veterinary Diagnostic Laboratories*, it is a standard for Members having such facilities that they be managed within the context of a formally stated animal health policy that indicates clearly the purposes for which laboratory services are required. This animal health policy typically includes specific mention of the disease agents for which a diagnostic or research capability is required and allows for the subsequent design and development of a laboratory capability that is fit for purpose. The design of the laboratory capability will guide decisions regarding the use or avoidance of particular direct and indirect laboratory test methods that may involve the handling, propagation, and storage of particular infectious agents or toxins in the laboratory. This process should be expected to result in a list of the biological materials, including each specific infectious agent that will be held by the laboratory.

Biological risk assessments are undertaken to inform and determine the policy and procedures that in turn give confidence that the laboratory procedures for each of the biological materials handled by the laboratory pose negligible danger to a country's animal and human populations. The assessments of biological risk are usually taken at a national or jurisdictional level and may lead to national or jurisdictional standards or regulations for biological risk management that are followed by all veterinary laboratories and animal handling facilities in that country or jurisdiction. Agencies and laboratories responsible for biological risk analysis may make use of data, information and guidance available in published technical documents such as specific chapters of the WOAH *Terrestrial Animal Health Code*, the *Aquatic Animal Health Code* and this *Terrestrial Manual* as well as publications from other internationally recognised bodies and organisations.

It is the intention of this chapter to provide countries and laboratories with a process that can be applied when developing standards, policies and procedures appropriate to their particular circumstances. It is additionally a requirement that the process be transparent to other Members that may have a legitimate interest in the effectiveness of the management of laboratory biological risks in the particular country. Although this chapter is applicable to veterinary laboratories and animal handling facilities, it is noted that in the international context, issues that have an impact on public health are also subject to binding international agreement. Consequently, the veterinary biological risk analysis process must deliver outcomes in support of the particular country's obligations regarding zoonotic diseases, such as under the World Health Organization (WHO) International Health Regulations (IHR) (WHO, 2005). For countries in the process of developing their national standard, this chapter provides guidance for identifying and assessing the country's animal health risks and related laboratory management strategies.

Laboratory biosafety describes the principles and practices for the prevention of unintentional release of or accidental exposure to biological agents and toxins. Laboratory biosecurity describes the physical control of biological agents and toxins within laboratories, in order to prevent their loss, theft, misuse, unauthorised access or intentional unauthorised release. These and other terms are defined in the Glossary of this *Terrestrial Manual.*

Laboratory risk assessments are used to identify the specific biosafety and biosecurity measures needed to contain and work safely with specific biological agents and toxins in a laboratory or animal facility. The common practice of linking a biological agent to a specific level of biocontainment arises from the concept of identifying biological agents and toxins as *biohazards* and classifying the individual agents into one of four risk groups based on the potential to cause disease in an individual and in a community. The criteria used in risk group classification schemes, which although similar are not consistent between countries, typically include pathogenicity, mode of transmission, host range, the presence of vectors, existing levels of population immunity, availability of appropriate prophylaxis or treatment, density and movement of the host population, and related environmental factors.

Independent of the biological agent "risk group" classification process, biosafety level designations (alternately termed physical containment levels) were historically developed to characterise laboratories based on a composite of physical design features, facility construction, equipment, operational procedures, and laboratory practices required for working safely with the range of biological agents and toxins that pose varying levels of risk to individuals and to a community. Laboratory facilities have been designated by WHO as: basic – Biosafety Level (BSL) 1 (basic teaching and research); basic – BSL 2 (primary health services, diagnostics, research); containment – BSL 3 (special diagnostics, research); and maximum containment – BSL 4 (dangerous pathogens) (WHO, 2004). The biosafety level classification system has been questioned in that uniform standards and definitions are not used globally, therefore comparison of laboratories using the numerically designated classification schemes of different countries may not be equivalent or representative.

It is critical to note that the classification of specific biological agents into risk groups was never intended to equate directly with similarly designated laboratory biosafety levels; instead, the link between a specific agent and specific individual biosafety measures was intended to be determined by an assessment of biological risk associated with the presence and handling of the individual biological agent and the associated procedures used in the particular facility or environment. It is the individual biosafety and laboratory biosecurity measure or composite of measures, rather than a designated biosafety level, that guides a laboratory in the safe and secure handling of any individual biological agent or toxin. These specific biosafety and biosecurity measures are identified during a *biological risk assessment* which takes into consideration a laboratory's organisation, the facility, and the surrounding environment in which the biological agent or toxin is to be handled. Over time the role of formal risk assessments in selecting appropriate biological risk mitigation measures has been minimised or over-looked in many laboratories, and replaced by generic assignment of biological agents based on their risk group classification into laboratory facilities defined by one of the four biocontainment levels. Such practices do not necessarily lead to appropriate strategies for the informed management of biological risks.

Moreover the expense of building and of maintaining high level containment laboratories can be impractical or prohibitive for some countries, or may simply not provide the most practical and feasible means of managing a specific biological risk. A laboratory-specific biological risk assessment and associated laboratory risk control decisions based on the country or region's animal health strategy, including consideration of endemic disease status, environment, animal movement, trade arrangements, and geopolitical borders, tends to be both more practical and effective.

This chapter defines the terminology and approaches used in biological risk analysis, and in doing so provides a practical approach for countries, jurisdictions and veterinary laboratories to develop, implement, and maintain appropriate biosafety and laboratory biosecurity measures leading to a functional and efficient biological risk management system.

B. BIOLOGICAL RISK ANALYSIS AND BIOLOGICAL RISK MANAGEMENT SYSTEM

Biological risk analysis includes identification of biohazards, a laboratory assessment followed by management of the associated biological risks, and biological risk communication. For veterinary laboratories, biological risk analyses focus on the potential for animal, human, and environmental exposures, including both intentional and unintentional release of biological agents and toxins from the laboratory. It is the laboratory's biological risk management system that ultimately provides laboratory managers, as well as the veterinary authorities of a country or jurisdiction, with a structured process for assessing, reviewing and controlling biological risks.

The laboratory's biological risk management system includes the policies, procedures, and operational components needed for identifying, determining the extent of, managing, and communicating disease and economic risks associated with a specific biological agent in the context of how that agent is handled, manipulated, and maintained in the laboratory.

It is the responsibility of the laboratory to ensure suitable methodologies for the allocation of actions resulting from biological risk assessments, including timelines, responsible persons, and that the associated reporting and approval mechanisms are identified, implemented, and maintained (CEN, 2011). This is accomplished through the development of a risk management policy appropriate to the nature and scale of the facility, activities, and associated biological risks. The policy (or policies) is designed to (a) protect staff, contractors, visitors, the community, surrounding animal populations, and the environment from unintentional or intentional release of or exposure to biological agents and toxins stored or handled within the facility; (b) reduce to acceptable levels laboratory risks that may result in release of or exposure to biological agents by conducting risk assessments of laboratory facilities and work practices, identifying appropriate risk control measures, implementing, and monitoring those measures for effectiveness; and (c) effectively informing and communicating to employees and relevant stakeholders the findings and obligations of the risk management system.

A successful biological risk management system will have clear and unequivocal commitment by laboratory management, who ensure that roles, responsibilities, and authorities related to biological risk management are defined, documented, and communicated to those who manage, perform, and verify work associated with biological agents and toxins in the laboratory. Laboratory management will ensure (a) the provision of adequate resources; (b) prioritisation and communication of biosafety and biosecurity policy; (c) integration of biosafety and biosecurity practices throughout the laboratory; and (d) a robust process of monitoring and evaluation that identifies opportunities for improvement, determines root causes where unsatisfactory situations arise and revises policies and procedures to prevent recurrence. The ongoing verification and continual improvement of a laboratory's effectiveness in managing its risks is a key component of a complete and effective biological risk management system.

Each laboratory should appoint a biological risk management advisor who will report directly to senior laboratory management and have authority to lead the development and implementation of the biological risk management system, be responsible for developing and maintaining documentation for all aspects of the system, and for monitoring the system within the laboratory or facility. The biological risk management advisor is an individual knowledgeable about the laboratory facility, laboratory procedures used, and biological agents and toxins likely to be encountered in the particular laboratory. In smaller laboratories the biological risk management advisor may also have other roles or duties, often quality management or safety management. The designated biological risk management advisor will have the authority delegated from senior management to call for the cessation of work that is not compliant with the laboratory's biological risk policies and procedures.

The key functions of biological risk analysis are: (a) biohazard identification (i.e. what can go wrong?); (b) biological risk assessment (i.e. how likely is the hazardous event to occur and how severe would be the harm?); (c) risk management (i.e. how can those risks be prevented or minimised to acceptable levels?); and (d) risk communication (i.e. how was the risk identified, characterised and controlled?). In addition there is a need for (e) verification with continual improvement (i.e. are the biosafety and laboratory biosecurity measures effective in controlling the biological risk and can they be improved?). The organisational structure, responsibilities, policies, and practices that provide for these activities, comprise a laboratory's biological risk management system. It is important that all relevant regulatory requirements are identified and fulfilled within the biological risk management system. Legal requirements include any national, federal, regional, state, provincial, city and local regulations with which the laboratory is obliged to comply.

1. Biohazard identification

The first step in the risk analysis process is identifying and documenting the potential laboratory biohazard(s). A *biohazard* can be any biological agent, toxin, or associated laboratory or animal facility procedure with the potential for causing harm or damage. During the biohazard identification process, it is necessary to identify biological agent characteristics that make the agent hazardous, and potentially make the agent attractive for malicious use or theft. Although not the focus of this chapter, it should additionally be noted that laboratories must be critically aware of all potential hazards (any source, situation, or act with the potential for causing harm) in the laboratory environment, and not just those that are specifically biological in nature. Examples would include electrical safety, physical safety or radiation hazards, and issues relating to utility failure, poor training, selection of suppliers, etc. that may not appear directly to link themselves to the biological agents and toxins, but that can result in release of a biological agent or toxin, as well as causing other harm.

A laboratory's risk management system should be complete in identifying and managing all hazards, including those outlined hereafter.

1.1. The inventory of biological materials held by and/or manipulated by the laboratory

All biological materials held by the laboratory must be known, recorded and individually addressed in the biological risk assessment process. The specific agents and toxins that a laboratory works with and the associated technical procedures used in that work must be recognised. This will be the primary focus of the biological risk assessment.

1.2. Diagnostic specimens

Veterinary diagnostic centres routinely receive specimens that have been submitted because they are suspect for any of a variety of diseases. While the infectious nature of the specimens is unknown, diagnostic case materials may contain a variety of unknown agents, some of which could be extremely hazardous to human health or pose a significant threat to animal populations. Veterinary diagnostic laboratories have the responsibility to implement appropriate biosafety and laboratory biosecurity measures to minimise the risk of occupational exposure of employees, or of release and spread to the population of pathogens that may be contained within diagnostic specimens. Initial laboratory processing of all unknown diagnostic specimens must be carried out with the assumption that an infectious agent or toxin likely exists in the specimens submitted. Until the specimen has been characterised as non-infectious, it is important that veterinary laboratories take adequate precautions to prevent exposure via percutaneous and mucous membrane routes, and particularly through inhalation and ingestion. Once a specific agent or toxin has been identified by the laboratory, further work is carried out using relevant biocontainment and risk controls.

1.3. Transportation and storage of pathogens

Requirements used for the safe and secure transportation of specimens are given in Chapter 1.1.3 *Transport of biological materials*. Storage of viable agents is a common laboratory and animal facility practice, and therefore a biohazard, in most if not all veterinary laboratories and animal facilities. The risks associated with accidental contact or unauthorised access to biological agents and toxins must be addressed within the storage facility and inventory system. As noted previously, it is an important biosecurity responsibility of veterinary laboratories and animal facilities to identify and to minimise any risk of release of pathogens into human and animal populations, either domestic or wild.

1.4. Physical and chemical hazards

Physical and chemical hazards associated with routine laboratory and animal-use manipulations cannot be ignored during biohazard identification exercises. The laboratory and animal facility must identify these hazards within their facility in order to ensure that their biosafety programmes adequately protect laboratory workers. Examples of hazards routinely found in veterinary laboratories include handling and disposal of glass, needles, and sharp instruments; burns from hot solids, liquids, or from radiation, and burns and asphyxiation risks associated with liquid nitrogen; explosion risks associated with incorrect or non-compatible storage of chemicals; and exposure or repeated exposure (dose–effect) to mutagenic, carcinogenic, and toxic chemicals through respiratory and percutaneous routes.

1.5. Laboratory animals

Work with laboratory animals is also an important laboratory hazard. Laboratory animals can generate large amounts of infectious agent, as well as pose risks associated with the potential for bites, scratches, kicks, and related injury to care-takers and laboratory workers. Expanded information on health and safety in laboratory animal facilities is available (Wood & Smith, 1999).

2. Biological risk assessment

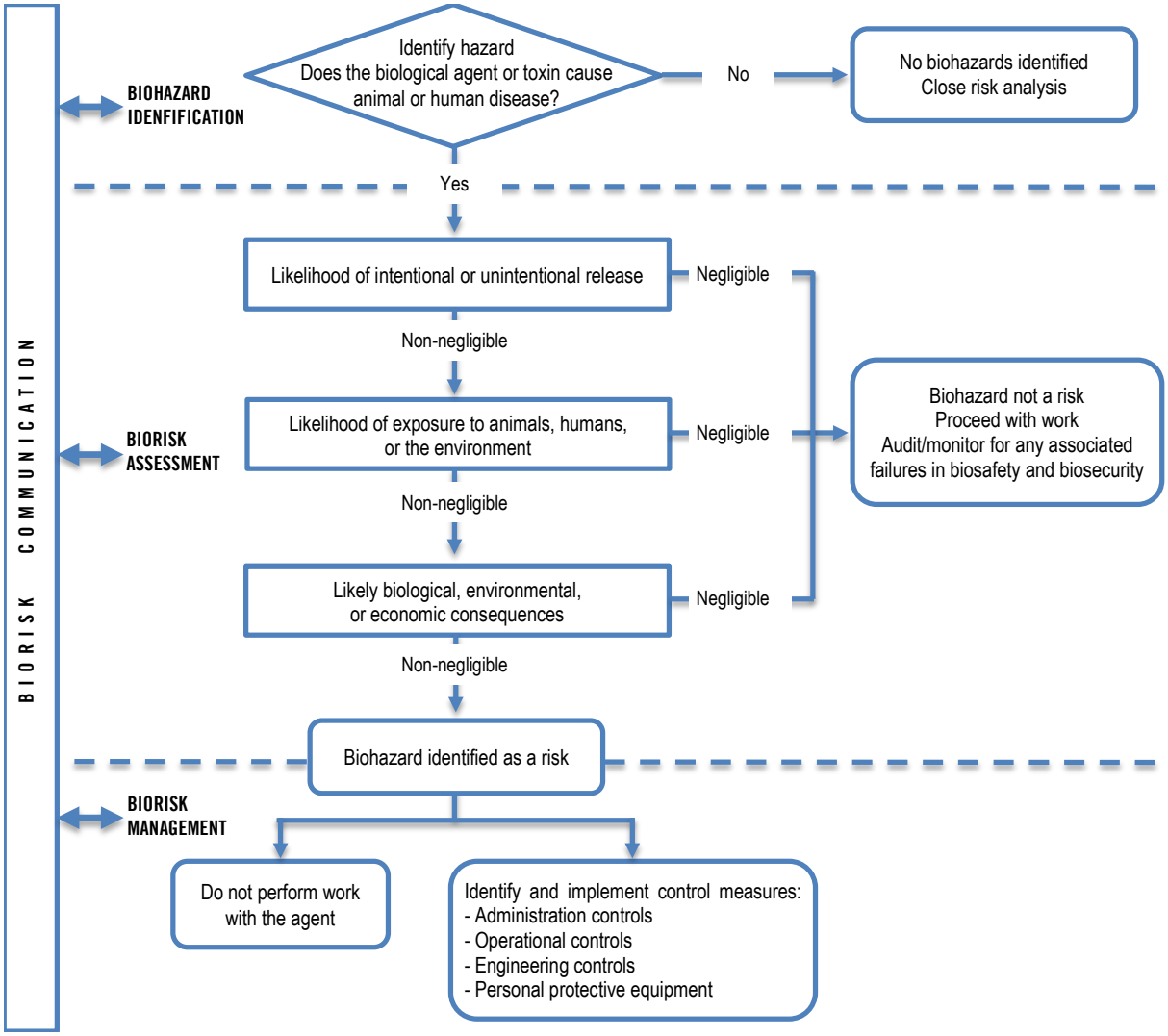
The laboratory's approach to biological risk assessment is a component of the laboratory's risk management policy that defines the scope, nature, and timing of assessments so that the process is proactive rather than reactive. Following identification of the biohazard, the next step in the biological risk assessment process is determining the likelihood and potential severity of consequences or harm associated with that biohazard. Severity (or harm) can be thought of as the biological, environmental, and economic impacts associated with a release of and exposure to the biohazard. The severity of harm associated with animal pathogens and toxins will include human and animal

disease, as well as economic losses associated with local, national, regional, and international restrictions on animal movement and on commerce associated with animals and animal products.

Risk is defined as a combination of the likelihood (probability) of the occurrence and the severity of harm (or consequence); the term biological risk is used where the source of harm is a biological agent or toxin. At this point in the biological risk analysis process (see Flowchart 1), the laboratory with the assistance of their biological risk management advisor, will evaluate the individual facility, human resources, protocols, methodologies and procedures to determine how the biohazard is to be handled and manipulated in their specific circumstances; in addition to assessing the surrounding environment, including identifying susceptible species and the specifics of the biological agent's transmission in order to determine the likelihood and severity of harm (see Appendix 1.1.4.2).

A comprehensive biological risk assessment includes evaluation of both biosafety and laboratory biosecurity practices. Biosafety addresses risks associated with exposure to, or accidental release of the biological material, while laboratory biosecurity addresses potential for theft, misuse, or deliberate release. A comprehensive risk assessment would include consideration of all relevant items that could be at risk for theft or misuse (e.g. electronics, computers, balances) that make the facility a target for theft. It is necessary to consider both biosafety and laboratory biosecurity to ensure that risk control measures implemented are not in conflict with each other and that any one control measure does not compromise others.

Flowchart 1: Biological risk analysis process



Note: The biological risk management process should address all laboratory processes and procedures associated with the specific hazard (biological agent or toxin). The biological risk assessment and biological risk control planning involves a team of individuals who understand the organisational aspects of the laboratory, the biology and pathogenesis of the agent, and the impacts of exposures and accidental or intentional release of the biological agent or toxin.

The biological risk assessment may be quantitative, using mathematical models (WOAH, 2010b), or may be qualitative (CEN, 2011; WOAH, 2010a). For the qualitative biological risk assessment approach discussed here, both likelihood and severity are given a non-numerical score or ranking, which allows a form of "quantifying" the biological risk by using qualitative definitions such as low, moderate, and severe or other non-numerical equivalents. The rankings determined for likelihood and severity of harm will help the laboratory further characterise its biological risks in order to determine the biosafety and laboratory biosecurity control measure(s), the necessary redundancy in controls, and the overall financial investment that will be appropriate to mitigate their specific biological risks.

Resource utilisation and financial investments in biological risk control measures should be proportionate to the biological risks identified in the assessment process ('protect pencils like pencils and diamonds like diamonds'). For example, one outcome of biological risk assessment is a very low likelihood score (e.g. unintentional release of the agent from laboratory containment via some specified process, such as waste treatment), with an extremely high severity score (e.g. release of a non-endemic biological agent with high transmissibility paired with high morbidity or mortality in a susceptible population, loss of trade status, and severe social and economic impacts). In such a case, the laboratory may determine that there are no available mitigation or combination of biosafety and biosecurity measures that would be sufficient to justify handling the biological agent in their facility. The same scenario occurring in a country or region where the agent is endemic may result in the same likelihood ranking, but carry a significantly lower severity ranking. This country could justify an investment for determining and then implementing appropriate biosafety and laboratory biosecurity measures to decrease the likelihood of an unintentional release to an acceptable minimum level.

Where it is determined for a specific biological agent or toxin that there is no severity of harm associated with exposure or release, the biological risk assessment can be concluded.

3. Biological risk management

Where the biological risk assessment identifies unacceptable biological risks, the laboratory is responsible for responding by not handling or storing the specified agent in its facility (elimination of the hazard); by using alternative technical procedures (substitution); or by identifying, implementing and maintaining appropriate biosafety and laboratory biosecurity measures. The response to a biological risk assessment requires documentation of the timelines for action, assignment of responsible persons, and the associated reporting and approvals. Dependent on the outcome of the biological risk assessment (likelihood and severity rankings), the laboratory managers working with the biological risk management advisor will identify which biosafety and biosecurity measure(s) are appropriate and feasible for use within the laboratory or animal facility in order to prevent release of and exposure to the biohazard. The principal routes for exposure and release of biological agents and toxins from laboratory environments include:

i) personnel via surface contamination, infection, or intentional acts allowing release;
ii) aerosol;
iii) liquid and solid waste;
iv) equipment and materials;
v) specimens and reagents;
vi) release via research animals or disease vectors.

To protect biological agents and toxins from unauthorised access or use, the laboratory should additionally consider laboratory security. In general, the components of laboratory security include

i) physical security (e.g. building structure, lockable doors);
ii) personnel (including steps taken to ensure an employee does not pose a safety or security risk);
iii) material control and accountability (inventory control and storage records);
iv) information and information technology security;
v) security of materials during transportation (ensuring the biological material is not subject to theft or diversion during transportation within a facility or between facilities).

In the absence of elimination or substitution as a possible risk control strategy, a strategy that includes administrative, operational, engineering, and personal protective equipment (PPE) controls is used to prevent exposures and accidental or intentional release. The different control approaches are complementary and are used in combination to accomplish appropriate risk reduction. A most basic biosafety and biosecurity programme will ultimately require implementation, at varying degrees, of all the different types of control strategies.

i) *Administrative controls:* qualified and suitable personnel; training and verification of competency of staff in the safe and secure handling of biological agents and toxins, in applicable technical procedures, and in use of PPE and equipment; health and safety programmes; prophylactic health care including vaccinations; emergency response and contingency plans; incident and accident investigation programmes; current biological agents and toxin inventory and inventory management requirements including access, storage, transfer, destruction, and audit; waste management policies; and security policies including facility security, visitor access, personnel security, access to biological agents and toxins; and information security.

ii) *Operational controls:* Standard Operating Procedures for all safety and laboratory biosecurity-relevant processes including Good Microbiological Technique (GMT); disinfection and decontamination practices; transport procedures; general laboratory safety; specimen and reagent handling and storage practices; waste management practices including disinfection and inactivation; emergency exercise drills; and accident/incident reporting, response, and review protocols.

iii) *Engineering controls:* physical features of the facility including ventilation and air-flow, barrier walls and shields, and separation of incompatible activities; equipment and equipment maintenance, calibration and certification; and physical security such as access restrictions, perimeter fences, facility and equipment locks with key control protocols, badge readers, detectors and sensors, or biometric devices. The laboratory must have measures to ensure that all changes to the facility associated with design, operation, and maintenance are documented and that documentation is used to update prior biological risk assessments that may be affected by the change. Engineering controls include the following principles of containment:

    a) *Primary containment layers* are those that enclose the biological agent or toxin within sealed containers or in a Class I, II or III biosafety cabinet. Biological biosafety cabinets must be installed and certified in accordance with the national or manufacturer's standards to ensure effective functioning. Class I cabinets provide personnel and environmental protection. The contents of Class I cabinets are not protected from environmental contamination. Class II cabinets draw a curtain of sterile air over the contents of the cabinet and exhaust through high efficiency particulate air (HEPA) filters in order to protect the contents of the cabinet, personnel, and the environment. Class III cabinets are gas-tight and designed for maximum containment. Class III cabinet engineering (e.g. attached gloves, dunk tanks, etc.) and protocols for use prevent direct contact with hazardous materials and air. In the case of infected animals, the agent is enclosed by physical containment in specially constructed rooms where all wastes are treated and air is filtered.

    b) *Secondary containment layer*s enclose infected materials and individuals working with infected materials within a closed and controlled physical environment that treats solids, fluids, and air using validated filtration and treatment procedures that remove or inactivate live agents.

    c) *Tertiary containment layer*s are those designed to prevent contact between biological agents and susceptible species using appropriate measures that physically restrict exposure to susceptible species.

iv) PPE: body protection (i.e. clothing), hand protection (i.e. gloves), eye protection, and respiratory protection.

Laboratory biosafety should be based on a solid foundation of good microbiological practices in the laboratory to which all laboratory work conforms. The essential requirements for any work with infectious agents or specimens likely to contain infectious agents, however innocuous the material may seem, are as follows:

i) The laboratory should be easy to clean, with surfaces that are impervious to water and resistant to chemicals used in the laboratory. There shall be a hand-wash basin, emergency shower, and eye wash station in each laboratory suite as appropriate for the chemicals and other hazards present. Procedures shall be established for frequent cleaning and disinfection of the work area during and at the end of the work period;

ii) Personnel access to the work area should be restricted (security measures such as controlled access may be necessary with higher risk agents);

iii) Basic PPE such as long-sleeved laboratory coats or gowns, closed-toe footwear, disposable gloves, and safety glasses, shall be worn in the laboratory and removed when leaving the laboratory. Masks, including face shields and oro-nasal respirators may be required as determined by the specific risk assessment;

iv) The laboratory door should be closed when work is in progress, and appropriate access restriction, warning, or biosafety signage clearly visible;

v) While forced ventilation is not a baseline requirement, appropriate ventilation shall be provided for the health and well-being of the workers and as required by risk assessment;

vi) Food (including chewing gum, candy, throat lozenges and cough drops) and drinks shall not be stored or consumed in laboratories; smoking or application of cosmetics shall not take place in the laboratory;

vii) Pipetting shall not be done by mouth;

viii) Care shall be taken to minimise the production of aerosols;

ix) Emergency response plans should be developed to deal with the biohazard of any safety or security incident. Items addressed in the plans should at a minimum include having effective disinfectants and instructions available for cleaning spills, removal and decontamination of contaminated protective clothing, washing of hands, and cleaning and disinfection of bench tops;

x) Used laboratory glassware and other contaminated material shall be appropriately identified (labelled) and stored safely. Materials for disposal shall be transported without spillage in robust containers. Waste material should be autoclaved, incinerated or otherwise decontaminated or inactivated before disposal. Reusable material shall be decontaminated by appropriate means;

xi) No infectious material shall be discarded down laboratory sinks or any other drain;

xii) Any accidents or incidents shall be recorded and reviewed with the biological risk management advisor to assist in continually improving the biological risk management system;

xiii) Workers shall be appropriately trained and verified as competent to perform the tasks assigned.

The risk assessment process is used in determining the appropriate biosafety and laboratory biosecurity controls required for the biohazard (biological agent or toxin) and laboratory or animal facility procedure in question.

4. Risk communication

Laboratory risk communication is a continuation of hazard identification, risk assessment and risk management processes, and is an integral component of incident or outbreak preparedness and response planning. With the understanding that the laboratory's stakeholders and the public are entitled to information that impacts on their own health and the health of their animals, risk communications are designed to inform the laboratory's stakeholders about technical practices and decisions used for handling biohazards and for responding to incidents that may arise from release of and exposure to those biohazards. As laboratories handling animal pathogens and toxins are a critical component of a country or region's veterinary infrastructure, it is critical that the laboratory biological risk management process be thorough, objective, transparent, and clearly communicated (Covello & Allen, 1988).

Effective risk communication should be designed to establish a common understanding among the laboratory and associated stakeholders of the biological risks, biological risk control measures (biosafety and laboratory biosecurity practices implemented), as well as the benefits of working with the identified biohazard. This common understanding not only builds trust, but is critical for effectively responding to potential incidents and enabling impacted individuals and agencies to make informed decisions when working with the laboratory. Risk communication should be provided in a format and language that is tailored to the intended audience, whether policy-makers, disease control authorities, animal care providers, or the public, in order to provide the information in a clear and understandable manner. Effective biological risk communication requires that the complexities of technical language, scientific data, assumptions, and the justification for assumptions used in the biological risk assessment be fully documented.

In general, an initial laboratory biological risk communication is directed toward the appropriate health and disease control authorities and will identify; (1) the biohazard (biological agent or toxin); (2) the benefits to the stakeholder gained by the laboratory working with the biohazard; (3) information indicating that a biological risk analysis was performed and is documented; and (4) information indicating that the laboratory has biosafety and laboratory biosecurity measures in place to mitigate against accidental or intentional release of the biological agent or toxin.

In preparedness for of an accidental or intentional release of the agent, the laboratory should additionally be prepared for incident and incident response communication. Among the documents that the laboratory should generate prior to initiating work with a biohazard are (1) documentation of the roles and responsibilities of individuals involved in drafting, reviewing, approving, and distributing laboratory information and official communications, (2) a contact list containing the names, phone numbers, email addresses or other information as

appropriate for those agencies and individuals to be notified, and (3) an incident response plan in the unlikely event of accidental or intentional release of the biological agent or toxin.

Contact lists should include (1) national, regional, and local disease control authorities (Veterinary Health and Public Health), as appropriate, (2) security authorities, as appropriate, for specific biothreat agents and risks, (3) the responsible physician or occupational health programme to be notified of human health-related agents, biological risks, and at-risk staff, and (4) stakeholders, including potentially impacted laboratory affiliates, e.g. shippers, rendering and waste disposal plants, janitorial staff, non-technical laboratory staff, potentially impacted local animal owners and industries.

5. Verification, corrective actions, and continual improvement

Biological risk management is an ongoing process in which specific biosafety and laboratory biosecurity measures are regularly monitored to ensure they are working as expected. The laboratory facility, management practices, and procedures should also be regularly reviewed to ensure that changes have not altered previously defined risks. Routine audits should be scheduled and conducted to document effectiveness of the implemented biosafety and laboratory biosecurity measures, to identify areas of noncompliance that need to be documented and corrected, and to identify areas for improvement. The process requires that the laboratory verify and document that the control measures implemented (e.g. administrative, operational, engineering, and PPE) effectively mitigate release of and exposure to the targeted biohazards. In a simple example: if during a laboratory assessment, the risk of release was defined as theft due to inadequate physical security, and the biosecurity control used was placement of a lock on the storage freezer, the laboratory administration would want to verify that the control implemented, locking the freezer, had mitigated the risk of theft. Assuming the administration found that the freezer key was kept on an accessible hook near the freezer, the risk of theft had not been adequately controlled and a corrective action would be implemented (e.g. additional or alternative choices of biosecurity control measures, such as implementing added policy and procedures managing access to the freezer key).

It is the responsibility of laboratory managers to continually review and improve the laboratory's effectiveness through the use of documented policy and procedures, training of personnel and verification of competence, self-audit and external audit, where appropriate, corrective and preventive actions, and routine management reviews. The cycle of assessing biological risks, implementing control measures, verifying effectiveness, and correcting any weaknesses follows the same pattern used in well-functioning quality management programmes. Chapter 1.1.5 *Quality management in veterinary testing laboratories* provides an overview of the subject; the CEN *Workshop Agreement on Laboratory Biological Risk Management* details the components of a comprehensive biological risk management system (CEN, 2011).

C. TECHNICAL GUIDANCE AND ASSESSMENT TOOLS

Technical advice and the level of detail needed for selecting individual laboratory and animal facility risk control measures is available through a number of published veterinary health and public health resources, including the WHO Biosafety Manual (WHO, 2004), CDC Biosafety in Microbiological and Biomedical Laboratories (CDC, 2009), Canadian Biosafety Standard and Guidelines (Government of Canada, 2013), Biological agents: Managing the risks in laboratories and healthcare premises (HSE, 2005), Laboratory Biosecurity Handbook (Salerno & Gaudioso, 2007), among others. Assessment tools such as the Food and Agriculture Organization of the United Nations (FAO) LMT (Laboratory Mapping Tool) and the Laboratory Assessment Checklists included in the WHO and CDC Biosafety Manuals, which are used for documenting a laboratory's capabilities and for monitoring compliance with laboratory management standards and good laboratory practices are additionally useful for both external and self-assessments.

D. CONCLUSION

The role of veterinary laboratories is to function as an integral component of a documented national animal health strategy to protect the health and well-being of local, national, regional, and global animal populations and associated commerce as well as protecting public health from biological risks of animal origin. The national animal health strategy will determine the biological materials, and particularly the infectious agents, for which the country's laboratories must maintain a capability.

Within the veterinary laboratory and animal facility environment there will be the inevitable presence and handling of biological materials that can pose biological risks for both animal and human populations. It is therefore of critical

importance that laboratory and animal facility managers ensure that biological risks in their facility are clearly identified, understood, controlled, and communicated to the appropriate stakeholders. It is likely, and recommended, that these risks will be managed within the context of national regulations so that laboratory biological risk management strategies are consistent within countries. The standards communicated in this chapter apply to the development of national standards for managing the biological risks associated with laboratories as much as to the development of biological risk management systems within individual laboratories.

The discipline of biological risk analysis, paired with a comprehensive biological risk management system, allows those responsible to assess and document the laboratory biosafety and biosecurity practices which are used to provide appropriate controls, thus assuring adequate biosafety and laboratory biosecurity. A complete and functioning laboratory biological risk management system will help ensure that the laboratory is in compliance with applicable local, national, regional, and international standards and requirements for biosafety and laboratory biosecurity.

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NB: First adopted in 1992 as Safety in the veterinary microbiological laboratory.
Most recent updates adopted in 2015.

APPENDIX 1.1.4.1.

STEPS IN BIOLOGICAL RISK ANALYSIS

1. Assemble a team for performing the risk assessment. Include individuals with knowledge and understanding of:

    i) The physical and biological properties of the agent or toxin (e.g. the infectious dose, routes of infection, susceptible species, environmental survivability, etc.),

    ii) The laboratory technologies and procedures to be used with the biological agent or toxin, associated technical competence, and laboratory facilities to be used,

    iii) Laboratory biosafety and biosecurity practices,

    iv) Risk analysis principles and practices.

    One team member may serve multiple functions, and qualified individuals from outside of the laboratory performing the analysis may be used. The quality of the risk analysis performed is directly related to the level of knowledge and understanding provided by the team members.

2. Define the scope of the biological risk analysis

    i) Biohazard Identification: identify the target biological agent or toxin. Perform a separate biological risk analysis for each relevant biological agent.

    ii) Define the laboratory environment in which the biological agent or toxin will be used:

        a) Identify technical procedures, methods and processes specifically to be used with the biological agent or toxin being evaluated (e.g. diagnostic specimens or reference materials, amplification in culture, centrifugation, ultrasound, pipetting, freeze–thaw, archival practices, concentrations and volumes of the biological agent or toxin, animal handling, etc.). These items define the laboratory environment relevant to the risk assessment, and document the potential sources of exposure and release from the laboratory environment.

        b) Identify existing laboratory resources, including management and technical competencies (e.g. technical training and proficiency programmes, quality management practices, health and safety management programmes, etc.). These items document existing and potential sources of risk control.

        c) Identify relevant laboratory facilities and associated resources (e.g. facility security, directional air-flow, autoclaves, incinerators, etc.). These items document existing and potential sources of risk control.

3. Develop and initiate the risk communication plan. The documentation and communication of risk analyses must be clear and complete. Because risk analysis supports decision-making where there is uncertainty in predicting events, it is critical that the process be transparent, objective, and clearly presented. It is useful to begin compiling the risk analysis report at the very beginning of the analysis in order to most effectively capture all relevant information, investigation, analysis, and findings.

4. Identify the severity of harm associated with any exposures or release of the biohazard from the laboratory. Severity should identify human health, animal health, and economic harm that would likely result from exposure and from release of the biological agent or toxin. Note that for a single agent, the economic cost of associated disease may vary considerably between a country in which the biological agent is endemic, and a

country that is free of the biological agent. Where specific morbidity, mortality, and economic estimates are available, the source and context of the information must be provided. For example, existing risk analysis performed for import or export in association with a country or region may be used as a valuable source of economic data.

5. Perform the biological risk assessment, assigning a likelihood ranking and a severity ranking for agent release and for exposure to susceptible animals and humans for **each** laboratory procedure involving the biological agent or toxin in the laboratory (e.g. specimen receipt, necropsy, amplification in culture, centrifugation, nucleic acid extraction, storage, archive, animal experimentation, etc.) Biosafety assessments address the likelihood and severity of inadvertent exposure and of release of the biological agent. Biosecurity assessments address theft, loss, and intentional misuse of a biological agent.

6. Identify appropriate risk control measures available to the laboratory, including those biosafety and biosecurity measures already in place and those which could be implemented. There are often several different control measures that when used alone or in combination can provide equivalent results at similar or widely different costs. Each biosafety and laboratory biosecurity measure or combination of measures must be evaluated independently in order to determine the relative effectiveness in reducing the overall risk of exposure and release. It is the responsibility of the laboratory managers with the local, national, and regional disease control authorities to determine the economic and logistical feasibility of different control measures and appropriately balance the risks and benefits associated with the presence and handling of the biohazard.

7. Document the information and approach used in the risk assessment. The documentation must be complete, including data, methods of analysis, results, discussion, explanatory notes, and conclusions, dates and responsible personnel. References should be provided when relevant scientific and laboratory data and information is used (e.g. infectious dose, routes of transmission, working concentrations, environmental stability, etc.). All assumptions used must be identified and justifications for the assumptions must be provided.

8. Maintain or implement the selected biological risk control measures (biosafety and biosecurity practices) in the laboratory.

9. Make a record of and communicate the complete risk analysis, including implementation of the biosafety and biosecurity measures to the appropriate authorities and stakeholders. There are multiple report formats and templates available for documenting risk analysis. Examples can be found in the WOAH *Handbook on Import Risk Analysis for Animals and Animal Products* (WOAH, 2010a; 2010b) and in the *Seven Cardinal Rules of Risk Communication* (Covello & Allen, 1988).

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APPENDIX 1.1.4.2.

CONSIDERATIONS USED IN EVALUATING AND IMPLEMENTING BIOLOGICAL RISK CONTROL MEASURES

Chapter 2.1.3 Managing biorisk: examples of aligning risk management strategies with assessed biorisks *provides illustrative examples of agent-specific risk assessments.*

Considerations used for identifying and assessing laboratory hazards	Determinant or level of risk for release from laboratory or exposure of staff	Examples of biological risk control measures
Epidemiology of the biological agent; routes of transmission, including aerosol, direct contact, fomites, vectors; infectious dose, susceptible species, and likely extent of transmission. Origin of the agent outside the host.	Route(s) of transmission determines possible mechanisms for exposures or release from a laboratory. Origin of samples: specimens derived from wildlife may contain human or animal pathogens not normally encountered. Geographical source of specimens.	Different routes of transmission require specific mitigation measures: - Aerosols: use of primary containment (e.g. biosafety cabinets), good microbiological technique (GMT), air filtration, directional airflow. -Surface contamination: disinfection, PPE including clothing and gloves, showering out of the laboratory. -Solid and liquid waste: waste treatment measures (e.g. autoclaving, chemical) -Fomites and materials exiting the laboratory: decontamination strategies
May cause human or animal disease; *Severity of harm for laboratory workers, public health, and animal health*.	Severe: potentially fatal disease, treatment or prophylaxis generally not available. Human: high individual or community risk. Animal: exotic or enzootic, subject to official control and that have high risk of spread from the laboratory into the environment and national/regional animal population.	Avoid release of the agent using a combination of administrative, operational, and engineering controls, and PPE. Considerations include stringent measures for biocontainment, decontamination and disinfection, redundancy of control measures used, GMT; mandatory training and competency for workers; mandatory employee health reporting programmes; mandatory laboratory security policies and procedures: redundancy of control measures, access control, due diligence in authorising personnel, inventory of seed and working stocks, intrusion detection, emergency response plans.
	Moderate: effective prophylaxis and treatments are generally available, but may be variable in effectiveness. Human: high individual risk, low community risk. Animal: exotic or enzootic, subject to official control and have moderate risk of spread from the laboratory.	Use a combination of administrative, operational, and engineering controls and PPE. Considerations include GMT such as effective infection control procedures, decontamination and disinfection, use of PPE and biosafety cabinets; employee health programmes (e.g. vaccination when relevant, health reporting); mandatory training and competency for workers; laboratory security policies and procedures. Access controls, escort for unauthorised individuals, inventory of seed stocks.

Considerations used for identifying and assessing laboratory hazards	Determinant or level of risk for release from laboratory or exposure of staff	Examples of biological risk control measures
	Low: effective prophylaxis and treatments are available. Human: moderate individual risk, low community risk. Animal: either exotic or enzootic, subject to official control and have low risk of spread from the laboratory. Human: no or low individual and population risk. Animal: enzootic, not subject to official control.	Considerations include routine use of GMT such as decontamination and disinfection, effective infection control procedures including use of dedicated laboratory clothing, biosafety cabinets; basic training and competency for workers. Waste management, including disinfection of laboratory wastes. Considerations include routine use of good microbiological practices (see Section B.3 of this chapter).
Impacts associated with animal population morbidity and mortality, and associated economic consequences (e.g. trade, food security, costs of disease control and movement controls, destocking or vaccination) dependent on whether the agent is exotic or endemic to the country or region.	Severe: Unacceptable costs nationally.	Avoid release of the biological agent using a combination of administrative, operational, and engineering controls, and PPE. Considerations include stringent biocontainment measures; specific features that are warranted by route(s) of exposure; PPE; GMT, decontamination and disinfection, primary containment systems; mandatory training and competency; mandatory laboratory security policies and procedures: redundancy of control measures, access control, due diligence in authorising personnel, inventory of seed and working stocks, intrusion detection, emergency response plans.
	Moderate: financial costs assessed on a case-by-case basis.	Use a combination of administrative, operational, and engineering controls, and PPE. Considerations include GMT such as decontamination and disinfection, effective infection control procedures including the use of PPE, and biosafety cabinets; air and effluent control; mandatory training and competency, laboratory security: access controls, escort for unauthorised individuals, inventory of seed stocks.
	Low: financial impact at manageable or existing levels.	Considerations include GMT such as decontamination and disinfection, effective infection control procedures including use of laboratory clothing and biosafety cabinets; basic training and competency.

Considerations used for identifying and assessing laboratory hazards	Determinant or level of risk for release from laboratory or exposure of staff	Examples of biological risk control measures
Nature of the laboratory procedures to be conducted in a facility (e.g. small- versus large-scale amplification, use and storage of the agent.	Moderate to severe • Procedures such as antigen or vaccine production that generate large amounts of organism. • Aerosols generated by laboratory procedures (homogenisation, sonication, centrifugation). • Sample history: agents on primary isolation/low passage number are often more virulent than laboratory-adapted strains.	GMT such as decontamination and disinfection, the use of effective infection control procedures including the use of proper primary containment systems to physically separate the process from the other work areas. Staff safety including agent/procedure-specific training and medical surveillance. The containment area(s) should be designed to contain spillage of the entire contents of the closed system. Inadvertent carriage from the area to be taken into consideration depending on the epidemiology of the disease and the impact on the animal disease situation in the country or region. Mandatory laboratory security policies and procedures: redundancy of control measures, access control, due diligence in authorising personnel, inventory of seed and working stocks, intrusion detection, emergency response plans
	Low: • Vector or intermediate host required in the life-cycle of the agent does not naturally occur or survive in the country or region	GMT such as decontamination and disinfection, effective infection control procedures including proper laboratory design, use of dedicated laboratory clothing, primary containment systems such as biosafety cabinets, and disinfection of laboratory wastes may be adequate. The risk of inadvertent carriage from the laboratory to be considered depending on the epidemiology of the disease and the impact on the animal disease situation in the country or region.
Use of animals in association with the biological agent or toxin.	A higher level of risk may arise when agents are inoculated into laboratory animals. The following factors should be considered in the risk assessment: i) Host species versus inoculated species; ii) Strain, treatment and concentration of the inoculum; iii) Route of inoculation; iv) Animal housing; v) Types of sampling during the experiment. Examples: • Production of biological reagents (e.g. antibody) in animals • Animal-based diagnosis, pathogenicity determinations • Research	Good animal handling and microbiological technique such as decontamination and disinfection, infection control procedures, protective clothing and proper equipment. Staff training and medical surveillance. The facility should be designed to minimise or prevent spread of the biological agent or toxin through contaminated air, laboratory materials, liquid or solid waste or animal carcasses. Pest control. The room is considered primary containment for large animals. For laboratory animals individual vented cages, isolators, or similar provide primary containment. Security considerations include prevention of intentional animal or agent release as a form of biothreat: parameter fences, identification, intrusion alarms, redundancy of control measures.

Note: The control measures provided are not meant to be comprehensive, but are examples of available control measures. Comprehensive information on applicable biosafety and biosecurity measures is available in internationally recognised technical manuals such as the WHO Biosafety Manual (WHO, 2004), the Biosafety in Microbiological and Medical Laboratories (CDC, 2009) and related guides.

CHAPTER 1.1.6.

VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES OF TERRESTRIAL ANIMALS

INTRODUCTION

Adequate validation and verification of the performance characteristics of diagnostic tests for infectious diseases are critical to ensuring that assays are applied and interpreted in a scientifically robust and defensible manner (Colling & Gardner, 2021). Since it was first adopted in 1996, the World Organisation for Animal Health (WOAH: founded as the OIE) Assay Development and Validation Pathway (Figure 1) has acted as the internationally recognised standard for the validation of veterinary diagnostic tests for infectious diseases.

Validation is a process that determines the fitness of an assay[1], which has been properly developed, optimised and standardised, for an intended purpose. All diagnostic assays regardless of whether they are used in the laboratory or as point-of-care tests should be validated for the species and specimen in which they will be used. Validation includes estimates of the analytical and diagnostic performance characteristics of a test. In the context of this chapter, an assay that has completed the first three stages of the validation pathway (see Figure 1 below), including performance characterisation, can be designated as "validated for the original intended purpose(s)". To maintain a validated assay status, however, the assay's performance should be carefully monitored under conditions of routine use, often by tracking the behaviour of assay controls within each run and through on-going assessment during routine diagnostic use in the targeted population over time. Should it no longer produce results consistent with the original validation data, the assay may be rendered unfit for its intended purpose(s).

Assays applied to individuals or populations have many purposes, such as aiding in: documenting freedom from disease in a country or region, preventing spread of disease through trade, contributing to eradication of an infection from a region or country, confirming diagnosis of clinical cases, estimating infection prevalence to facilitate risk analysis, identifying infected animals toward implementation of control measures, and classifying animals for herd health or immune status post-vaccination. A single assay may be validated for one or more intended purposes by optimising its performance characteristics for each purpose, e.g. setting diagnostic sensitivity (DSe) high, with associated lower diagnostic specificity (DSp) for a screening assay, or conversely, setting DSp high with associated lower DSe for a confirmatory assay (see Section A.1 Definition of the intended purpose(s) of an assay*).*

This chapter focuses on the criteria that must be fulfilled during assay development and validation of all assay types and the metrics used to characterise test performance. The inclusion of assay development as part of the assay validation process may seem counterintuitive, but in reality, three of the required validation criteria (definition of intended purpose[s], optimisation, and standardisation) that must be assessed in order to achieve a validated assay, comprise steps in the assay development process. Accordingly the assay development process seamlessly leads into an assay validation pathway, both of which require fulfilment of validation criteria. The guiding principles described herein also apply to infectious diseases that are not WOAH listed. More detailed guidance is provided in a series of recommendations for validation of diagnostic tests (Section 2.2 Validation of diagnostic tests, *chapters 2.2.1 to 2.2.8) that are tailored for several fundamentally different types*

1 "Assay," "test method," and "test" are synonymous terms for purposes of this chapter, and therefore are used interchangeably.

of assay (e.g. detection of antibodies, antigen and nucleic acids) and provide more information on specific issues related to the validation of diagnostic assays (Halpin et al., *2021). For specific information for wildlife species, refer to Chapter 2.2.7* Validation of diagnostic tests for infectious diseases applicable to wildlife (*Jia* et al., *2020; Michel* et al., *2021*). *The information provided in chapter 2.2.7, which is specific to wildlife species, might also be useful for domestic animal test validation, for example, where the number or availability of samples is limited.*

An up-to-date compilation of the relevant validation standards (WOAH and non-WOAH) and guidance documents for all stages of diagnostic test validation and proficiency testing, including design, analysis, interpretation as well as clear reporting and case studies are provided in the WOAH Scientific and Technical Review *issue Diagnostic Test Validation Science (Vol. 40, April 2021). Published standards for peer-reviewed reporting of accuracy studies (STARD) are available for infectious diseases of human, terrestrial (paratuberculosis, Bayesian latent class model) (Bossuyt* et al., *2015; Gardner* et al., *2011; Kostoulas* et al., *2017) and aquatic animals (Gardner* et al., *2019; Kostoulas* et al., *2021). Verification (Kirkland & Newberry, 2021) and comparability studies (Reising* et al., *2021) are briefly described at the end of this chapter for assays that have completed at least stage 2 of the WOAH pathway. There is a pressing need to develop validation guidelines and standards for the rapidly increasing use of point-of-care tests (POCTs). Typically, POCTs are used in the field under varying environmental conditions, on a range of sample types collected in non-sterile settings, by operators with a diverse range of experience, training and proficiency. Field-testing conditions, including extreme variations in temperature and humidity, as well as other variables such as water and reagent quality, an inadequate cold chain, operator ability, and poor or non-existent quality assurance systems, can all contribute to lower test accuracies than those reported by POCT manufacturers or obtained in an accredited laboratory (see Figure 1 Stage 5). The consequences of a positive test result and the need for confirmatory testing and reporting by an accredited laboratory in particular when performing a test for an exotic disease need to be embedded in existing testing policies and guidelines. POCT-specific standards and recommendations, such as the point-of-care key evidence tool (POCKET) checklist for multi-dimensional evidence reporting; scorecards and guidelines for POCT evaluation; and guidelines on quality practices in non-instrumented POCTs (i.e. those that do not require a specific piece of equipment) and ISO/TS 22583:2019 Guidance for supervisors and operators for POCT devices provide guidance for healthcare workers. In some countries, such as Germany, POCTs that detect notifiable and reportable animal diseases require formal authorisation by the national licensing authority. In other countries, POCT accreditation with organisations such as the WOAH and national testing authorities is encouraged but not mandatory (Halpin* et al., *2021; Hobbs* et al., *2020; and Section 2.5* Robustness and ruggedness *below*).

PRELIMINARY CONSIDERATIONS IN ASSAY DEVELOPMENT AND VALIDATION

All laboratories should comply with the requirements of Chapter 1.1.5 *Quality management in veterinary testing laboratories* (Newberry & Colling, 2021). This will minimise the influence of factors that do not depend on the test itself such as instrumentation, operator error, reagent choice (both chemical and biological) and calibration, reaction vessels and platforms, water quality, pH and ionicity of buffers and diluents, incubation temperatures and durations, and errors in the technical performance of the assay. Comprehensive and well-designed experiments are required to develop and optimise assays with favourable analytical characteristics. The underlying principles are broadly applicable to all assay types and, when conducted with appropriate rigour, provide the foundations for high-quality diagnostic tests that are fit for their intended purpose(s) Bowden *et al.* (2021). In their review of WOAH recommended diagnostic tests, Cullinane & Garvey (2021) concluded that enzyme-linked immunosorbent assay (ELISA) and molecular assays were the most commonly used WOAH-recommended tests, which is why examples in the following sections focus on these methods (Mayo *et al.,* 2021).

The first step in assay development is to define the purpose of the assay, because this guides all subsequent steps in the validation process. Assay validation criteria are the characterising traits of an assay that represent decisive factors, measures or standards upon which a judgment or decision may be based. By considering the variables that affect an assay's performance, the criteria that must be addressed in assay validation become clearer. The variables can be grouped into categories such as the: (a) sample – individual or pooled, matrix composition, and host/organism interactions affecting the target analyte quantitatively or qualitatively; (b) assay system – physical, chemical, biological and operator-related factors affecting the capacity of the assay to detect a specific analyte in

the sample; and (c) test result interpretation – the capacity of a test result, derived from the assay system, to predict accurately the status of the individual or population relative to the purpose for which the assay is applied.

Selection, collection, preparation, preservation and management of samples are critical variables in design and development of an assay to ensure valid test results. Other variables such as transport, chain of custody, tracking of samples, and the laboratory information management system are also key sources of variation/error that become especially important when the assay is used for routine testing. Integrity of laboratory-based experimental outcomes during assay development and validation is only as good as the quality of the samples used. Anticipating factors that can negatively impact sample quality must precede launching an assay validation effort. Reference samples used in assay development and validation should be in the same matrix that is to be used in the assay (e.g. serum, tissue, whole blood) and representative of the species to be tested by the assay. The reference materials should appropriately represent the range of analyte concentration to be detected by the assay. Virtual biobanks have become relevant resources with respect to reagents and samples during test development and validation. For example, once the genomic sequence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) became available, European Virus Archive Global (EVAg) Members rapidly developed virus stocks, diagnostic tests and positive controls and, within 10 weeks, over 1500 products had been distributed worldwide for diagnostic or research purposes (Watson *et al.,* 2021). Chapter 2.2.6 *Selection and use of reference samples and panels* provides an overview about selection and use of reference samples and panels to address relevant validation parameters such as repeatability, reproducibility, DSe and DSp etc. Information on sample collection, preparation, preservation, management, and transport is available in Chapters 1.1.2 *Collection, submission and storage of diagnostic specimens* and 1.1.3 *Transport of biological materials*.

The matrix in which the targeted analyte is found (serum, faeces, tissue, etc.) may contain endogenous or exogenous inhibitors that may interfere with the performance of the assay. This is of particular concern for enzyme-dependent tests such as polymerase chain reaction (PCR) or ELISA. Other factors that affect the concentration and composition of the target analyte (particularly antibody) in the sample may be mainly attributable to the host and are either inherent (e.g. age, sex, breed, nutritional status, pregnancy, immunological responsiveness) or acquired (e.g. passively acquired antibody, active immunity elicited by vaccination or infection). Non-host factors, such as contamination or deterioration of the sample, also potentially affect the ability of the assay to detect the specific targeted analyte in the sample. It is also important that biological reagents are free of extraneous agents that might otherwise lead to erroneous results.

THE CRITERIA OF ASSAY DEVELOPMENT AND VALIDATION

Accuracy and precision are two independent parameters which ultimately define the performance of a diagnostic test. Sensitivity, specificity and functions of these variables (e.g. likelihood ratios), change with the cut-off and represent accuracy while repeatability and reproducibility are measures of precision. A reliable test is accurate and precise. Assay performance is affected by many factors beginning with optimisation of the assay. After initial optimisation for an intended purpose, characteristics of the performance of the assay will be tested. The assay may need additional optimisation or may be found to be fit for purpose based on the results of the validation work. WOAH's Secretariat for Registration of Diagnostic Kits (SRDK) has a test validation and certification process where new tests, which are deemed to be fit for purpose(s) can be registered[2].

Criteria for Assay Development and Validation
i) Definition of the intended purpose(s)
ii) Optimisation
iii) Standardisation
iv) Repeatability
v) Analytical sensitivity
vi) Analytical specificity
vii) Thresholds (cut-offs)
viii) Diagnostic sensitivity
ix) Diagnostic specificity
x) Reproducibility
xi) Fitness for the intended purpose(s)

2 https://www.woah.org/en/what-we-offer/veterinary-products/diagnostic-kits/the-register-of-diagnostic-kits/

A. ASSAY DEVELOPMENT PATHWAY

1. Definition of the intended purpose(s) for an assay

Criteria for interpretation of results	
Positive predictive value	(PV^+)
Negative predictive value	(PV^-)
Positive likelihood ratio	(LR^+)
Negative likelihood ratio	(LR^-)

ISO/IEC 17025 (2017) for testing and calibration laboratories states that the laboratory shall use appropriate methods and procedures for all laboratory activities (Newberry & Colling, 2021). In other words, the assay must be 'fit for purpose'. Failure to define the purpose of testing *a priori* will likely lead to errors in both the design of the assay and in the determination of critical test parameters with the potential to invalidate the entire assay development and validation process and ultimately result in a test that does not meet the user's needs. Such errors may occur when, for example, an inappropriate reference population is selected that is heterologous to the population for which the test is being developed.

The qualitative and quantitative assessment of capacity of a positive or negative test result, e.g. predictive value and likelihood ratio to predict with high confidence the infection or exposure status of the animal or population of animals is the ultimate consideration of assay validation (Section B.4.2). This capacity is dependent on development of a carefully optimised and standardised (Section A.2.3) assay that, through accrual of validation data, provides confidence in the assay's ability to perform according to the intended purpose (Table 1).

In order to ensure that test results provide useful diagnostic inferences about animals or populations with regard to the intended purpose, the validation process encompasses initial development and assay performance documentation, as well as on-going assessment of quality control and quality assurance.

Figure 1 shows the assay validation process, from assay design through the development and validation pathways to implementation, deployment, and maintenance of the assay.

The first step of assay development is selection of an assay type that is appropriate and that likely can be validated for a particular use (fitness for purpose).

The most common purposes listed in the WOAH *Terrestrial Manual* are to:

1) Contribute to the demonstration of freedom from infection in a defined population (country/zone/compartment/herd) (prevalence apparently zero):
   - 1a) 'Free' with and/or without vaccination,
   - 1b) Re-establishment of freedom after outbreaks
2) Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes.
3) Contribute to the eradication of disease or elimination of infection from defined populations.
4) Confirm diagnosis of suspect or clinical cases (includes confirmation of positive screening test).
5) Estimate prevalence of infection or exposure to facilitate risk analysis (surveys, herd health status, disease control measures).
6) Determine immune status of individual animals or populations (post-vaccination).

Table 1. Test purposes and relative importance of diagnostic sensitivity (DSe), diagnostic specificity (DSp), positive predictive value (PV^+), negative predictive value (PV^-), likelihood ratio of a positive test result (LR^+) and likelihood ratio of a negative test result (LR^-)

Purpose	Examples and measures of diagnostic accuracy depending on test purpose (Reid *et al.,* 2022)
1a) Historical freedom (with or without vaccination)	In a population that is historically free of a particular disease/pathogen, the prevalence is zero (or close to zero). To be fit for purpose, the test or test algorithm aims to minimise chances of false-positive results and ideally requires **high DSp**, **high PV^+ and high LR+.** This can be achieved by a single test with a high DSp or serial testing(*).
1b) Re-establishment of freedom after outbreak	During the course of a successful disease control programme, a gradual shift in prevalence from high (during the peak of the outbreak) to low (at the tail end of an outbreak) can be expected. During the early stages of a proof-of-freedom testing programme, when disease prevalence remains at non-negligible levels, a fit for purpose test needs a **high DSe**, **high PV^- and high LR^-.** This approach minimises the chances of false-negative results and allows detection of positive individuals. This can be achieved by a single test with a high DSe or parallel testing(*). At the end of the disease control programme when the remaining infected animals have been removed from the population, disease prevalence will be very low and so the proof-of-freedom testing algorithm will likely need to be altered to increase DSp (and thus improve PV^+ and LR^+) similar as in 1a.
2) Certify freedom from infection or agent in individual animals or products for trade/movement purposes	For the purpose of trade and movement the probability of false-negative results needs to be minimised. Otherwise, infected animals could be traded or moved with the potential to spread infection into non-infected, healthy populations. As the test is applied on individuals, no or little information is available about the prevalence or pre-test probability of infection. To be fit for purpose the test or test algorithm aims to minimise chances of false-negative results and ideally requires **high DSe**, **high PV^- and high LR^-**. This can be achieved by a single test with a high DSe or parallel testing(*).
3) Contribute to the eradication of disease or elimination of infection from defined populations	This purpose follows a similar pattern as in 1b, where prevalence is expected to decrease from high to low over time in a defined population.
4) Confirm diagnosis of suspect or clinical cases (includes confirmation of positive screening test)	The goal of a confirmatory test is to minimise the chances of a false-positive result. *Confirmation of clinical cases* For the purpose of confirmation of a clinical case ideally a test with a **high DSp, high PV^+** and **high LR^+** is needed. Because of the clinical manifestation of the disease and expected high pathogen load DSe is not considered to be as relevant. *Confirmation of positive screening samples* Screening tests are applied on healthy populations. They usually have a high DSe to ensure infected individuals are not missed. Only if confirmed by a confirmatory test with a high DSp the animal is considered positive. In this case the confirmatory test* needs to have a **high DSp**, **high PV^+** and **high LR^+**. This approach follows the series testing algorithm(*).
5) Estimate prevalence of infection or exposure to facilitate risk analysis	Epidemiologists require reliable estimates for test accuracy to design sampling plans for prevalence studies, surveys, herd health status and disease control measures. Using a screening test with a high DSe followed by a confirmatory test with a high DSp is a common approach for prevalence estimations.
6) Determine immune status a) in individual animals post-vaccination b) estimate sero-prevalence post-vaccination (research and monitoring of vaccine efficacy)	For this group the aim is to have a **high DSp, PV^+** and **LR^+**. A false-positive result could have fatal consequences because such an animal could in fact not be vaccinated/protected. The higher the accuracy of the test the more precise will be the estimate of post-vaccine seroconversion in individuals and populations. An example is the fluorescent antibody virus neutralisation (FAVN) test to assess the immune status of dogs and cats post-vaccination against rabies virus. For international travel a result of > 0.5 IU/ml is considered to represent acceptable protection.

(*)Multiple testing

Multiple testing consists of using more than one test to determine the infection status of an animal. The most common algorithms are testing in series or in parallel. For example, if two tests are used in series a sample is considered positive only if the first and the second test are positive. Series testing increases DSp but decreases DSe and increases PV^+ and LR^+. Confirmatory testing follows the series testing approach because a positive result from a screening test (high DSe) needs to be confirmed by a second test with a high DSp. The confirmatory test needs to have at least the same DSe as the screening test otherwise they could generate false-negative results, which would be considered a true-negative in this algorithm. Confirmatory testing increases DSp but decreases DSe, and increases PV^+ and LR^+. If two tests are used in parallel a sample is considered positive if any of the tests or both tests are positive. Parallel testing increases DSe but decreases DSp and increases PV^- and LR^-. In addition, for multiple testing algorithms to be effective screening and confirmatory tests should not be conditionally dependent (for example measure the same analyte, such as two ELISAs using the same antigen). Positive dependence in test sensitivity reduces the sensitivity of parallel test interpretation and a positive dependence in test specificity reduces the specificity of serial interpretation (Gardner *et al.,* 2000).

2. Assay development – the experimental studies

2.1. Test method design and proof of concept

Prior knowledge, thought and planning need to go into designing all steps of a new assay destined for validation, or an existing assay that is being modified. Assistance is offered in the recommendations for validation of diagnostic tests[3], which cover best practices for development and validation of assays for detection of various analytes e.g. antibody, antigen, and nucleic acid detection, Chapters 2.2.1 *Development and optimisation of antibody detection assays*, 2.2.2 *Development and optimisation of antigen detection assays*, and 2.2.3 D*evelopment and optimisation of nucleic acid detection assays*, respectively.

Development of any assay is dependent on analyte reference samples that reflect the target analyte, the matrix in which the analyte is found, and the population for which the assay is intended to be used. The reference samples may be sera, fluids (including meat juices) or tissues that contain the analyte of interest or a genomic construct consistent with the target analyte. These reference materials are used in experiments conducted throughout the development process and carried over into the validation of the assay.

Factors that affect the analytical characteristics of diagnostic assays are numerous and may vary according to each assay type, e.g. the main factors affecting the analytical characteristics of serological and molecular assays are described in Bowden *et al.* (2021).

For molecular assays, DSe is more dependent on the ability to obtain the target analyte in a processed sample from an animal that has the disease, than on the inherent ability of the assay to detect very low concentrations of analyte. Although an assay, such as real-time PCR, may be extremely sensitive analytically, this may not always translate to high DSe, due to the potential shortcomings of sampling (small volume, inhibitory substances, variations in the clinical spectrum of disease in an individual animal and efficiency of extraction). The inhibition of Taq polymerase due to sample characteristics may cause false-negative results in a test that otherwise has high analytical sensitivity (ASe). In addition, when evaluating two separate, real-time PCR assays for African swine fever virus (ASFV), it was found that both had comparable ASe (equivalent to 14 genomic copies of ASFV) when using a high-quality plasmid construct containing ASFV VP72 as a template. However, when evaluating various samples collected from clinically diseased pigs, the resulting DSe was determined to be 83% for one assay and 92% for the other (data not shown). The amplicon size of the assay with the lower DSe (250 base pairs or bp) was significantly larger than that of the other assay (75 bp). In general, amplification efficiency would be expected to decrease with increasing amplicon size. This characteristic becomes more pronounced when testing viral nucleic acids extracted from clinical samples, the quality of which may be affected by varying degrees of degradation. Such an outcome had not been evident when using plasmid as the template during determination of the assay's ASe. Furthermore, as an assay such as real-time PCR is so highly sensitive analytically, great care must be taken to prevent carry-over contamination with previously amplified template. Such contamination would cause a false-positive result in an assay that is otherwise also considered very highly specific (analytically) (Bowden & Wang 2021).

3 https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.02.00_INTRODUCTION.pdf

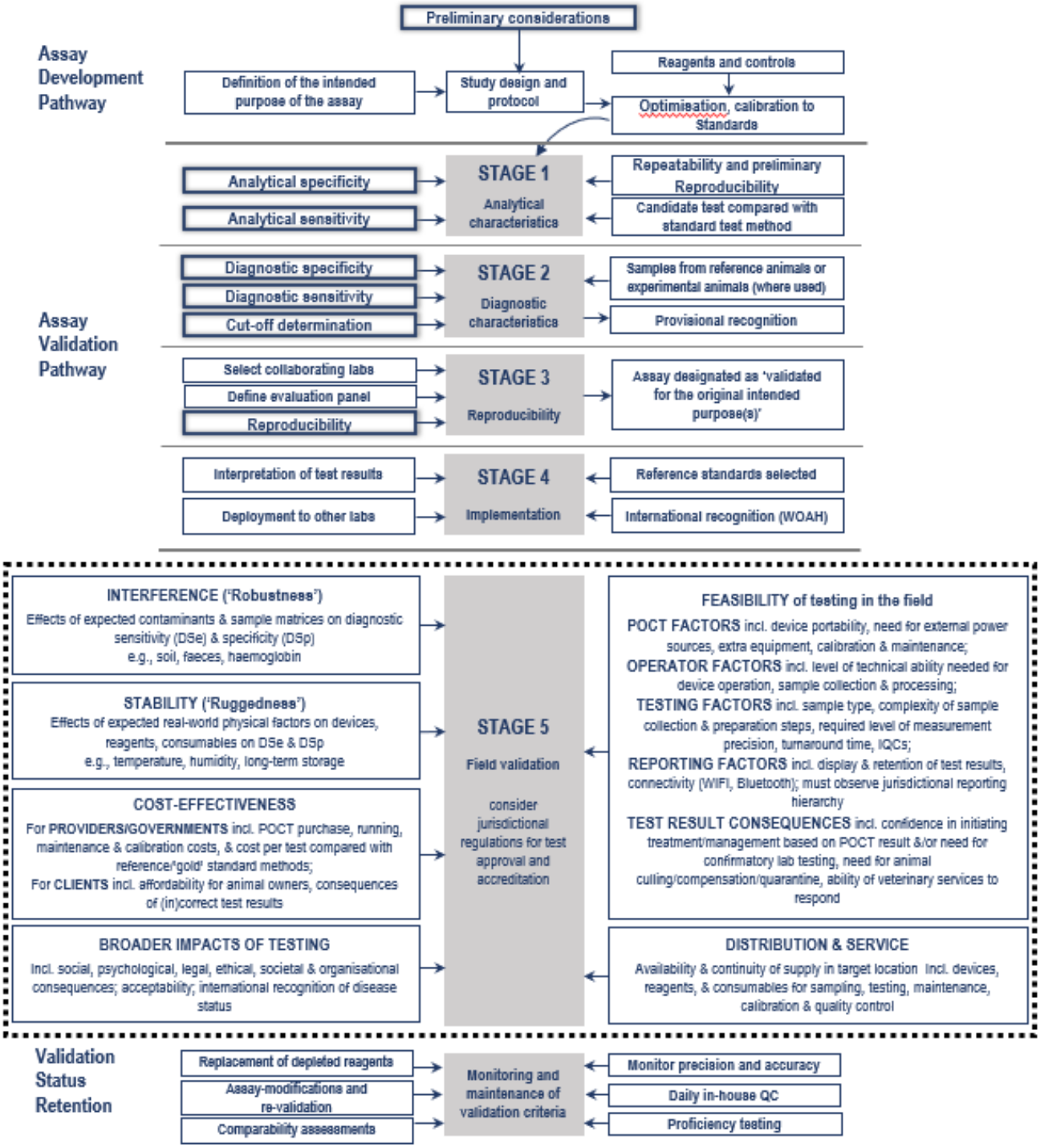

Fig. 1. Modified general assay development and validation pathways (Stages 1–4 and validation status retention) with validation criteria highlighted in bold typescript within shadowed boxes.
Specific criteria for the field validation of POCTs are added in Stage 5 within the dotted box (Halpin et al., 2021); (IQCs = internal quality control sample).

2.2. Standardisation and optimisation

Optimisation is the process by which the most important physical, chemical and biological parameters of an assay are evaluated and adjusted to ensure that the performance characteristics of the assay are best suited to the intended application. It is useful to select at least three well-defined reference samples, representing the analyte ranging from high positive to negative (e.g. strong positive, weak positive and negative). These samples ideally should represent known infected and uninfected animals from the population that will become the target of the assay. Obtaining such reference samples, however, is not

always possible, particularly for nucleic acid and antigen detection assays. The alternative of preparing reference samples spiked with cultured agents or positive sera is inferior as these samples do not truly represent the naturally occurring matrix-agent interaction (see also chapter 2.2.6). When no other alternative exists, spiking a sample with a known amount of the analyte or agent derived from culture, or diluting a high positive serum in negative serum of the same species may be all that is available. In either case, it is imperative that the matrix, into which the analyte is placed or diluted, is identical to, or resembles as closely as possible the samples that ultimately will be tested in the assay. Ideally, reference samples have been well characterised by one or preferably at least two alternate methodologies. These samples can be used in experiments to determine if the assay is able to distinguish between varying quantities of analyte, distinguish the target from closely related analytes, and for optimising the reagent concentrations and perfecting the protocol. In principle, for all assay types, it is highly desirable to prepare and store a sufficient amount of each reference sample in aliquots for use in every run of the candidate assay as it is evaluated through the entire development and validation process. Switching reference samples during the validation process introduces an intractable variable that can severely undermine interpretation of experimental data and, therefore, the integrity of the development and validation process.

The labour-intensive process of optimising an assay is fundamental and critical to achieving a reliable and predictable assay performance. Scientific judgment and use of best scientific practices, as provided in Bowden *et al.* (2021), are recommended to guide optimisation of all elements of assay development and validation. The approach outlined provides a solid foundation for development of a reliable assay. Often, prototype assays are developed using reagents and equipment at hand in the laboratory. However, if the assay is intended for routine diagnostic use in multiple laboratories, standardisation becomes critical. Every chemical and buffer formulation must be fully described. All reagents must be defined with respect to purity and grade (including water). Acceptable working ranges must be established and documented for parameters such as pH, molarity, etc. Standards for quality, purity, concentration and reactivity of biologicals must be defined. Shelf lives and storage conditions must also be considered for both chemicals and biologicals. Acceptable ranges for reaction times and temperatures also need to be established. Essential equipment critical to assay performance must be described in detail, including operational specifications and calibration. Process (quality) control should be an integral part of optimisation and considered from the very beginning rather than, as is often the case at the end of assay development. In addition to the above, downstream aspects such as data capture, data manipulation and interpretation may also require standardisation and optimisation. Finally, all of these parameters, once optimised, must be fully described in the test method protocol.

During optimisation of an assay, it is important to take note of procedural steps and assay parameters that have a narrow range in which the assay performs optimally, as these are the critical points that ultimately affect an assay's reliability (see Section A.2.7). For some assay types, specific steps in the procedure may have more impact than other steps on the final assay performance (see Section B.5 below and Chapter 2.2.8 *Comparability of assays after changes in a validated test method* for additional information on establishing comparability when reagents or processes are changed; also Reising *et al.,* 2021).

A variety of analyte reference samples and other process controls that are routinely included in any assay system are identified in the following sections. These provide critical assay monitoring functions that require special attention during assay optimisation. In addition, attention must be paid to the proper preparation and storage of all biological reagents and reference materials to ensure stability (see Chapter 1.1.2; Watson *et al.,* 2021).

2.3. Operating range of the assay

The operating range of an assay is the interval of analyte concentrations or titres over which the method provides suitable accuracy and precision. Accuracy is the closeness of a test value to the expected (true) value (mean or median) for a reference standard reagent of known concentration or titre. Precision is the degree of dispersion (variance, standard deviation [SD] or coefficient of variation [Cv]) within a series of measurements of the same sample tested under specified conditions. Laboratory sources of variation that affect assay precision include: 1) within a single test run, 2) between concurrent runs, 3a) between assay runs at different times in the same day or on different days under similar conditions, 3b) between assay runs on different days with different operators, 4) between laboratories. In this chapter, categories 1–3 are estimates of repeatability, and category 4 is synonymous with reproducibility. The repeatability of results of operators in different laboratories is likely to be lower (higher SD, higher Cv) than that of operators working in the same laboratory. During development of the assay, the lower and upper limits of the operating range are determined. To formally determine this range, a high positive reference sample is

usually selected (ideally, this sample will be the same one from among the three samples described in Section A.2.3 below). This high positive sample is serially diluted to extinction of the assay's response in an analyte-negative matrix of the same constitution as the sample matrix from animals in the population targeted by the assay. The results are plotted as a 'response-curve', with the response (e.g. optical density, cycle threshold, counts of pathogens, etc.) a function of analyte concentration (amount). The curve establishes the working range of the assay. If the range is found to be unacceptable for the intended purpose, additional optimisation may be needed. The typical calibration curve for most assays is sigmoidal in shape. The data are transformed to approximate a linear relationship between response and concentration using a suitable algorithm (Findlay & Dillard, 2007).

2.4. Inhibitory factors in the sample matrix

Each different matrix to be used in an assay must be used in the validation process. Some sample matrices include inhibitory factors that interfere with the performance of specific types of assays. Serum, particularly if haemolysed, may contain factors toxic to the cells used in viral neutralisation assays, while endogenous substances found in some tissues and fluids can interfere with or inhibit ligand-binding and enzymatic-based assays such as ELISAs. Faeces, autolysed tissues and semen samples tend to contain more interfering substances and are therefore more problematic for assay performance than are serum, blood or fresh tissues. For molecular assays, inhibitors of enzymes in the reaction mix, interferents and degradants may be present in the matrix. Selectivity is essentially the test for detection in the presence of inhibitors. Sample matrix variation is one of the most important, but may be among the least acknowledged, sources of error in analytical measurements. Assessment of ASp should use matrices relevant to the intended purpose, such as solid tissue, whole blood and swabs. Interfering substances (inhibitors) can originate from the samples, e.g. haemoglobin (Schrader *et al.,* 2012; Wilson, 1997), from the environment, or from the process of sample collection and transport (e.g. media and anticoagulants) (Druce *et al.,* 2012; Garcia *et al.,* 2002; Gibb *et al.,* 1998; Miyachi *et al.,* 1998; Yokota *et al.,* 1999;). For example, whole blood is one of the most important and common samples submitted for Hendravirus (HeV) real-time RT-PCR testing. It was found that lithium heparin, a common anticoagulant, had a significant impact on HeV real-time RT-PCR testing, producing false-negative results. In contrast, the anticoagulant ethylenediamine tetra-acetic acid (EDTA) had a comparatively low inhibitory effect. Furthermore, for the real-time RT-PCR assay for detecting bluetongue virus (BTV), diluting blood 1/10 (volume per volume or v/v) in phosphate-buffered saline resulted in improved sensitivity, evidenced by lower Ct values, in comparison to undiluted blood. A recent review of the different types of internal controls available for monitoring the inhibition of real-time PCR-based assays (Yan *et al.,* 2020) provides information on internal control strategies as a routine quality management component in veterinary molecular testing. Data collected during the validation process concerning assay performance using the sample matrices being targeted will allow for a risk-based decision as to whether an inhibition control should be included for each sample or whether the test system is unlikely to be affected by inhibition. If inhibitory substances are a significant problem, an inhibition control must be included for each test sample (Section 1.2; Bowden *et al.,* 2021).

2.5. Robustness

Robustness refers to an assay's capacity to remain unaffected by minor variations in test situations that may occur over the course of testing. Assessment of robustness should begin during assay development and optimisation stages. The deliberate variations in method parameters may be addressed in experiments after optimal conditions for an assay are established. However, when multi-factorial titrations of reagents are used for optimising the assay, indications of a compromised robustness may surface. If slight differences in conditions or reagent concentrations cause unacceptable variability, the assay most likely will not be robust. Early knowledge of this situation elicits a critical decision point for determining whether to continue with validation of the assay would be worthwhile, because if an assay is not robust within one laboratory under rather ideal conditions, it is unlikely to be reproducible when transferred to other laboratories.

The factors most likely to affect assay robustness include pH, temperature, batch of reagents or brand of microtitre plates and aqueous or organic matrix factors (Bowden *et al.,* 2021; Dejaegher & Vander Heyden, 2006). Once optimisation is complete, the robustness of the assay becomes part of the assessment of repeatability. For point-of-care tests (POCTs) (see Figure 1 Stage 5), ruggedness (is expressed as the lack of influence on test results of operational and environmental variables of the analytical method) assessments are also necessary because POCT performance should not be easily affected by operator proficiency, fluctuations in temperature, humidity, sunlight, or other environmental factors.

2.6. Calibration of the assay to standard reagents

2.6.1. International and national reference standards

Ideally, WOAH or other international reference standards, containing a known concentration or titre of analyte, are the reagents to which all assays are standardised (see WOAH Guides[4] and also chapter 2.2.6). Such standards are prepared and distributed by WOAH Reference Laboratories or other international reference laboratories. National reference standards are calibrated by comparison with an international reference standard whenever possible; they are prepared and distributed by a national reference laboratory. In the absence of an international reference standard, a national reference standard becomes the standard of comparison for the candidate assay. These standards are highly characterised through extensive analysis, and preferably the methods for their characterisation, preparation, and storage have been published in peer-reviewed publications (Watson *et al.,* 2021).

2.6.2. In-house standard

An in-house reference standard generally should be calibrated against an international or national standard. In the absence of either of these calibrators and to the extent possible, the in-house standard is highly characterised in the same manner as international and national standards (see chapter 2.2.6). This local in-house standard therefore becomes the best available standard, and is retained in sufficient aliquoted volumes for periodic use as the standard to which working standards are calibrated.

2.6.3. Working standard

One or more working standards, commonly known as analyte or process controls, are calibrated to an international, national, or in-house standard, and are prepared in large quantities, aliquoted and stored for routine use in each diagnostic run of the assay.

2.7. 'Normalising' test results to a working standard

Due to the inherent variation in raw test results that are often observed between test runs of the same assay or among laboratories using the same or similar assays, it is almost impossible to compare directly (semi-) quantitative data. To improve markedly the comparability of test results both within and between laboratories, one or more working standard reagent(s) are included in each run of an assay. Raw test values for each test sample can then be converted to units of activity relative to the working standard(s) by a process called 'normalisation'. The 'normalised' values may be expressed in many ways, such as a per cent of a positive control (e.g. in an ELISA), or as the estimated concentration, e.g. genomic copies or titre of an analyte derived from a standard curve such as a cycle threshold (Ct) value in a hydrolysis probe assay. It is good practice to include working standards in all runs of the assay during assay development and validation because this allows 'normalisation' of data, which provides a valid means for direct comparison of results between runs of an assay. It is mandatory to control the (absolute) variation of the normalisation standards as otherwise normalisation can introduce a bias. For more information, see chapters 2.2.1, 2.2.2 and 2.2.3; Bowden *et al.* (2021).

2.8. Preliminary study of the repeatability

Assessment of repeatability should begin during assay development and optimisation stages. Early knowledge of this situation elicits a critical decision point for determining whether it is worthwhile to continue with validation of the assay.

Repeatability is further verified during Stage 1 of assay validation (Section B.1.1). When the optimised test is run under routine laboratory or field conditions (Stage 4 of assay validation), repeatability is continually monitored as part of process control procedures for the duration of the life of the assay (see Section B.5.1).

4 Available at: https://www.woah.org/en/what-we-offer/veterinary-products/reference-reagents/

B. ASSAY VALIDATION PATHWAY

"Validation" is a process that determines the fitness of an assay that has been properly developed, optimised and standardised for an intended purpose(s). Validation includes estimates of the analytical and diagnostic performance characteristics of a test. In the context of this document, an assay that has completed the first three stages of the validation pathway (Figure 1), including performance characterisation, can be designated as "validated for the original intended purpose(s)". Lack of statistically robust numbers of samples from infected and non-infected animals is frequently observed with new and emerging zoonotic disease and can be a major obstacle to obtaining reliable accuracy estimates (Colling *et al.,* 2018; Stevenson *et al.,* 2021). In these circumstances tests with acceptable analytical sensitivity (ASe) and analytical specificity (ASp), repeatability and promising but preliminary DSe and DSp and reproducibility results based on a limited number of samples can be provisionally recognised by national authorities or trading partners until results from further testing confirm overall fitness for purpose. Well characterised samples have been used successfully for different purposes in interlaboratory comparison studies to assess fitness for purpose (Chapter 2.2.6; Gardner *et al.,* 2021). New platforms that can detect multiple pathogens simultaneously, e.g. multiplex technologies, high-throughput sequencing, biomarker assays and point-of-care tests represent new challenges for purpose-oriented validation studies (Bath *et al.,* 2020; Halpin *et al.,* 2021; Reid *et al*., 2021; van Borm *et al.,* 2016).

1. Stage 1 – Analytical performance characteristics

Ideally, the design of studies outlined in the following sections should be done with assistance of a statistician and a disease expert to ensure that the sample size and experimental approach are valid. It is possible to design experiments that efficiently provide information on likely within- and between-laboratory sources of variation in assay precision[5], which will define the performance characteristics of the assay. The choice of organisms, strains or serotypes to assess analytical sensitivity and specificity should reflect current knowledge and therefore inform the best possible experimental design for targeting specific analytes.

1.1. Repeatability

Repeatability is the level of agreement between results of replicates of a sample both within and between runs of the same test method in a given laboratory. Repeatability is estimated by evaluating variation in results of replicates. The number of replicates should preferably be determined in consultation with a statistician with a suggested minimum of three samples representing analyte activity within the operating range of the assay. Each of these samples is then aliquoted into the appropriate number of individual vessels as identical replicates of the original sample containing the original analyte and matrix concentration (see chapter 2.2.6). Each replicate is then run through all steps of the assay, including creating the working dilution, as though it were a test sample derived from the population targeted by the assay. It is not acceptable to prepare a final working dilution of a sample in a single tube from which diluted aliquots are pipetted into reaction vessels, or to create replicates from one extraction of nucleic acid rather than to extract each replicate before dilution into the reaction vessels. Such 'samples' do not constitute valid replicates for repeatability studies. Between-run variation is determined by using the same samples in multiple runs involving two or more operators, done on multiple days. Bowden & Wang (2021) provide an example for inter-assay repeatability of three ASFV real-time PCR assays that were evaluated using a weak-positive ASFV sample. The testing, including the extraction of viral DNA, was conducted on ten different days by different operators in the same laboratory. The variation in replicate results can be expressed as standard deviations, coefficients of variation (standard deviation ÷ mean of replicates), or other possible options (see chapter 2.2.4 *Measurement uncertainty* for assessments of repeatability).

1.2. Analytical specificity

ASp is the ability of the assay to distinguish the target analyte (e.g. antibody, organism or genomic sequence) from non-target analytes, including matrix components. The assessment is qualitative and the choice and sources of sample types, organisms and sequences for the ASp evaluation should reflect test purpose and assay type. See chapters 2.2.1, 2.2.2 and 2.2.3 for guidance for antibody, antigen and nucleic acid assays, respectively. For example, assessing the analytical specificity of a foot and mouth disease

5 Precision may be evaluated in several ways by testing the same replicated sample: 1) within a plate or plates in a run of the assay, 2) between plates run concurrently within a run of the assay, 3a) between assay runs at different times in the same day or on different days under similar conditions, 3b) between assay runs on different days with different operators, 4) between laboratories. In this chapter, precision categories 1–3 are estimates of repeatability, and precision category 4 is synonymous with reproducibility. Levels 3a and 3b are also known as intermediate precision.

(FMD) PCR could be performed by testing reference samples known to be positive for vesicular stomatitis virus, swine vesicular disease and/or malignant catarrhal fever. Analytical specificity assessments cannot determine the full range of potential cross-reacting analytes present in the population, or account for population-level sample variability; as such, determining the analytical specificity is not a surrogate for assessment of diagnostic specificity. ASp is documented during Stage 1 validation, and cross-reactions identified (Ludi *et al.,* 2021). Cross-reactivity (ASp less 100%) may be acceptable depending on the proposed use of the assay. The impact of cross-reactivity is further documented during Stage 2 (establishment of DSp) and assessed at Stage 4 implementation.

1.2.1. Selectivity

Selectivity refers to the extent to which a method can accurately quantify the targeted analyte in the presence of: 1) interferents such as matrix components (e.g. inhibitors of enzymes in the reaction mix; 2) degradants (e.g. toxic factors); 3) nonspecific binding of reactants to a solid phase (e.g. conjugate of an ELISA adsorbed to well of microtiter plate); and 4) antibodies to vaccination that may be confused with antibodies to active infection. Such interferents may cause falsely reduced or elevated responses in the assay that negatively affect its analytical specificity. Vessman *et al.* (2001) is a useful overview of selectivity as defined for analytical chemistry from which a modification described herein was deduced for application to diagnostic tests.

1.2.2. Exclusivity

Exclusivity is the capacity of the assay to detect an analyte or genomic sequence that is unique to a targeted organism, and excludes all other known organisms that are potentially cross-reactive. This would also define a confirmatory assay. For example, an assay to detect avian influenza virus (AIV) H5 subtypes should be assessed for cross-reaction with non-H5 AIV subtypes. Specificity testing should also include other organisms that cause similar clinical signs, to demonstrate the utility of the assay for differential detection of the target organism.

1.2.3. Inclusivity

Inclusivity is the capacity of an assay to detect several strains or serovars of a species, several species of a genus, or a similar grouping of closely related organisms or antibodies thereto. It characterises the scope of action for a screening assay, e.g. a group-specific bluetongue (BTV) ELISA that detects antibodies to all BTV serotypes or an NSP FMD ELISA that detects antibodies to all seven FMD serotypes .

1.3. Analytical sensitivity

The limit of detection (LOD) is a measure of the ASe of an assay. The LOD is the estimated amount of analyte in a specified matrix that would produce a positive result at least a specified percent of the time. Typically, estimated LOD will be based on spiking of the analyte into the target matrix. The choice of analyte(s) (e.g. species, strains) is part of the ASe definition and should be reported properly. These experiments may be designed for precise and accurate estimation of the probability point (e.g. 50% or 100%), but in some circumstances a conservative estimate of the LOD (e.g. 100%) may be acceptable. For example, in a titration using tenfold dilutions all replicates at all dilutions might show either 100% or 0% response. There are two choices at that point. The last dilution showing 100% response may be accepted as a conservative estimate of the lower limit of detection. A more accurate estimate may be obtained by a second stage experiment using narrower intervals in the dilution scheme focusing on the region between 100% and 0%. Methods for statistical evaluation of LOD data are in the Chapter 2.2.5 *Statistical approaches to validation*.

1.4. Analytical accuracy of ancillary tests or procedures

Some test methods or procedures may be qualified for use as analytical tools in the diagnostic laboratory. These usually are secondary tests or procedures that are applied to an analyte that has been detected in a primary assay. The purpose of such analytical tools is to further characterise the analyte detected in the primary assay. Examples of such adjunct tests include virus neutralisation to type an isolated virus, molecular sequencing and MALDI-TOF-MS (matrix assisted laser desorption ionisation time of flight mass spectrometry) for bacteria (Ricchi *et al.,* 2016).

Such ancillary tests must be validated for analytical performance characteristics (Sections A.2 through B.1.3, above). However, they differ from diagnostic tests because they do not require validation for diagnostic performance characteristics (Sections B.2 through B.4, below) if their results are not used to establish a final diagnosis with regard to the intended purpose. The analytical accuracy of these tools may be defined by comparison with a reference reagent standard, or by characteristics inherent in the tool itself (such as endpoint titration). Inthese examples, the targeted analyte is further characterised quantitatively or qualitatively by the analytical tool.

2. Stage 2 – Diagnostic performance of the assay

Diagnostic sensitivity
Percentage of known infected animals that test positive in the assay; infected animals that test negative are considered to have false-negative results.

Diagnostic specificity
Percentage of known uninfected animals that test negative in the assay; uninfected animals that test positive are considered to have false-positive results.

Animal samples used to assess DSe and DSp come from four main sources: (1) reference banks with samples of known infection status, (2) outbreak or surveillance samples where animal status is unknown but population status (infected or not infected) is known, (3) neither animal nor population status known, and (4) experimental challenge studies (see Table 3 for advantages and limitations of each method – in the Table, groups 2 and 3 are combined for brevity). DSe (proportion of samples from known infected animals that test positive in an assay) and DSp (the proportion of samples from known uninfected animals that test negative in an assay) are the primary performance indicators established during validation of an assay (see chapters 2.2.1., 2.2.2., 2.2.3). These estimates are the basis for calculation of other parameters from which inferences are made about test results (e.g. predictive values and likelihood ratios of positive and negative test results). Therefore, it is imperative that estimates of DSe and DSp are as accurate as possible. Ideally, they are derived from testing a panel of samples from animals of known history and infection status relative to the disease/infection in question and relevant to the country or region in which the test is to be used. An estimate of the area under the receiver operating characteristic (ROC) curve is a useful adjunct to DSe and DSp estimates for a quantitative diagnostic test because it assesses its global accuracy across all possible assay values (Greiner *et al.,* 2000; Zweig & Campbell, 1993). This approach is described in chapter 2.2.5.

Case definition, e.g. what constitutes an infected animal and what constitutes an uninfected animal, respectively, e.g. clinical signs, fever, results from a reference test, etc.

Reference samples: The designated number of known positive and known negative reference samples will depend on the likely values of DSe and DSp of the candidate assay and the desired confidence level for the estimates (Table 2 and Jacobson, 1998). Table 2 provides two panels of the theoretical number of samples required, when either a 5% or 2% error is allowed in the estimates of DSe or DSp. Many samples are required to achieve a high confidence (typically 95%) in the estimates of DSe and DSp when a small error margin in the estimate is desired. For example, comparison of a 2% vs 5% error for a likely DSe or DSp of 90% and 95% confidence shows a considerable increase (864 *vs* 138) in the number of samples required. For the most economically important listed diseases, 99% confidence might be preferred. Logistical and financial limitations may require that less than the statistically required sample size will be evaluated, in which case the confidence interval calculated for DSe and DSp will indicate less diagnostic confidence in the results. Sample size also may be limited by the fact that reference populations and WOAH reference standards may be lacking (see chapter 2.2.5 for further details). Therefore, it may be necessary to use fewer samples initially. It is, however, highly desirable to enhance confidence and reduce error margin in the DSe and DSp estimates by adding more samples (of equivalent status to the original panel) as they become available.

Table 2. Theoretical number of samples from animals of known infection status required for establishing diagnostic sensitivity (DSe) and specificity (DSp) estimates depending on likely value of DSe or DSp and desired error margin and confidence

Estimated DSe or DSp	2% error allowed in estimate of DSe and DSp			5% error allowed in estimate of DSe and DSp		
	Confidence			Confidence		
	90%	95%	99%	90%	95%	99%
90%	610	864	1493	98	138	239
92%	466	707	1221	75	113	195
94%	382	542	935	61	87	150
95%	372	456	788	60	73	126
96%	260	369	637	42	59	102
97%	197	279	483	32	45	77
98%	133	188	325	21	30	52
99%	67	95	164	11	15	26

The following are examples of reference populations and methodologies that may aid in estimating performance characteristics of the test being validated.

2.1. Reference animal populations

Ideally, selection of reference animals requires that important host variables in the target population are represented in animals chosen for being infected with or exposed to the target agent, or that have never been infected or exposed (Table 4). The variables to be noted include but are not limited to species, age, sex, breed, stage of infection, vaccination history, and relevant herd disease history (for further details see chapter 2.2.6). After the initial detection of a novel disease, reference samples may not exist or be in limited quantities. In these situations, samples from experimental challenge studies might be the only available sample for initial validation of assays.

2.1.1. Negative reference samples

True negative samples, from animals that have had no possible infection or exposure to the agent, may be difficult to locate. It is often possible to obtain these samples from countries or zones that have eradicated or have never had the disease in question. Such samples may be useful as long as the targeted population for the assay is sufficiently similar to the sample-source population.

2.1.2. Positive reference samples

It is generally problematic to find sufficient numbers of true-positive reference animals, as determined by isolation of the pathogen. It may be necessary to resort to samples from animals that have been identified by another test of sufficiently high accuracy, such as a validated nucleic acid detection assay. The candidate test is applied to these reference samples and results (positive and negative) are cross-classified in a 2 × 2 table. This has been called the “gold standard model” as it assumes the reference standard is perfect. A sample calculation is shown in Table 4 in Section B.2.5). Situations where a perfect reference is available for either positive or negative animals, and one where the reference is perfect for both are described for diagnostic test validation by Heuer & Stevenson (2021).

2.2. Samples from animals of unknown status

In some situations, the infection status of a population may be known. If the population is known to be non-infected, then all animals in that population are also assumed to be non-infected. In an infected population, not all animals will be infected especially if the disease is not highly contagious. Deducing the population’s status is more challenging if the infection is subclinical or covert or if animals are protected by vaccination

or prior exposure to a pathogen. When the so-called reference standard (true infection status) is imperfect, which is the rule with any diagnostic tests used to test field samples, estimates of DSe and DSp for the candidate assay based on this standard will be flawed. A way to overcome this problem is to perform a latent class analysis (LCA) of the joint results of two or more tests assuming neither test is perfect (e.g. Johnson *et al.,* 2019).

Latent-class models do not rely on the assumption of a perfect reference test but rather estimate the accuracy of the candidate test and a reference test using the joint test results (Branscum *et al.,* 2005; Enoe *et al.,* 2000; Georgiadis *et al.,* 2003; Hui & Walter, 1980). If a Bayesian framework is used for the LCA, prior knowledge about the DSe and DSp of the reference test and the candidate test can be incorporated into the analysis. The infection status of source populations can also be specified in a Bayesian LCA and inclusion of animal samples from known non-infected populations can enhance the ability of these models to estimate DSp and other parameters in the model (e.g. DSe and prevalence) more precisely than would occur if only data from two or more infected populations are used. Three key assumptions must normally be satisfied in a LCA: (1) when data from multiple populations are used, each population prevalence should be different; (2) the DSe and DSp of the test are constant across test populations; and (3) the tests are conditionally independent. However, if conditional dependence exists between the index and reference tests (e.g. they both measure a similar analyte), latent class models (LCM) with different dependence structure have been developed to model the conditional dependence among tests.

Because Bayesian latent class models are complex and require adherence to critical assumptions, statistical assistance should be sought to help guide the analysis and describe the sampling from the target population(s), the characteristics of other tests included in the analysis, the appropriate choice of model and the estimation methods should be based on peer-reviewed literature (see chapter 2.2.5 for details and Cheung *et al.,* 2021). Sample size calculations for a LCA require assistance from a statistician or epidemiologist with experience with the techniques.

Table 3. Source populations for test validation samples

Reference sample banks (e.g. from Ref. Labs)	Field populations with animals of unknown infection status	Experimental challenge trials
Advantages • Infection status known • Simple statistical analysis **Limitations** • Often not available for rare or new diseases • May not be representative of animals to be tested in the future • Clinical status, results of other tests and demographic information may be missing	**Advantages** • Representative of animals to be tested in future • Latent class models (LCM) used for data analysis: infection status of population and covariates can be added • Assessment of accuracy of multiple assays is possible **Limitations** • LCM requires expertise and training, and adherence to model assumptions	**Advantages** • Infection status based on challenge group • Assessment of "diagnostic window" of an assay **Limitations** • Samples from challenge trials may not mirror samples collected in the field • Often a secondary outcome in a pathogenesis study • Route of exposure, infectious dose, and other experimental conditions can be influential • Best suited to acute infections • Ethical considerations

2.3. Experimentally infected or vaccinated reference animals

Samples obtained sequentially from experimentally infected or vaccinated animals are useful for determining the kinetics of antibody responses or the presence/absence of antigen or organisms in samples from such animals (Table 3). However, multiple serially acquired pre- and post-exposure results from individual animals are not acceptable for establishing estimates of DSe and DSp because the statistical requirement of independent observations is violated. Single time-point sampling of individual experimental animals can be acceptable (e.g. one sample randomly chosen from each animal). Nevertheless, it should be noted that for indirect methods of analyte detection, exposure to organisms under experimental conditions, or vaccination, may elicit antibody responses that are not quantitatively and qualitatively typical of natural

infection in the target population (Jacobson, 1998). The strain of organism, dose, and route of administration to experimental animals are examples of variables that may introduce error when extrapolating DSe and DSp estimates to the target population. In cases when the near-impossibility of obtaining suitable reference samples from naturally exposed animals necessitates the use of samples from experimental animals for validation studies, the resulting DSe and DSp measures should be considered as "proof-of-concept" results and less than ideal estimates of the true DSp and DSe.

2.4. Selection of a cut-off (threshold) value for classification of test results

To obtain DSe and DSp estimates of the candidate assay, which is measured on a continuous scale, the test results must first be reduced to two (positive or negative) or three (positive, intermediate [doubtful] or negative) categories of test results. This is accomplished by insertion of one or two cut-off points (threshold or decision limits) on the scale of test results. The selection of the cut-off(s) should reflect the intended purpose of the assay and its application, and must support the required DSe and DSp of the assay. Options and descriptive methods for determining the best way to express DSe and DSp are available (Branscum *et al*., 2005; Georgiadis *et al.,* 2003; Greiner *et al.,* 1995; 2000; Jacobson, 1998; Zweig & Campbell, 1993; and chapter 2.2.5). If considerable overlap occurs in the distributions of test values from known infected and uninfected animals, it is impossible to select a single cut-off that will accurately classify these animals according to their infection status. Rather than a single cut-off, two cut-offs can be selected that define a high DSe (e.g. inclusion of 99% of the values from infected animals), and a high DSp (e.g. 99% of the values from uninfected animals) (Greiner *et al.,* 1995).

The main difficulty in establishing cut-offs based on diagnostic performance characteristics is the lack of availability of the required number of well-characterised (reference) samples. Alternatives are discussed in Section B.2.6 on provisional acceptance of an assay during accrual of data to enhance estimates of DSe and DSp.

2.5. Calculation of DSe and DSp based on test results of reference samples

A typical method for determining DSe and DSp estimates is to test the reference samples in the new assay, and cross tabulate the categorical test results in a 2 × 2 table. In a hypothetical example, assume the test developer has selected a sample size for DSe and DSp for the new assay under the assumption that the most likely values are 97% (DSe) and 99% (DSp), respectively, with a desired confidence of 95% for both estimates. The desired error margin in the estimates was set at 2%. Table 4 indicates that 279 samples from known infected animals are required for the DSe assessment, and 95 known negative samples are needed for establishing the DSp estimate. Assume that the samples were then run with the new assay. Table 4 is a hypothetical set of results from which DSe and DSp estimates have been obtained.

Table 4. Diagnostic sensitivity and specificity estimates calculated from hypothetical set of results for samples tested from known infected and non-infected populations

		Number of reference samples required*	
		Known positive (279)	Known negative (95)
Test results	Positive	270 TP	FP 7
	Negative	FN 9	TN 88
		Diagnostic sensitivity* TP/(TP + FN) **96.8%** (94.0 – 98.5%)**	Diagnostic specificity* TN/(TN + FP) **92.6%** (85.4 – 97.0%)**

*Based on Table 2 for an assay with the following parameters:
1) Prior to testing, estimated DSe of 97% and DSp of 99%
2) 95% = required confidence in DSe and DSp estimates
3) 2% = Error margin in the estimates of DSe and DSp
TP and FP = True Positive & False Positive, respectively
TN and FN = True Negative and False Negative, respectively
**95% exact binomial confidence limits for DSe and DSp calculated values
(see chapter 2.2.5 for information on confidence limits)

In this example, the DSe estimate is as anticipated, but the DSp is much lower (92%) than the anticipated value of 99%. As a consequence, the width of the confidence interval for DSp is greater than expected. Re-inspection of Table 2 indicates that 707 samples are necessary to achieve an error margin of ± 2% at a DSP of 92% but such an increase in sample size might not be feasible (see chapter 2.2.5 for further details).

2.6. Provisional assay recognition[6]

There are situations where it is not possible or desirable to fulfil Stage 2 of the Validation Pathway because appropriate samples from the target population are scarce and animals are difficult to access (such as for transboundary infectious diseases or wildlife diseases).

Experience has shown that the greatest obstacle for continuing through Stage 2 of the Validation Pathway is the number of defined samples required to calculate DSe and DSp. The formula is well known and tables are available for determining the number of samples required to estimate various levels of DSe and DSp, depending on the desired error margin and the level of confidence in the estimates (Table 2 and Jacobson, 1998). The formula assumes that the myriad of host/organism factors that may affect the test outcome are all accounted for. Since that assumption may be questionable, the estimated sample sizes are at best minimal. For a disease that is not endemic or widespread, it may be impossible, initially, to obtain the number of samples required, but over time, accrual of additional data will allow adjustment of the cut-off (threshold) or if no adjustment is needed, enhance confidence in the estimates.

Provisional recognition defines an assay that has been assessed through Stage 1 for critical assay benchmark parameters (ASe, ASp and repeatability) with, in addition, a preliminary estimate of DSp and DSe based on a small select panel of well-characterised samples containing the targeted analyte and a preliminary estimate of reproducibility. This represents partial completion of Stage 2. Preliminary reproducibility estimates of the candidate assay could be done using the select panel of well-characterised samples to enhance provisional acceptance status for the assay. The candidate test method is then duplicated in laboratories in at least two different institutes, and the panel of samples is evaluated using the candidate assay in each of these laboratories, using the same protocol, same reagents as specified in the protocol, and comparable equipment. This is a scaled-down version of the reproducibility study in Stage 3 of assay validation. In following this procedure of provisional recognition the test protocol must not be varied.

Provisional recognition
Test with acceptable ASe and ASp, repeatability and promising preliminary DSe and DSp based on a small select panel of well-characterised samples containing the targeted analyte and a preliminary estimate of reproducibility. Results can be provisionally recognised until adequate samples sizes for both DSe and DSp are obtained (refer to Table 2) and a more extensive reproducibility study is done to confirm overall fitness-for-purpose.

Provisional recognition of an assay by state or national authorities means that the assay has not been evaluated for diagnostic performance characteristics. As such, the laboratory should develop and follow a protocol for adding and evaluating samples, as they become available, to fulfil this requirement. Ideally, this process should be limited to a specific timeframe in which such an accrual would be directed toward fulfilling Stages 2 and 3 of the validation pathway, and to particular situations (emergencies, minor species, no other test available, etc.)

3. Stage 3 – Reproducibility and augmented repeatability estimates

3.1. Reproducibility

Reproducibility is the ability of a test method to provide consistent results, as determined by estimates of precision, when applied to aliquots of the same samples tested in different laboratories, preferably located in distinct or different regions or countries using the identical assay (protocol, reagents and controls). To assess the reproducibility of an assay, each of at least three laboratories should test the same panel of samples (blinded) containing a suggested minimum of 20 samples, with identical aliquots going to each laboratory (see chapter 2.2.6). This exercise also generates preliminary data on non-random effects attributable to deployment of the assay to other laboratories. In addition, within-laboratory repeatability estimates are augmented by the replicates used in the reproducibility studies. Measurements of precision

6 Provisional recognition does not imply acceptance by WOAH. It does, however, recognise an informed decision of authorities at local, state, national or international levels of their conditional approval of a partially validated assay.

can be estimated for both the reproducibility and repeatability data (see chapter 2.2.4 for further explanation of the topic and its application). Factors affecting testing reproducibility among laboratories and practical examples of proficiency testing and interlaboratory comparison testing are provided by Johnson & Cabuang (2021) and Waugh & Clark (2021). A case study with FMD for selection and use of reference panels is presented in Ludi *et al.* (2021) and the value of virtual biobanks for transparency purposes with respect to reagents and samples used during test development and validation is reported by Watson *et al.,* 2021.

For POCTs, reproducibility should be evaluated under the conditions of intended use.

3.2. Designation of a validated assay

On completion of Stage 3 validation, assuming the earlier stages have been fully and satisfactorily completed, the assay may be designated as "validated for the original intended purpose". Retention of this designation is dependent on continual monitoring of the assay performance, as described in Section 5.1.

4. Stage 4 – Programme implementation

The successful deployment of an assay provides additional and valuable evidence for its performance according to the expectations. Moreover, the (true) prevalence of the diagnostic trait in the target population is an important factor that needs to be accounted for as described below.

4.1. Fitness for use

While this chapter deals with validation and fitness for purpose from a scientific perspective, it should also be noted that other practical factors might impact the utility of an assay with respect to its intended application. These factors include not only the diagnostic suitability of the assay, but also its acceptability by scientific and regulatory communities, acceptability to the client, and feasibility given available laboratory resources. For some diseases, multiple assays might be available for use in combination in disease control and surveillance programmes and hence, an assay's utility might need to be assessed by evaluating incremental changes in DSe, DSp and predictive values of the combined tests.

An inability to meet operational requirements of an assay also may make it unfit for its intended use. Such requirements may include performance costs, equipment availability, level of technical sophistication and interpretation skills, kit/reagent availability, shelf life, transport requirements, safety, biosecurity, sample throughput, turn-around times for test results, aspects of quality control and quality assurance, and whether the assay can practically be deployed to other laboratories. Test kits used in the field are highly desirable from an ease-of-use viewpoint, but because they are performed outside the confines of a controlled laboratory environment, they require added precautions to maintain fitness for purpose (Crowther *et al.,* 2006; Halpin *et al.,* 2021). Examples supporting each of the six purposes are provided in Table 1 *Test purposes and relative importance of diagnostic sensitivity* (*DSe*), *diagnostic specificity* (*DSp*)*, positive predictive value* (*PV*$^+$), *negative predictive value* (*PV*$^-$)*, likelihood ratio of a positive test result* (*LR*$^+$) *and likelihood ratio of a negative test result* (*LR*$^-$).

4.2. Interpretation of test results

Predictive values of test results: The positive predictive value (PPV) is the probability that an animal that has tested positive is in fact positive with regard to the true diagnostic status. The negative predictive value (NPV) is the probability that an animal that has tested negative is in fact negative with regard to the true diagnostic status.

Predictive values of test results are an application of Bayes' theorem and are calculated as follows:

$$PPV = \frac{P\ x\ DSe}{P\ x\ DSe + (1-P)\ x\ (1-DSp)} \quad \text{and} \quad NPV = \frac{(1-P)\ x\ DSp}{P\ x\ (1-DSe) + (1-P)\ x\ DSp}$$

Where:

PPV = Predictive value of a positive test result

NPV = Predictive value of a negative test result

P = Prevalence of infection

DSe = Diagnostic sensitivity

DSp = Diagnostic specificity

In contrast to DSe and DSp, predictive values are influenced by the true prevalence of the true infection status of the target population. In other words, predictive values are not inherent characteristics of a specific diagnostic test, but are a function of its DSe and DSp and the local prevalence of infection in a defined population at a given point in time.

Predictive values are of great importance to field veterinarians for the interpretation of results. For example, a PPV of 0.9 means that an animal reacting positive to the test has 90% chance of being indeed infected and 10% probability of being a false positive.

The predictive value of a positive result also has great importance for the veterinary services in charge of the management of control or eradication programmes. If we consider the inverse of the PPV (i.e. 1/PPV) it gives the information on how much money is spent in the culling of true and false positives for each true positive animal detected by the surveillance activity. In other words, if the PPV is 0.67, it means that two positive animals out of three are true positives and the remaining is a false positive. Since during the application of a control programme, the prevalence of infection is continually changing, the monitoring of the PPV is a way of evaluating the costs of the programme.

Furthermore, during the application of a control programme it is usually advisable to change the sensitivity of the tests employed, based on the variation of prevalence of infection in the target population and on the objective of the programme, the PPV may be used to make the changes in DSe and DSp based on economic considerations. In other words, when the need for a change in DSe and DSp of the test arises, a number of putative cut-offs may be set along the ROC curve of the test validation and the relevant values of DSe and DSp for each cut-off may be used to evaluate the expected cost for the culling of each infected animal.

If the purpose is establishing evidence for freedom from disease, the NPV is the more important measure. The NPV critically depends on DSe. Whilst predictive values can be a useful tool in diagnostic test interpretation, they are highly dependent on the prevalence of disease in the population. Predictive values calculated in populations with high prevalence, or at the peak of an outbreak where disease prevalence is high, are not applicable in populations with low prevalence or at the tail end of an outbreak where disease prevalence is markedly decreased.

The likelihood ratio (LR) indicates the diagnostic power of a given test result and can be used to assist in test interpretation. The likelihood ratio is an inherent characteristic of the test; it depends solely on the combined diagnostic sensitivity and diagnostic specificity and therefore does not vary with prevalence. Likelihood ratios are extremely powerful, as they can be used to calculate the 'post-test' probability of disease, given the observed quantitative test result, and the diagnostician's assessment of the probability of infection, prior to the test being performed (Caraguel & Colling, 2021).

The LR is calculated as the ratio of the likelihood of a given test result occurring in infected individuals to the likelihood of the same test result occurring in non-infected individuals. If the LR is lower than one, the test result supports the absence of the infection, i.e. this test result is less likely to occur in infected animals than in non-infected animals. A LR equal to '1' means that the test result has no diagnostic power (i.e. it is as likely to occur in infected animals as it is in non-infected animals). The further the LR is away from one, towards either zero or infinity, the stronger the evidence provided by the test result. In the clinical context, test outputs with an LR > 10 or < 0.1 are considered good diagnostic evidence of the infection being either present or absent, respectively.

$$LR^{+} = \frac{DSe}{1 - DSp} \quad \text{and} \quad LR^{-} = \frac{1 - DSe}{DSp}$$

Where:

LR^{+} = Likelihood ratio of a positive test result

LR^{-} = Likelihood ratio of a negative test result

DSe = Diagnostic sensitivity

DSp= Diagnostic specificity

LR ranges from zero to infinity. If the LR of a given test result is greater than one, this test result supports the presence of the infection. LRs can be applied at the test cut-off or at different ranges of results.

4.3. International recognition

Traditionally, assays have been recognised internationally by WOAH when they are designated as prescribed or alternate tests for trade purposes. This has often been based on evidence of their usefulness on a national, regional or international basis. For commercial diagnostic kits that have gone through the WOAH procedure for validation and certification of diagnostic assays, the final step is listing of the test in the WOAH Register. Tests listed in the Register are certified as fit for a specific purpose if they have completed Validation Stages 1, 2 and 3 followed by supportive review from a panel of independent experts. The Register is intended to provide potential kit users with an informed and unbiased source of information about the kit and its performance characteristics for an intended purpose (Gifford *et al.,* 2021). The Register is available on the WOAH website (see footnote 2).

4.4. Deployment of the assay

Ultimate evidence of the usefulness of an assay is its successful application(s) in other laboratories and inclusion in national, regional and/or international control or surveillance programmes. Reference laboratories play a critical role in this process (Brown *et al.,* 2021). In the natural progression of diagnostic and/or technological improvements, recently validated assays may become the new standard method to which other assays will be compared. As such, they may progressively achieve national, regional and international recognition. As a recognised standard, these assays will also be used to develop reference reagents for quality control, proficiency and harmonisation purposes. These reference reagents may also become international standards.

An assessment of the reproducibility should be repeated when the test is transferred from the development laboratory to the field, whether for use in local laboratories or in field applications. Predictable changes, e.g. extremes of temperature and levels of operator experience, should be assessed as additional sources of variation in assay results that may affect estimates of reproducibility.

5. Monitoring assay performance after initial validation

5.1. Monitoring the assay

To retain the status of a validated assay it is necessary to assure that the assay as originally validated consistently maintains the performance characteristics as defined during validation of the assay. This can be determined in a quality assurance programme characterised by carefully monitoring the assay's daily performance, primarily through precision and accuracy estimates for internal controls, as well as outlier tendencies (1.1 Repeatability). The performance can be monitored graphically by plotting measurements from assay controls in control charts[7] (Crowther *et al.,* 2006). Deviations from the expected performance should be investigated so corrective action can be taken if necessary. Such monitoring provides critical evidence that the assay retains its "validated" designation during the implementation phase of the assay. Reproducibility is assessed through external quality control programmes such as proficiency testing (Johnson & Cabuang, 2021) (3.1 Reproducibility). Should the assay cease to produce results consistent with the original validation data, the assay would be rendered unfit for its intended purpose. Thus, a validated assay must be continuously assessed to assure it maintains its fitness for purpose (Waugh & Clark, 2021).

5.2. Modifications and enhancements – considerations for changes in the assay

Over time, modifications of the assay will likely be necessary to address changes in the intended purpose, analytes targeted (i.e. modification of the assay to adjust diagnostic performance) or technical modifications to improve assay efficiency or cost-effectiveness. For a change in intended purpose of the assay, then a revised validation from Stage 2 onwards is obligatory.

If the assay is to be applied in another geographical region and/or population, revalidation of the assay under the new conditions is recommended. Lineages or sub-lineages of an infectious agent, derived from animals in different geographic locations, are known to vary requiring revalidation of the assay for the specified target population. This is especially true for nucleic acid detection (NAD) systems as it is very common for point mutations to occur in many infectious agents (especially RNA viruses). Mutations, which may occur within the primer or probe sites can affect the efficiency of the assay and even invalidate the

7 *Control chart:* A graphical representation of data from the repetitive measurement of a control sample(s) tested in different runs of the assay over time.

established performance characteristics. It is also advisable to regularly confirm the target sequence at the selected genomic regions for national or regional isolates of the infectious agents. This is especially true for the primer and probe sites, to ensure that they remain stable and the DSe and DSp for the assay are not compromised. Similar issues can arise with immunologically based assays for antigen or antibody.

A similar situation may occur with emergence of new subtypes of existing pathogens. In these circumstances, existing assays may need to be modified.

5.2.1. Technical modifications and comparability assessments

Technical modifications to a validated assay such as changes in instrumentation, extraction protocols, and conversion of an assay to a semi-automated or fully automated system using robotics will typically not necessitate full revalidation of the assay. Rather, a methods comparison study is done to determine if the relatively minor modification to the assay affected the previously documented performance characteristics of the assay. Comparability can be established by running the modified procedure and original procedure side-by-side, with the same panel of samples in both, over several runs. The panel chosen for this comparison should represent the entire operating range of both assays. If the results from the modified procedure and originally validated method are determined to be comparable in an experiment based on a pre-specified criterion, the modified assay remains valid for its intended purpose. See chapter 2.2.8 for description of experiments that are appropriate for comparability testing, chapter 2.2.6 on reference sample panels, Bowden & Wang, 2021 and Reising *et al.* 2021.

5.2.2. Biological modifications and comparability assessments

There may be situations where changes to some of the biologicals used in the assay may be necessary and/or warranted. This may include changes to the test specimen itself (e.g. a change in tissue to be tested or perhaps testing of a different species altogether). It may include changes to reagents (e.g. the substitution of a recombinant antigen for a cell culture derived antigen or one antibody conjugate for another of similar immunological specificity in an ELISA). The difficulty in making any modification lies in determining whether the change requires a complete revalidation of the assay at both bench and field levels. At the very least, any modification requires that the appropriate Stage 1 'analytical requisites' be assessed. The more difficult decision relates to Stage 2 'diagnostic performance'. To assist here, the original (reference) assay should initially be compared to the modified (candidate) assay in a controlled trial using a defined panel of positive and negative diagnostic samples. See chapter 2.2.8 and Reising *et al*. (2021) for a description of comparability assessment. If the comparability assessment does not suggest a change in diagnostic performance, the modified assay may be phased into routine use. If, on the other hand, differences in DSp and DSe are observed, the modified assay would require additional Stage 2 or field validation before being adopted.

> A **comparability study** is required when a change has been made in a test protocol of a validated test to ensure that test performance is comparable.

5.2.3. Replacement of depleted reagents

When a reagent such as a control sample or working standard is nearing depletion, it is essential to prepare and repeatedly test a replacement before such a control is depleted. The prospective control sample should be included in multiple runs of the assay in parallel with the original control to establish their proportional relationship. It is important to change only one reagent at a time to avoid the compound problem of evaluating more than one variable.

5.3. Enhancing confidence in validation criteria

Because many host variables have an impact on the diagnostic performance of assays, it is highly desirable over time to increase the number of reference samples or samples suitable for latent class analysis. The sampling design, collection, transportation, and testing environment for the new samples should be the same as used for the original validation study. Increases in sample numbers improves the precision of the overall estimates of DSe and DSp, and may allow calculations of DSe estimates by factors such as age, stage of disease, and load of organisms. Practical experiences show that regular updates of validation dossiers with new data is difficult. Participation in proficiency testing rounds using relevant panels with the latest strains may help to assist in proving ongoing assay accuracy and precision.

5.3.1. Data management

Long-term storage of data, review of validation data and on-going verification is an important component for enhancing confidence that assays are still performing with acceptable diagnostic accuracy. To achieve that goal, Laboratory Information Management Systems (LIMS) are required that integrate validation and diagnostic data to provide regular updates of assay performance. At a minimum, data management systems should facilitate: (1) storage of validation data in central locations, (2) review and/or sharing of data when, for example, assays are deployed to external laboratories (Stage 4 of the WOAH Assay Validation Pathway (see Figure 1), (3) on-going accrual and integration of diagnostic results to update the diagnostic characteristics of an assay (Stage 3 (see Figure 1), and (4) storage of Internal Quality Control (IQC) and External Quality Control (EQC) data for use in reviewing assay sensitivity (Stage 1, (see Figure 1), especially when changes to reagents or equipment occur.

Access and utilisation of LIMS for the purposes of test validation and ongoing verification remains limited, as they require significant investment and Information Technology expertise to maintain.

6. Verification of existing validated assays

If a laboratory is considering the use of a validated commercial kit or a candidate assay based on published literature with validation data, some form of verification will be required to determine whether the assay complies with either the kit manufacturer's or the author's assertions, with respect to Stage 1 validation criteria, in the context of the intended application. This may require a limited verification of both ASp and ASe using available reference materials, whether they be external and/or locally acquired from the target population. Once the laboratory is confident that the assay is performing as described from an analytical perspective, then proceeding to a limited Stage 2 validation should be considered in the context of the intended application and target population before the assay is put into routine diagnostic use (Kirkland & Newberry, 2021).

A **verification study** is required when a validated test is used in a new laboratory to ensure that results from the original validation study can be verified and overall test performance is comparable.

7. New technologies

The use of high-throughput sequencing (HTS) and opportunities for its application to diagnosis are growing rapidly; the major purposes are unbiased sequencing for pathogen discovery and targeted sequencing for detection and further characterisation. If the assay is used for detecting previously unidentified microorganisms, such as during an outbreak investigation, then the primary purpose is diagnostic. If the assay is used to further characterise a previously identified pathogen or to follow the molecular epidemiology of the pathogen during an outbreak, then its general purpose can be described as an ancillary test. In the absence of a known target analyte, following a traditional validation pathway is not possible. Monitoring quality metrics such as depth of coverage, uniformity of coverage, GC bias, base-call quality scores, decline in signal intensity or read-length, mapping quality and the inclusion of internal controls are used to assess relative performance of various HTS assays (Halpin *et al.*, 2021; van Borm *et al.,* 2016).

Another complexity in diagnostic test validation is the development of multiplexed assays such as bead-based assays and multiplexed real-time RT-PCR/PCR where more than one target is identified. Test accuracy of each of those targets in different concentrations and distribution is important and challenging to validate.

8. Conclusions

Adequate validation and verification of the performance characteristics of diagnostic tests for infectious diseases is critical to ensuring that assays are applied and interpreted in a scientifically robust and defensible manner (Colling & Gardner, 2021). Since it was first adopted in 1996, the WOAH Assay Development and Validation Pathway (Figure 1) has acted as the internationally recognised standard for the validation of veterinary diagnostic tests for infectious diseases.

Whilst the WOAH standard outlines a comprehensive approach to test validation, experience has demonstrated that assay developers continue to face a number of critical challenges in complying with many stages of the pathway. In identifying these obstacles, opportunities for improvement in diagnostic test validation standards, approaches and extension activities have been identified and are summarised in Table 5 (Reid *et al.,* 2022).

Table 5. Summary of challenges and opportunities for diagnostic test validation

Challenges	Opportunities
• What is the purpose of the test?	• Clearly define the purpose(s) and relevant associated parameters, e.g. screening test requires high diagnostic sensitivity and confirmatory test requires high diagnostic specificity
• Define scope and limitations of test	• What can be expected from the test (scope) and what cannot be expected (limitations)?
• Case definition: what constitutes an infected animal and what constitutes a non-infected animal?	• For example: positive in reference test(s), characteristic lesions, experimental infection, sample taken from an individual from a historically negative population, etc. if infection status is not known Bayesian latent class model (BLCM) may be applicable
• Is reference test imperfectly accurate or likely inferior to the new test under evaluation	• Use BLCM for estimation of diagnostic sensitivity and specificity, likelihood ratios, and other relevant parameters, e.g. prevalence
• Species and specimens	• Define species and specimen for which test will be validated, e.g. domestic chicken, nasopharyngeal swabs
• Source and target population	• Is source population (where test was validated) similar to target populations (where test will be applied)?
• Design, analysis, interpretation and reporting of validation/verification studies (lack of original validation data)?	• *Terrestrial Manual* chapters 1.1.6, 2.2.1–2.2.8, WOAH certification and registration process, STARD, ParaTB, Aquatic, BLCM, ISO/IEC 17025:2017, validation templates from national and international organisations & workshops provided by WOAH Reference Laboratories and Collaborating Centres
• Lack of samples (new and emerging, rare diseases, subclinical, wildlife diseases etc.)	• Reference panels of well described samples, if available, and samples for Interlaboratory Comparison (Network collaboration, "Vetlab"), Provisional Recognition

The thoroughness with which assays should be validated might seem a daunting task for diagnostic laboratories and research teams. However, resources are available to help plan and guide validation and verification studies, including relevant chapters of the WOAH *Terrestrial Manual*, the recent special issue of the WOAH *Scientific and Technical Review* on Diagnostic Test Validation (Colling & Gardner, 2021**)** and guidance documents published by national and regional accreditation bodies. International leaders in the field of diagnostic test validation, including the WOAH and their associated Reference Laboratories and Collaborating Centres, as well as national regulatory bodies, have an important role to play in continuing to develop the standards and systems required to ensure that assay developers have the resources required, and incentives, to meet their responsibilities to perform and report well designed and transparent validation studies. End users of diagnostic tests must also be supported to take responsibility to understand and verify assay performance in their own laboratories, and clearly communicate the uncertainty associated with diagnostic test results to their stakeholders.

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* *

NB: There is a WOAH Collaborating Centre for
Diagnostic Test Validation Science in the Asia-Pacific Region (please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-3).
Please contact the WOAH Collaborating Centre for any further information on validation.

NB: FIRST ADOPTED IN 1996 AS PRINCIPLES OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES.
MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 1.1.7.

STANDARDS FOR HIGH THROUGHPUT SEQUENCING, BIOINFORMATICS AND COMPUTATIONAL GENOMICS

INTRODUCTION

High throughput sequencing, bioinformatics and computational genomics (HTS-BCG) in animal health and food safety investigations should be used in accordance with standards for laboratory testing, just as any other laboratory tool or procedure. As HTS-BCG is a relatively new procedure, the purpose of this chapter is to assist laboratories by defining standards that will allow inclusion of the capability into a laboratory's scope of operations in a way in which the users of the results can have confidence.

A. GENERAL CONSIDERATIONS

Sequence information is playing an increasingly important role in the diagnosis and management of microbial infections, including in the characterisation of infectious agents, their possible phenotypic characteristics and their epidemiology. Consequently it is incumbent on laboratories to adopt policies and practices for generating, analysing and managing genomic sequence data that are based on accurate information and rigorously interpreted.

Increasingly, for full identification and characterisation of a microorganism, there is an expectation that essential features of its genome should be described. For viruses, this may be the whole genome, while for bacteria and parasites, it may be only partial sequences. However as sequencing technology is developing so rapidly, within a short time whole genome sequences for these larger microorganisms may also be routinely generated after suitable bioinformatics procedures have been developed.

The standards described here apply to the generation of genomic sequence data during investigations of infections of single animals, animal populations and their environment. They apply to the generation, management and use of such data within the accepted practices of veterinary investigations and within a laboratory's quality assurance system.

B. THE CONDUCT OF VETERINARY INVESTIGATIONS INCORPORATING HTS-BCG

Sequence data of microorganisms, such as is generated by HTS or metagenomics approaches, is only a tool, although a powerful one, to assist in the investigation of issues regarding animal health and food safety. Appropriate experts should perform the analysis of sequence data. The interpretation of that data in relation to the disease investigation should be led by suitably qualified veterinarians, consistent with the standard requirements for diagnosis of animal disease.

The sequence and sequence analysis of infections associated with cases, outbreaks and investigations of animal disease and food safety by laboratories should be recorded and analysed together with all other information relating to the reporting and recording of such cases and outbreaks. These data should be considered a necessary part of such reports and records.

HTS-BCG can be deployed for a range of purposes in the detection of infectious agents and their characterisation, either in biological material such as diagnostic or surveillance specimens or propagated in cultures or as isolates. For primary diagnostic applications, the users of the technology should consider the purposes of their testing in relation to the normal purposes of testing as defined in Chapter 1.1.6 *Validation Of diagnostic assays for infectious diseases of terrestrial animals*. HTS-BCG may also be applied as a confirmatory assay for organisms detected in some other primary assay, or to provide additional characterisation of such organisms.

Further to these general purposes of testing, HTS-BCG offers specific opportunities for:

i) The detection, identification and characterisation of previously unidentified microorganisms;

ii) The improved diagnosis of known diseases;

iii) The improved diagnosis of emerging or re-emerging diseases with known or unknown aetiology;

iv) The development of single 'universal' diagnostic assays, able to identify any potential pathogen;

v) The simultaneous and quick detection of multiple agents in diseases with multifactorial aetiologies;

vi) The increased capability to study the evolutionary dynamics of pathogens at the farm, local, national and global levels;

vii) The deeper understanding of the epidemiology of infectious diseases and the phylogeography of infectious agents;

viii) The enhanced traceability of infectious diseases and modes of pathogen transmission including applications in forensic epidemiology;

ix) More extensive characterisation of 'populations' of known pathogens (e.g. relevant minority strains, escape mutants) that in turn facilitates the design of better vaccines, antivirals, etc.;

x) Better links between pathogen genotype and phenotypes enabled through full genome sequence of multiple strains (including reference strains) of a single agent.

C. STANDARDS FOR THE USE OF HTS-BCG

1. Selection of a technology platform or service

Laboratories may choose to establish a HTS-BCG capability in-house, contract commercial suppliers of services or submit specimens to designated Reference Centres.

Where the laboratory establishes its own capability there are a number of commercially available sequencing platforms for the purpose of generating sequence information from test samples. The choice of platform should be based on a consideration of the intended purpose or combination of purposes as outlined in Section B above.

Of primary concern is that the technology selected is fit for the intended purpose, that it is appropriate for the production of sequence information from the types of genome intended for study. Other considerations may take into account the time required to conduct a sequencing run, including sample preparation; ancillary equipment needed in addition to the actual sequencing device; the capital cost of the purchase and set up of all necessary equipment and the cost of annual licences or service agreements, including manufacturer's recommended maintenance schedule; the availability of supporting expertise from the supplier; the cost of reagents for a sequencing run and the likely availability of reagents in the country concerned; the staff requirements and training required to operate the equipment and to conduct the associated bioinformatic analyses and the data management requirements. Currently available systems have been reviewed (Belák *et al.,* 2013; Granberg *et al*., 2016; Marston *et al*., 2013), but new models and technologies can be expected to become available frequently.

Where a laboratory or veterinary service contracts an external provider to supply HTS-BCG services, they should ensure that the service provider meets the standards defined in this chapter.

2. Sampling and reporting

HTS-BCG is a new technological tool in the management of diseases of animals and its use should be adopted within the context of tried and accepted processes for the management of animal health and food safety including clinical or epidemiological field investigations and the sampling of animals, animal populations or other epidemiologically relevant situations. The use of the technology should be appropriate to the purpose of the investigation, and the sampling strategy and the specimens taken should be appropriate for that investigation, based on an understanding of the pathogenesis and epidemiology of the infection under study or the likely pathogenesis and epidemiology of any novel infectious agent suspected. Such investigations should be under the supervision of appropriately qualified veterinarians.

In laboratories where HTS-BCG is used it should be managed within the context of the laboratory's quality assurance system. Hence the results of HTS-BCG must be interpreted in the context of the pathogenesis and epidemiology of the infection in the animal species under study. Results should be reported by appropriately qualified veterinary investigators with the authority to make diagnoses of animal diseases under the laboratory's quality assurance system and in the jurisdiction where the investigation is conducted.

3. Specimens and sample preparation

Specimens should be collected and submitted to the testing laboratory in accordance with the standards communicated in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens.* The normal comprehensive information regarding the individual animal, the case or reason for sampling and the relevant epidemiological information should be recorded in the laboratory's accessions processes, as for any submission to the laboratory.

As with other laboratory processes ensuring the integrity of the specimen and the samples to be tested is critical. Nucleic acids, either DNA or RNA, need to be extracted from the samples. In some cases, enrichment strategies to increase the ratio of pathogen to host nucleic acids can be used to maximise the sensitivity of the technique, however care must be taken to avoid biasing the outcome in the context of the intended purpose. Precautions to ensure the integrity and quality of nucleic acids must be followed similarly to any other molecular technique (e.g. polymerase chain reaction [PCR]) as already described in Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases*. Once nucleic acids are extracted from the samples, they need to be further manipulated (e.g. reverse transcription of RNA into complementary DNA) in order to be used in HTS. Different technological platforms require specific sets of reagents in order to generate the final material ("libraries") ready for sequencing. Commercial kits are available for this purpose.

HTS is an extremely sensitive technology and even few molecules of nucleic acid could be detected. Hence, precautions to avoid cross-contaminations must be followed as in the case of many other molecular techniques used to detect nucleic acids (e.g. PCR). Separation of work areas from the possibility of cross contamination with nucleic acid from other molecular investigations is an essential requirement. In addition, HTS very frequently involves "multiplexing" of several samples in a single reaction. Individual samples are "tagged" during one of the stages of sample preparation by the use of short index sequences linked to nucleic acid molecules. Index sequences must be of sufficient quality and design to be relied on as a signature for the tagged library for HTS use in order to avoid artefacts during bioinformatics analysis of sequencing data obtained.

Every application of HTS-BCG technology should include positive and negative controls appropriate to the investigation and that have been incorporated through the sample preparation processes of the sequencing run as well as the actual run on the technology platform. Appropriate controls should be used to verify each step of the procedure including nucleic acid quality, library preparation, cross-contamination (including multiplexing) sensitivity and reproducibility.

As with any other diagnostic method, confirmation of results would require resampling of the original specimen, which therefore has to be protected from cross-contamination and be stored appropriately.

4. Generation of sequence data

While HTS platforms differ widely in their details, basic principles of quality control relevant to the technology can be followed, and generic recommendations for acceptable quality metrics can be made. Suitable control measures might include the use of positive, negative and no-template controls run in replicates of the test and a quality

scoring system. Sequencing quality metrics provide suitable parameters for the validation and monitoring of platform performance. Most platforms offer the possibility to spike controls in reagents and to use the control's QC metrics to monitor platform and reagent performance. Additional technology specific performance metrics can be used to monitor platform performance and to identify aberrant sequencing runs.

Quality metrics for the evaluation of the analytical performance of HTS-based tests, include:

i) Depth of coverage. This indicates the number of sequence reads providing information about a given nucleotide. When ongoing quality monitoring shows that the coverage depth at a given nucleotide is below the validated minimum coverage, confirmation should be provided using alternate methods (e.g. Sanger sequencing) or additional sequencing.

ii) Uniformity of coverage. This parameter describes how the depth of coverage is distributed over the test's target region(s). Deviations of uniformity of coverage from the validated range potentially indicate errors in the testing process.

iii) GC bias. The GC content (relative abundance of G and C nucleotides) of a target region affects the efficiency of sequencing reactions and will affect the uniformity of coverage. Where possible, the amount of GC bias in the test's target region(s) should be determined during validation and monitored to evaluate test performance.

iv) Base call quality scores. These are platform-derived reflections of the signal-to noise ratio and reflect the probability that the base call was correct. An acceptable raw base call quality threshold should be established during validation, and incorporated in bioinformatics filters to eliminate poor quality data during analysis.

v) Decline in signal intensity or read length. Depending on the exact application, HTS platform and chemistry, sequence reads have a typical distribution of read length and signal intensity. The expected signal intensity across reads (or read length distribution) should be established during validation and monitored for each run. Deviations in the distribution of read lengths may indicate problematic datasets.

vi) Mapping quality. This is a measure of uncertainty that a read is mapped properly to a genomic position within the target region. Acceptable values (e.g. proportion of reads mapping to the target) should be established during validation of bioinformatics workflows and the proportion of reads not mapping to the target can be monitored during each run.

vii) Internal controls. Most platforms offer the possibility to spike an internal control at very low frequency during the sequencing run. The quality metrics of those reads can to be compared to previously reported quality metrics.

5. Bioinformatics

An absolute requirement for any laboratory intending to establish a HTS-BCG capability is the employment of specialised bioinformatics skills. Even if platforms with supporting software for specific analyses in defined clinical situations were to become available the use of such packages would not remove the responsibility of the laboratory to be able to competently analyse its own data.

The bioinformatic analysis assembling the pathogen genomic sequence from the raw data and the subsequent secondary analysis are the critical elements in HTS-BCG. Hence the approaches used must be transparent and a declaration of the software packages, software versions, and reference databases or sequences used should be a component of every report of sequence analysis. Software programs used for these analyses must be readily available (commercially or open access) in order to be evaluated by the international community.

As with any laboratory procedure, attention must be given to quality assurance. The test method should include criteria for acceptance or rejection of each run based on the satisfactory analyses of the controls. Sequencing data must be documented to have satisfied minimum quality scores and coverage for each nucleotide of the assembled final consensus sequence obtained.

The appropriateness of chosen bioinformatics software for particular analyses can be evaluated through testing its performance against standard data sets containing data relating to agents expected to be present in the specimens to be tested.

6. Data management

The data generated from HTS-BCG operations are essential to reach the diagnosis or other scientific purpose of the investigation, such as agent characterisation, and are an integral component of the process. As such it is an essential requirement of laboratories to have policies, processes and supporting systems to curate, manage and store the data generated.

Different HTS technology platforms produce raw data in different formats and stage of pre-analysis, so it is necessary for laboratories to have policies and processes specific to the technology platform in use. Data management systems will include aspects of which data to keep, and the length of time for which they will be kept, and the back-up strategies to protect against accidental loss or deliberate erasure. Metadata describing the generation and analysis of the sequence data is essential, so that the process itself can be analysed or repeated.

Where a sequence analysis leads to an output of animal health significance, especially one of trade or international significance, it is an absolute requirement that the data on which the analysis was performed be kept available for audit or confirmatory analysis for a period of time commensurate with the significance of the animal health finding. This is particularly important where the finding may be disputed. Failure to be able to produce the required data for independent analysis could be taken to invalidate the finding.

Sequence data should be stored in a manner in which there is a clear link to the metadata associated with the specimen that was the subject of the analysis. As is standard practice in laboratory investigations, such metadata includes information regarding the animal sampled, its ownership and location, and accompanying clinical and epidemiological information regarding the animal population.

7. Validation of test systems for designated purposes

The concepts of test validation as stated in chapter 1.1.6 are broadly applicable to HTS-BCG. All procedures including sample processing (nucleic acid extraction, library preparation, tagging, target enrichment), sequencing, bioinformatics and reporting should be documented in SOPs before validation can start. Stage 1 validation data must be developed to confirm the analytic sensitivity (Se) and specificity (Sp) of the technique, and its repeatability. For sequencing based tests, analytic sensitivity can be defined as the likelihood that the assay will detect the targeted sequence variations, if present, at a given probability (e.g. 95% confidence), while analytical specificity can be defined as the probability that an assay will not detect a sequence variation when none are present at a given probability. Furthermore, each type of specimens has its own characteristics that have to be considered, e.g. nasal swab, sera or faeces. Well described samples with known concentrations of target analyte or non-target analytes and matrix components can be used to assess the analytical performance. This should include, as a minimum, serial dilutions of each type of specimen containing defined organisms to document the limits of detection of designated whole genomes or genetic sequences representative of the type for which the HTS-BCG capability will be used in the laboratory. For viral disease investigations, test specimens could be prepared to contain representative viruses of the full range of viral families from which agents may be present in test specimens of the type to be investigated in routine operations. Documentation of the laboratory's HTS-BCG system to detect these viruses will be established. The same principles apply to genetic markers, bacteria or other organisms for which the HTS-BCG capability will be used in planned routine operations. In all these runs designed to establish sensitivity and specificity, the sample preparation steps should be part of each assessment as these steps are likely to be critical to all aspects of overall test performance.

Several factors complicate the validation of NGS tests as primary diagnostic assays including:

i) The weight of the analytical and diagnostic validation required (chapter 1.1.6);

ii) The operational cost of the technology;

iii) The challenges of validation of a data analysis workflow;

iv) The high need for investment in hardware and expertise;

v) The time taken to obtain a result (currently days compared to hours for specific molecular diagnostics such as real-time PCR).

Confirmatory adjunct or secondary diagnostic assays need to be validated only for their analytical performance, e.g. analytical sensitivity and specificity, and repeatability and initial reproducibility (stage 1) and not to the full diagnostic extent (diagnostic sensitivity and specificity, stage 2).

It is recognised that it may not always be practical to produce large data sets on test performance such as would normally allow calculation of test diagnostic sensitivity and specificity, but other aspects of validation such as demonstration of test reproducibility among laboratories conducting similar investigations should be undertaken.

8. Quality assurance

Testing using HTS-BCG for the purposes of investigations of animal health and food safety should be conducted in accordance with the requirements of the laboratory's quality assurance system, the features of which will meet the standards listed in Chapter 1.1.5 *Quality management in veterinary testing laboratories.* Where the laboratory is accredited, the testing should be part of the laboratory's scope of accreditation.

Standard data sets against which the usefulness of bioinformatics software packages can be verified have been developed. Laboratories using HTS-BCG should ensure that their software packages for bioinformatics meet expected performance criteria against data standards.

Where proficiency testing strategies have been developed, laboratories using HTS-BCG should participate.

9. Interpretation of results

HTS-BCG can be used for a variety of purposes ranging from pathogen discovery to diagnosis or in-depth characterisation of known infectious agents. Consequently, the interpretation of the results obtained will be in the context of the specific clinical and epidemiological situation, reassured by satisfactory performance against all specified controls and quality assurance parameters. As with any other laboratory tests, these considerations are one among a number of parameters to be taken into account.

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NB: There is a WOAH Collaborating Centre for Viral Genomics and Bioinformatics (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-3). Please contact the WOAH Collaborating Centre for any further information on HTS-BCG.

NB: FIRST ADOPTED IN 2016.

CHAPTER 1.1.8.

PRINCIPLES OF VETERINARY VACCINE PRODUCTION

SUMMARY

A reliable supply of pure, safe, potent, and effective vaccines is essential for maintenance of animal health and the successful operation of animal health programmes. Immunisation of animals with high quality vaccines is the primary means of control for many animal diseases. In other cases, vaccines are used in conjunction with national disease control or eradication programmes.

The requirements and procedures described here are intended to be general in nature and to be consistent with published standards that are generally available for guidance in the production of veterinary vaccines. The approach to ensuring the purity, safety, potency, and efficacy of veterinary vaccines may vary from country to country depending on local needs. However, proper standards and production controls are essential to ensure the availability of consistent, high quality products for use in animal health programmes.

As the pathogenesis and epidemiology of each disease varies, the role and efficacy of vaccination as a means of control also varies from one disease to another. Some vaccines may be highly efficacious, inducing an immunity that not only prevents clinical signs of the disease, but may also prevent infection and reduce multiplication and shedding of the disease-causing agent. Other vaccines may prevent clinical disease, but not prevent infection or the development of the carrier state. In other cases, immunisation may be completely ineffective or only able to reduce the severity of the disease. Thus the decision whether to recommend vaccination as part of an animal disease control strategy requires a thorough knowledge of the characteristics of the disease agent and its epidemiology, as well as the characteristics and capabilities of the various available vaccines. There is also growing public interest in the beneficial implications for animal welfare of the use of veterinary vaccines as a means of disease control. In any case, if vaccines are used, successful performance requires that they be produced in a manner that ensures a uniform and consistent product of high quality.

As for all medicines, vaccine production starts within research and development (R&D) facilities, carrying out all the preclinical studies which are intended to demonstrate the quality of the products, including the safety and the efficacy. All these studies are generally carried out according to international reference standards such as good laboratory practice (GLP) for preclinical studies and good clinical practice (GCP) for clinical studies.

Before release of a vaccine for use in a country, relevant regulatory approval must be requested from and be assessed and authorised by the competent authority to ensure compliance with local product regulatory requirements. Starting materials to be used, manufacturing steps, in-process controls and controls on the finished product before release by a responsible person should be described in the dossier for relevant regulatory approval, as should be the necessary tests to demonstrate quality, safety, and efficacy of the vaccine.

After the relevant regulatory approval has been granted by a competent authority, the industrial production can be launched in a manufacturing site which is authorised by the competent authority in accordance with national requirements and having the relevant equipment, facilities and personnel for production and controls. The manufacturing site should be inspected on a regular basis by experienced official inspectors.

Quality assurance is an integral part of the production of pure, safe and efficacious vaccines. This chapter outlines critical check points, with more details provided in Chapters 2.3.3 Minimum

requirements for the organisation and management of a vaccine manufacturing facility*, 2.3.4* Minimum requirements for the production and quality control of vaccines*, and 2.3.5* Minimum requirements for aseptic production in vaccine manufacture*. It is a step-wise and iterative process. Compliance with the full standards described in these chapters can be achieved through risk analysis and step-wise process improvement.*

NOMENCLATURE

The nomenclature for veterinary biological products varies from country to country. For example, in the United States of America (USA) the term 'vaccine' is used for products containing live[1] or inactivated viruses or protozoa, live bacteria, or nucleic acids. Products containing killed bacteria and other microorganisms are identified as bacterins, bacterial extracts, conventional or recombinant subunits, bacterintoxoids, or toxoids, depending on the type of antigen they contain. For example, products containing antigenic or immunising components of microorganisms may be called 'subunits' or 'bacterial extracts', and those produced from the inactivation of toxins are called 'toxoids'. In the European Union (EU), Immunological Veterinary Medicinal Products are defined as 'products administered to animals in order to produce active or passive immunity or to diagnose the state of immunity', see Directive 2001/82/EC, as amended by Directive 2004/28/EC. For this chapter, however, the term 'vaccine' will include all products designed to stimulate active immunisation of animals against disease, without regard to the type of microorganism or microbial toxin from which they may be derived or that they contain. This use is more consistent with international nomenclature. 'Vaccine' will not be used in this discussion in reference to biological products recommended for passive immunisation, immunomodulation, treatment of allergies, or diagnosis.

VACCINE TYPES OR FORMS

Vaccines may be prepared as live or inactivated (killed) products. Some live vaccines are prepared from low virulence, mild, field isolates of a disease-causing agent that have been found to be safe and effective when administered by an unnatural route or under other conditions where exposure to the microorganism will immunise rather than cause disease. Other live vaccines are prepared from isolates of disease-causing agents that have been modified by passage through laboratory animals, culture media, cell cultures, or avian embryos to select a variant of reduced virulence. The development of recombinant DNA (rDNA) procedures has provided some unique opportunities for vaccine production. Modified live vaccines may now be specifically produced by deletion of virulence-related genes from a microorganism. Others are produced by the insertion of genes that code for specific immunising antigens from a disease-causing microorganism into a nonvirulent vector microorganism. Nucleic-acid-mediated vaccines containing plasmid DNA have been developed. The DNA is usually in plasmid form and codes for immunising antigens from disease-causing microorganisms.

Killed products may contain: 1) Cultures of microorganisms that have been inactivated by chemical or physical means; 2) Inactivated toxins; or 3) Subunits (antigenic parts of microorganisms) that have been extracted from cultures or that have been produced through rDNA procedures.

Both live and inactivated vaccines may contain different antigenic components and may be formulated with adjuvants, stabilisers, antimicrobial preservatives and diluents. Adjuvants are designed to enhance the immunising efficacy of the vaccine. Those used frequently are typically water-in-oil emulsions (either single or double), made with mineral or vegetable oil and an emulsifying agent.

Other adjuvants, such as aluminium hydroxide gel or saponin, are also used. In addition to these traditional adjuvants, vaccines are being developed that include additional ingredients that induce immunomodulatory effects in the host animal and serve to enhance the efficacy of the product. These ingredients may include immunogenic components of microorganisms such as killed bacteria, which stimulate the immune response to other fractions contained in the vaccine, or cytokines, which may be used to regulate specific aspects of the immune system and are included in rDNA constructs used in products manufactured through biotechnology.

1 The generic term "live" (usually modified or attenuated) is used throughout this *Terrestrial Manual* to differentiate from inactivated organisms, although it is recognised that in the case of viruses they cannot be considered truly alive.

Many products obtained by biotechnology have now received relevant regulatory approval, and more are being developed. Products of rDNA technology do not differ fundamentally from conventional products. Therefore, existing laws and regulations are fully applicable to these new products.

Each competent authority with power to regulate organisms and products derived from recombinant techniques should ensure that the public health and the environment are protected from any potentially harmful effect. Veterinary vaccines derived through rDNA technology may be divided into three broad categories. The division is based on the products' biological properties and on the safety concerns they present.

Category I consists of non-viable or killed products that pose negligible risk to the environment and present no new or unusual safety concerns. Such products include inactivated microorganisms, either whole or as subunits, created by using rDNA techniques.

Category II products contain live microorganisms modified by adding or deleting one or more gene(s). Added genes may code for marker antigens, enzymes, or other biochemical by-products. Deleted genes may code for virulence, oncogenicity, marker antigens, enzymes, or other biochemical by-products. The relevant regulatory approval application must include a characterisation of the DNA segments added or deleted, as well as a phenotypic characterisation of the altered organism. The genetic modifications must not result in any increase in virulence, pathogenicity, or survivability of the altered organism in comparison with the wild-type form. It is important that the genetic modification does not cause deterioration in the safety characteristics of the organism.

Category III products make use of live vectors to carry recombinant-derived foreign genes that code for immunising antigens. Live vectors may carry one or more foreign gene(s) that have been shown to be effective for immunising target host animals. The use of DNA vaccines containing recombinant-derived foreign genes that code for immunising antigens (plasmid DNA vaccines) constitutes a new approach to vaccine development. The proper categorisation of this type of rDNA-derived product will be established as biological properties and safety characteristics are determined. These new vaccines may find application in a wide variety of situations much as conventional products have.

VACCINE PRODUCTION

1. Quality assurance

Quality assurance is a wide-ranging concept that covers all matters that individually or collectively influence the quality of a product. It is the total sum of the organised arrangements made with the object of ensuring that medicinal products are of the quality required for their intended use, ranging from process control, improvement and inspection, testing of the quality, efficacy and safety of the vaccines to assurance achieved through competent authority procedures. It is a step-wise and iterative process, and compliance with the standards described in these chapters can be achieved through risk analysis and step-wise process improvement. The basic concepts of quality assurance, good manufacturing practice (GMP), quality risk management and quality control are inter-related. See chapter 2.3.4 for full details.

2. Production facilities

Facilities used for the production of vaccines should be designed to protect the purity of the product throughout the production process, to safeguard the health of the personnel, and provide secure containment of any disease causing agents.

For each vaccine, there should be a detailed production plan that describes where each step in the production process will occur. This plan should be documented in a detailed standard operating procedure (SOP) or by providing a building blueprint and accompanying blueprint legend. Each room in the establishment should be uniquely identified, and all functions performed and all microorganisms involved should be specified for each room. Disinfection procedures, monitoring of equipment and other procedures used in the operation of the facilities to prevent contamination or errors during production should also be documented. This plan should be updated as new products or microorganisms are added to the facility, or other changes or improvements in procedures are developed.

The requirements for vaccine production facilities are described in more detail in chapter 2.3.3.

3. Documentation of the manufacturing process and record keeping

An Outline of Production (a highly detailed description of how the vaccine is produced and tested for release), a series of SOPs, or other documents should also be prepared to describe the procedure for the manufacture and testing of each product produced in an establishment.

Criteria and standards for acceptance for use of source materials should be clearly and accurately documented.

Guidelines for the preparation of such documents for veterinary vaccines are published by competent control authorities. This documentation is intended to define the product and to establish its specifications and standards. It should ensure, along with the blueprints and blueprint legends (or production plan and SOPs), a uniform and consistent method of producing the product and should be followed in the preparation of each batch/serial (one master batch record for each product).

The producer should establish a detailed record-keeping system capable of tracking the performance of successive steps in the preparation of each biological product. Records kept should indicate the date that each essential step was taken, the name of the person who carried out the task, the identity and quantity of ingredients added or removed at each step, critical measurements such as temperature and time, and any loss or gain in quantity in the course of the preparation. Records should be maintained of all tests conducted on each batch/serial. All records relevant to a batch/serial of product should be retained for at least 2 years after the expiry date on the label, or in line with the requirements of the competent control authority.

Details of documentation required at a manufacturing site are described in chapter 2.3.3.

4. Production

Because of the wide variety of products, the frequently large number of stages involved in the manufacture of vaccines and the nature of the biological processes, each stage must be constantly monitored. Adherence to validated operating procedures and in-process controls is critical.

The specifications and source of all product ingredients should be recorded in approved documents. The Outline of Production must be approved by the competent authority and is used for inspection and regulatory or legal action. Approval, by the regulatory authority, of documents that describe critical details of manufacture and testing is recommended. All ingredients of animal origin that are not subject to a validated sterilisation procedure should also be tested to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses as specified in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*. The country of origin should be known. Measures should be implemented by the manufacturer to avoid the risk of transmissible spongiform encephalopathy (TSE) agent contamination by ingredients of animal origin.

Some control authorities discourage the use of preservatives, especially antibiotics as a means of controlling adventitious contamination during production and prefer the use of strict aseptic techniques to ensure purity. However, they may allow the use of preservatives, particularly in multidose containers, to protect the product during use. These control authorities usually limit any addition of antibiotics in the manufacture of the product to cell culture fluids and other media, egg inocula, and material harvested from skin or possibly other tissues. Some control authorities prohibit the use of penicillin or streptomycin in vaccines administered by aerosol or parenterally. If the antibiotics used are not recommended for use in the target species, they should be shown to have no harmful effects in the vaccinated animals and not result in the contamination of food derived from vaccinated animals. Many countries ban thimerosal due to environmental concerns.

Details of vaccine production required at a manufacturing site, including requirements for starting materials, cell bank systems and seed-lot systems are described in chapter 2.3.3.

5. Process validation

Prior to obtaining relevant regulatory approval for any new product, each establishment should produce in its facilities three consecutive production batches/serials of completed product with satisfactory test results, to evaluate the consistency of production. The process used should follow the manufacturing procedure used to demonstrate efficacy and field safety as specified in the Outline of Production or equivalent documents. Some

authorities require that the size of each of the three batches/serials should be at least one-third the size of the average batch/serial that will be produced once the product is in production, so as to be typical.

The manufacturer should test each of these batches/serials for purity, safety, and potency as provided in approved documentation. Applicable standard requirements and test procedures, for example those described in United States Code of Federal Regulations [CFR] Title 9 Part 113, in the Annex to EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or as described in this *Terrestrial Manual* may be used. Satisfactory test results should be demonstrated for all three batches/serials prior to approving the production of the product in the facilities and its release for marketing. Each subsequent batch/serial should be tested in the same manner with satisfactory results prior to release for marketing.

6. Stability tests

It is important to monitor the stability of each product through a programme to determine on-going stability.

Conditions of storage affecting the quality of the product should be taken into account as evaluated in the relevant regulatory approval, including light, temperature and the adhesive/absorptive properties of containers. All vaccines are sensitive to heat to some extent, but some are more sensitive than others. There is increasing interest in the development of vaccines that can tolerate adverse storage conditions. In this *Terrestrial Manual*, thermotolerant (see Glossary of Terms) is defined as the ability of vaccines to retain protective immunogenicity after exposure to temperatures above the storage temperature required according to the manufacturer's recommendations. Various electronic devices and heat-sensitive indicators are available to monitor cold chain temperatures. Specific thermotolerance claims should be supported by data from time–temperature studies undertaken under the relevant storage or transport conditions.

7. Tests to demonstrate safety and efficacy of a vaccine

All laboratory procedures and tests should be conducted in compliance with an international standard such as Good Laboratory Practice (GLP), see chapter 2.3.4. Similarly tests in animals should comply with Good Clinical Practice (GCP), including compliance with international standards for animal care and use. Submission of the results of the tests described below would normally be required in a dossier supporting a request for relevant regulatory approval.

7.1. Safety tests

7.1.1. Target animal safety tests

Harmonised international guidelines for safety tests are published by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products (VICH) in VICH GL 44 Target animal safety for veterinary live and inactivated vaccines (http://www.vichsec.org/guidelines/biologicals/bio-safety/target-animal-safety.html). An overdose test is required for live vaccines shown to retain residual pathogenicity by induction of disease-specific signs or lesions. In general other vaccines do not require overdose testing.

For vaccines that require a single life-time dose or primary vaccination series only, the primary vaccination regimen should be used. For vaccines that require a single dose or primary vaccination series followed by booster vaccination, the primary vaccination regimen plus an additional dose should be used. The vaccination programme should be supported by the efficacy study design.

The intrinsic safety of vaccines should be demonstrated early in product development and documented as part of the regulatory approval dossier. Safety studies during development should include the safety of a single dose for all products, as well as the safety of an overdose and of repeated single doses for vaccines that require more than one dose during the lifetime of the animal. Additional data are derived for live vaccines from non-reversion to virulence studies and by assessing risk to the environment and in-contact animals, as discussed below. Safety should be demonstrated in each species and by each route for which the product is indicated. Safety in pregnant animals should be determined in separate, controlled studies using animals at appropriate stages of gestation.

For inactivated virus or bacterial products, where host animals are used for potency testing, some level of safety may be determined by measuring local and systemic responses following vaccination and before challenge in the potency tests. Further evidence concerning the safety of products is derived from field safety trials (discussed below). Vaccines derived through biotechnology should be evaluated as discussed in the classification of biotechnology-derived vaccines and release of live rDNA vaccines below.

7.1.2. Increase in virulence tests

With live vaccines, there is concern that the organism might be shed from the host and transmitted to contact animals, causing disease if it retains residual virulence or reverts to virulence with repeated host passages. Guidelines for testing are published by VICH: GL 41: Examination of live veterinary vaccines in target animals for absence of reversion to virulence (http://www.vichsec.org/guidelines/biologicals/bio-safety/target-animal-safety.html).

All live vaccines should be tested for non-reversion to virulence by means of passage studies. Vaccine organisms are propagated *in vivo* by inoculating a group of target animals of susceptible age, with master seed, not finished product; this inoculation uses the natural route of infection for the organism that is most likely to result in infection and reversion or a recommended route of administration of the vaccine manufactured from this master seed. The vaccine organism is recovered from tissues or excretions and is used directly to inoculate a further group of animals, and so on. After not less than four passages, i.e. use of a total of five groups of animals, the isolate must be fully characterised, using the same procedures used to characterise the master seed. Regulatory authority opinion varies in whether or not it is acceptable to propagate *in vitro* between passages organisms that otherwise cannot be passaged five times because of their degree of attenuation. The vaccine organism must retain an acceptable level of attenuation after propagation in this way.

7.1.3. Assessing risk to the environment

The ability of each live vaccine to shed, to spread to contact target and non-target animals, and to persist in the environment must be evaluated to provide information for assessing the risk of the vaccine to the environment, taking into account human health. In some cases this may be done in conjunction with the increase in virulence tests. In the case of live vaccines strains that may be zoonotic, the risk for humans should be assessed. These and additional considerations are especially important in the case of products based on biotechnology or recombinant DNA techniques; more information about such products is provided in other sections.

7.2. Efficacy tests

7.2.1. Laboratory efficacy

The efficacy of veterinary vaccines should be demonstrated by statistically valid vaccination–challenge studies in the host animal, using the most sensitive, usually the youngest, animals for which the product is to be recommended. Data should support the efficacy of the vaccine in each animal species by each vaccination regimen that is described in the product label recommendation. This includes studies regarding the onset of protection when claims for onset are made in the product labelling and for the duration of immunity. The tests should be performed under controlled conditions starting, wherever possible, with seronegative animals. Where validated potency tests are available, target species vaccination–challenge studies may not be required if predictive serological test results are available. The application of procedures to replace, reduce, and refine animal tests (the 'three Rs rule') should be encouraged whenever possible.

Efficacy studies should be conducted with final product vaccine that has been produced at the highest passage level from the master seed that is permitted in the Outline of Production, or equivalent approved documentation. This will have specified the minimum amount of antigen per dose that must be in the final product throughout the entire authorised shelf-life. Where a range of antigen level per dose is permitted, the antigen level per dose in the vaccine tested for efficacy must be at or below the minimum permitted amount. The precise challenge method and the

criteria for determining protection vary with the immunising agent, should have clinical significance, and should be standardised whenever possible.

Field efficacy studies may be used to confirm the results of laboratory studies or to demonstrate efficacy when vaccination–challenge studies are not feasible. However, it is generally more difficult to obtain statistically significant data to demonstrate efficacy under field conditions. Protocols for field studies are more complex, and care must be taken to establish proper controls to ensure the validity of the data. Even when properly designed, field efficacy studies may be inconclusive because of uncontrollable outside influences. Problems may include: a highly variable level of challenge; a low incidence of disease in non-vaccinated controls; and exposure to other organisms causing a similar disease. Therefore, efficacy data from both laboratory and field studies may be required to establish the efficacy of some products, as well as '*a posteriori*' field trials linked to vaccinovigilance.

7.2.2. Interference tests

Consideration must be given to possible interference between two different vaccines from the same manufacturer in the same container/ as a combined dose, or recommended to be given to the same animal within a 2-week period. The safety and the efficacy of this association should be investigated. Such studies are often termed "lack of interference studies" and should evaluate both interference of X on Y and X by Y. In some cases this can be determined serologically.

7.2.3. Field tests (safety and efficacy)

7.2.3.1. All vaccines

All veterinary vaccines administered to animals should be tested for field safety and, if feasible, for efficacy in the field, before being authorised for general use. Field studies are designed to demonstrate safety and efficacy under conditions of normal environment, care and use, and should detect unexpected reactions, including mortality that may not have been observed during the development of the product. Under field conditions there are many uncontrollable variables that make it difficult to obtain good efficacy data, but demonstration of safety is more reliable. The tests should be done on the host animal, at a variety of geographical locations, using appropriate numbers of susceptible animals. The test animals should represent all the ages and husbandry practices for which the product is indicated; unvaccinated controls must be included. The product tested should be two or more typical production batches/serials. A protocol should be developed indicating the observation methods and the recording methods.

7.2.3.2. Additional requirements for live rDNA products

The release of live rDNA microorganisms (Categories II and III) for field testing or general distribution as an approved product may have a significant effect on the quality of the human and animal environment. Before release is authorised, the manufacturers of the vaccine should conduct a risk assessment to evaluate the impact on the human and animal environment. In the USA, for example, a procedure is adopted that could be used as a model system in other countries. This is based on a "Summary Information Format" that summarises all sources and considerations of risk, including the results of laboratory testing, characterisation or sequencing of the master seed, and results of studies that address sources of risk to other species and the environment. The information included should allow the regulatory authority to conduct the risk assessment. If the results of the risk assessment are satisfactory, the regulatory authority will issue a formal document called a "Finding of No Significant Impact".

The EU has adopted a similar system. It is performed as follows:

A risk assessment is carried out that should contain the following information:

i) the purpose and need for the proposed action;

ii) the alternatives considered;

iii) a list of the government agencies, organisations, and persons consulted;

iv) the affected environment and the potential environmental consequences.

The topics discussed should include:

i) the characteristics of the vaccine organism;

ii) human health risks;

iii) animal health risks for both target and nontarget animals;

iv) persistence in the environment, and increase in virulence.

If the risk assessment results in a finding by competent authorities that the proposed release of the recombinant vaccine into the environment for field trials or general distribution would not have a significant impact on the environment, a notice should be published and distributed to the public announcing this and that the risk assessment and findings are available for public review and comment. If no substantive comments are received to refute the findings, competent authorities may authorise the field testing or grant the relevant regulatory approval for general distribution.

The preparation of a risk assessment and the findings made from the assessment may also include the scheduling of one or more public meetings if a proposed action has ecological or public health significance. Such meetings should be announced through a public notice. Interested persons should be invited to make presentations, along with presentations by the producer of the product, and government personnel. The transcripts of such meetings should become part of the public record.

If, in the course of preparing a risk assessment, competent authorities conclude that the proposed action may have a significant effect on the environment, an environmental impact statement (EIS) should be prepared. The EIS provides a full and fair discussion of the significant environmental impacts, and informs decision-makers and the public of any reasonable alternatives that would avoid or minimise the adverse impacts. Environmental documents are considered in the CFR Title 40 part 1508. The EU has issued guidelines under Directive 2001/18/EC: *Guideline on Live Recombinant Vector Vaccines for Veterinary Use,* see http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004590.pdf

8. Updating the Outline of Production

Before production procedures are changed, they should be approved by the regulatory authority. This is generally done by submitting an amended Outline of Production or equivalent documentation. Establishments should have internal review procedures to evaluate all changes in production before they are initiated.

In cases where a significant production step is altered, revisions may require additional data to support the purity, safety, potency, efficacy or stability of the product. In countries with regulatory systems that include confirmatory testing the final product at national laboratories, revisions may entail testing of the revised product before changes are approved by competent authorities.

QUALITY CONTROLS IN VACCINE PRODUCTION

1. Principle

Quality control is concerned with sampling, specifications and testing as well as the organisation, documentation and release procedures. Quality control ensures the necessary and relevant tests are carried out, and that materials and equipment are not released for use, nor products released for sale or supply, until their quality has been judged satisfactory. Quality control is not confined to laboratory operations, but must be involved in all decisions and specifications that may concern the quality of the product. The independence of quality control from production is considered fundamental to the satisfactory operation of quality control. Details of quality control are described in the chapter 2.3.4.

2. Batch/serial release for distribution

Prior to release, the manufacturer must test a representative sample of each batch/serial for purity, safety, and potency, as well as perform any other tests described in the firm's approved documentation of the manufacturing process for that product. In countries that have national regulatory programmes that include official control authority re-testing (check or confirmatory testing) of final products, samples of each batch/serial are submitted for testing in government laboratories by competent authorities prior to release for sale. If unsatisfactory results are obtained for tests conducted either by the manufacturer or by competent authorities, the batch/serial should not be released. In such cases, subsequent batches/serials of the product may be given priority for check testing by competent authorities.

2.1. Batch/serial purity test

Purity is determined by testing for a variety of contaminants, often defined by regulations. Tests to detect contaminants are performed on a representative sample of each batch/serial of final product prior to release.

Purity test procedures have been published, for example in CFR Title 9 part 113, in the annex to EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or in this *Terrestrial Manual* (chapter 1.1.9), for the detection of extraneous viruses, bacteria, mycoplasma and fungi. Examples include tests for: *Salmonella*, *Brucella,* chlamydial agents, haemagglutinating viruses, avian lymphoid leucosis (virus), pathogens detected by a chicken inoculation test, or a chicken embryo inoculation test, lymphocytic choriomeningitis virus, cytopathic and haemadsorbing agents, and pathogens detected by enzyme-linked immunosorbent assay, polymerase chain reaction, or the fluorescent antibody technique.

2.2. Batch/serial safety test

VICH Guidelines 50 (inactivated vaccines) and 55 (live vaccines) provide for a waiver of the target animal batch/serial safety tests in recognition of the 3R principles. VICH Guideline 59 also provides for a waiver of the laboratory animal batch/serial safety tests (LABST) in recognition of the 3R principles. As stated in VICH Guidelines 50, 55 and 59 (http://www.vichsec.org/guidelines/biologicals/bio-safety/target-animal-safety.html), these batch safety tests may be waived by the regulatory authority when a sufficient number of production batches have been produced under the control of a seed lot system and found to comply with the test, thus demonstrating consistency of the manufacturing process. Some regulatory authorities still require safety tests for the release of each batch/serial and typical tests are described in CFR Title 9 part 113, in this *Terrestrial Manual* and elsewhere. Standard procedures are given for safety tests in mice, guinea-pigs, cats, dogs, horses, pigs, and sheep and are generally conducted using fewer animals than are used in the safety tests required for regulatory approval. Batches/serials are considered satisfactory if local and systemic reactions to vaccination with the batch/serial to be released are in line with those described in the regulatory approval dossier and product literature. Some authorities do not permit batch/serial safety testing in laboratory animals, requiring a test in one of the target species for the product. The European Pharmacopoeia no longer requires a batch safety test in target animal species for the release of vaccine batches/serials nor a general safety test (abnormal toxicity test) in mice or guinea pigs.

2.3. Batch/serial potency test

Batch/serial potency tests, required for each batch/serial prior to release, are designed to correlate with the host animal vaccination–challenge efficacy studies. Potency assays must be properly validated for both live and killed products. For inactivated viral or bacterial products, potency tests may be conducted in laboratory or host animals, or by means of quantitative *in-vitro* methods that have been validated reliably to correlate *in-vitro* quantification of important antigen(s) with *in-vivo* efficacy. The potency of live vaccines is generally measured by means of bacterial counts or virus titration. Recombinant DNA or biotechnology-based vaccines should also be tested. Live genetically modified organisms can be quantified like any other live vaccine by titration, and expressed products of recombinant technology are quantified by *in-vitro* tests, which may be easier to perform compared with tests on naturally grown antigens because of the in-process purification of the desired product.

When testing a live bacterial vaccine for release for marketing, the bacterial count/titre must be sufficiently greater than that shown to be protective in the immunogenicity (efficacy) study to ensure that

at any time prior to the expiry date, the count/titre will be at least equal to that of the batch/serial used in the immunogenicity test. When testing a live viral vaccine for release, the virus titre must, as a rule, be sufficiently greater than that shown to be protective in the immunogenicity test in order to ensure that at any time prior to the expiry date, the titre will be at least equal to that used in the immunogenicity test. Some control authorities specify higher bacterial or viral content than these. It is evident that the appropriate release titre is primarily dependent on the required potency and secondarily dependent on the rate of decay of the bacteria or viruses in the vaccine, as indicated by the stability test.

Standard Requirements have been developed and published by competent authorities for potency testing several vaccines. These tests can be found in CFR Title 9 part 113, in the European Pharmacopoeia, and in this *Terrestrial Manual*.

3. Other tests

3.1. Tests on the finished product

Depending on the form of vaccine being produced, certain tests may be indicated and should be provided as appropriate in the approved documentation of the manufacturing process. These tests may concern: the level of moisture contained in desiccated/lyophilised products, the level of residual inactivant in killed products, the complete inactivation of killed products, pH, the level of preservatives and permitted antibiotics, physical stability of adjuvants, retention of vacuum in desiccated/lyophilised products, and a general physical examination of the final vaccine. A loss of potency may result when residual inactivating agent in a killed liquid product used as a diluent for a desiccated/ lyophilised live fraction reduces the viability of the live organisms because of virucidal or bactericidal activity. Each batch/serial of liquid killed vaccine that is to be used as a diluent for live vaccines must, therefore, be tested for virucidal or bactericidal activity prior to release.

Tests for these purposes may also be found in CFR Title 9 part 113, in EU Directive 2001/82/EC (as amended), in the European Pharmacopoeia, or in this *Terrestrial Manual*.

3.2. Tests on other products

3.2.1. Purity

Purity is determined by testing for a variety of contaminants. Tests to detect contaminants are performed on samples of master seeds, primary cells, master cell stocks (MCS), ingredients of animal origin if not subjected to sterilisation (e.g. fetal bovine serum, bovine albumin, or trypsin).

Procedures used to ensure that fetal or calf serum and other ingredients of bovine origin are free of pestiviruses should be of high concern and well documented. Tests to be used to minimise the risk of impurity vary with the nature of the product, and should be prescribed in the approved documentation of the manufacturing process.

3.2.2. Tests for the detection of TSE agents

As tests for the detection of TSE agents in ingredients of animal origin have not been developed, vaccine manufacturers should document in their Outlines of Production or SOPs the measures they have implemented to minimise the risk of such contamination in ingredients of animal origin. This relies on three principles: first, verification that sources of all ingredients of animal origin in production facilities are from countries recognised as having the lowest possible risk of bovine spongiform encephalopathy; second, that the tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE agents; third, where relevant, that the processes applied to the material have been validated for inactivation of TSE agents in accordance with the *Terrestrial Code*. Methods of production should also document the measures taken to prevent cross contamination of low risk materials by higher risk materials during processing.

MARKET MONITORING

1. Performance monitoring

Holders of relevant regulatory approval or manufacturers are required to maintain an adverse reaction notification system and an effective mechanism for rapid product recall. These should both be subject to audit by regulatory bodies. In many countries, the manufacturer must notify all adverse reactions immediately to the regulatory authority, along with any remedial action taken. An alternative used in some countries is that if at any time, there are indications that raise questions regarding the purity, safety, potency, or efficacy of a product, or if it appears that there may be a problem regarding the preparation, testing or distribution of a product, the manufacturer must immediately notify the regulatory authorities concerning the circumstances and the action taken.

After release of a product, its performance under field conditions should continue to be monitored by competent authorities and by the manufacturer itself. Consumer complaints may serve as one source of information; however, such information should be investigated to determine whether the reported observations are related to the use of the product. Users of veterinary vaccines should be informed of the proper procedures for making their complaints. The manufacturer of the product should be informed of all complaints received by competent authorities. Competent authorities should also ascertain whether they have received other similar complaints for this product and, if so, whether the manufacturer has taken appropriate action. Control laboratories designated by the competent authority may test samples of the batch/serial of product involved, if necessary.

Exporting countries and importing countries should ensure that regulatory approval holders or manufacturers establish a reliable system to monitor adverse reaction notification (vaccinovigilance, post-approval monitoring) is established to identify, at the earliest stage, any serious problems encountered from the use of veterinary vaccines. Vaccinovigilance should be on-going and an integral part of all regulatory programmes for veterinary vaccines, especially live vaccines. The regulatory approval holder or manufacturer plays a big part in the conduct of this continuous overall vaccinovigilance evaluation. When it is determined that a product has a quality defect, immediate action should be taken to notify animal health authorities, to remove the product from the market and, if possible, to inform the end users.

2. Enforcement

National programmes established to ensure the purity, safety, potency, and efficacy of veterinary vaccines must have adequate legal authority to ensure compliance with relevant regulatory approval conditions for the product and other programme requirements. The goal should be to obtain voluntary compliance with established regulatory requirements. However, when violations occur, competent authorities must have adequate legal authority to protect animal and human health and the public interest. Authority for detention, seizure, and condemnation of products found to be worthless, contaminated, dangerous, or harmful is essential for this purpose. Under such authority, product may be detained for a period of time, and if during that time compliance cannot be achieved, competent authorities may seek legal authorisation for seizure and condemnation.

The authority to remove or suspend establishment and/or product regulatory approvals, obtain injunctions, and stop the sale of product is also needed. Civil penalties or criminal prosecution may also be necessary for serious or deliberate violations.

INSPECTION OF PRODUCTION FACILITIES

Establishments that are approved to produce veterinary biologicals should be subject to in-depth inspections of the entire premises by national competent authorities to ensure compliance with the Outline of Production and blueprints and legends, SOPs, or other documentation related to the manufacturing process. These inspections should be carried out on a regular basis and should allow the assessment of the manufacturing sites with regards to GMP standards.

These inspections may include such items as: personnel qualifications; record keeping; general sanitation and laboratory standards; production procedures; operation of sterilisers, pasteurisers, incubators, and refrigerators; filling, desiccating, and finishing procedures; care and control of animals; testing procedures; distribution and marketing; and product destruction.

Details regarding the inspection of production facilities and requirements for inspectorates are described in chapter 2.3.4.

FURTHER READING

The following are some suggested texts that contain guidelines on aspects of vaccine production.

CODE OF FEDERAL REGULATIONS (OF THE UNITED STATES OF AMERICA) (CFR) (2000). Title 9, Parts 1–199. US Government Printing Office, Washington DC, USA. http://www.gpo.gov/fdsys/pkg/CFR-2006-title9-vol1/pdf/CFR-2006-title9-vol1-chapI.pdf or ELECTRONIC CODE OF FEDERAL REGULATIONS, accessed at http://www.ecfr.gov/cgi-bin/text-idx?SID=a96ece744f88b16cc39202d9cbc5bdae&tpl=/ecfrbrowse/Title09/9tab_02.tpl

EUROPEAN PHARMACOPOEIA 7.0. (2012). European Directorate for the Quality of Medicines and Health Care (EDQM), Council of Europe, Strasbourg, France.

ESPESETH D.A. (1993). Licensing Veterinary Biologics in the United States. The First Steps Towards an International Harmonization of Veterinary Biologicals; and Free circulation of vaccines within the EEC. *Dev. Biol. Stand.,* **79**, 17–25.

ESPESETH D.A. & GOODMAN J.B. (1993). Chapter 13. *In:* Licensing and Regulation in the USA. Vaccines for Veterinary Application. Butterworth Heinemann, London, UK, 321–342.

EUROPEAN COMMISSION (2006). The Rules Governing Medicinal Products in the European Union. Eudralex. Volumes 1–9. European Commission Enterprise and Industry DG; Directorate F – Consumer goods. Latest versions only available at http://pharmacos.eudra.org/F2/eudralex/index.htm.

GAY C.G. & ROTH H.J. (1994). Confirming the safety characteristics of recombinant vectors used in veterinary medicine: a regulatory perspective. Recombinant vectors in vaccine development. *Dev. Biol. Stand.*, **82**, 93–105.

ROTH H.J. & GAY C.G. (1996). Specific safety requirements for products derived from biotechnology. *In:* Veterinary Vaccinology, Pastoret P.-P., Blancou J., Vannier P. & Verschueren C., eds. Elseviers Science Publishers B.V. Amsterdam, The Netherlands.

PASTORET P.P., BLANCOU J., VANNIER P. & VERSCHUEREN C., EDS (1997). Veterinary Vaccinology. Elsevier Science, Amsterdam, The Netherlands.

PIC/S GUIDE AVAILABLE AT THE FOLLOWING ADDRESS: WWW.PICSCHEME.ORG

USDA-APHIS[2]-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1999). Categories of Inspection for Licensed Veterinary Biologics Establishments. Veterinary Services Memorandum No. 800.91. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1999). Veterinary Biological Product Samples. Veterinary Services Memorandum No. 800.59. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

USDA-APHIS- VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1995). Guidelines for Submission of Materials in Support of Licensure. Veterinary Biologics Memorandum No. 800.84. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1995). Veterinary Biologics General Licensing Considerations No. 800.200, Efficacy Studies. USDA-APHIS-Veterinary Biologics, 4700 River Road, Riverdale, Maryland 20737, USA.

2 United States Department of Agriculture (USDA), Animal and Plant Health Inspection Services (APHIS). USDA-APHIS-Center for Veterinary Biologics Home Page: http://www.aphis.usda.gov/vs/cvb/index.html

USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1995). Veterinary Biologics General Licensing Considerations No. 800.201, Back Passage Studies. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

USDA-APHIS-VETERINARY SERVICES (1964–1994). Standard Assay Methods, Series 100–900. National Veterinary Services Laboratories, Ames, Iowa 50010, USA.

USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1984). Basic License Requirements for Applicants. Veterinary Biologics Memorandum No. 800.50. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA

USDA-APHIS-VETERINARY SERVICES-CENTER FOR VETERINARY BIOLOGICS (1988). Guidelines for the Preparation and Review of Labeling Materials. Veterinary Services Memorandum No. 800.54. Center for Veterinary Biologics, 510 S. 17th Street, Suite 104, Ames, Iowa 50010, USA.

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NB: FIRST ADOPTED IN 1996. MOST RECENT UPDATES ADOPTED IN 2022.

APPENDIX 1.1.8.1.

RISK ANALYSIS FOR BIOLOGICALS FOR VETERINARY USE

GENERAL CONSIDERATIONS

All products, including biologicals for veterinary use, derived from animals have some capacity to transmit animal disease. The level of this capacity depends on the inherent nature of the products, their source, the treatment that they might have undergone, and the purpose for which they are intended. Biologicals for *in-vivo* use in particular will have the highest probability of exposure to animals and as such present the highest risk. Products used for *in-vitro* purposes can introduce disease into animal populations through deliberate or inadvertent use *in vivo*, contamination of other biologicals, or spread by other means. Even products for diagnosis and research have the potential for close contact with animals. Exotic micro-organisms, some highly pathogenic, which may be held for research and diagnostic purposes in countries free from infection or the diseases they cause, could possibly contaminate other biological products.

Veterinary Authorities of importing countries shall make available specific procedural requirements for relevant regulatory approval of biologicals for veterinary use. They may limit supply to registered institutions or *in-vitro* use or for non-veterinary purposes where such assurance cannot be provided.

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APPENDIX 1.1.8.2.

RISK ANALYSIS FOR VETERINARY VACCINES

INTRODUCTION

Risk analysis for veterinary vaccines has to be founded on the principles of quality assurance, which includes quality control, in the production of veterinary vaccines. These recommendations are focused mainly on the risk related to the contamination of vaccines by infectious agents particularly in regard to the risk of importing exotic diseases. The major risk of introducing a disease into a country is through importation of live animals or animal products and rarely through veterinary vaccines. Veterinary vaccines can however be contaminated by disease agents if master seeds, strains, cell cultures, animals or ingredients of animal origin such as fetal calf serum used in production are contaminated or if cross contamination occurs during the production process.

PRINCIPLES

Exporting countries and importing countries should agree on a system of classification of risks associated with veterinary vaccines taking into account factors such as purification procedures which have been applied.

Exporting countries and importing countries should agree on risk analysis models to address specific issues and products. Such risk analysis models should include a scientific risk assessment and formalised procedures for making risk management recommendations and communicating risk. The regulation of veterinary vaccines should include the use of either qualitative or quantitative models.

Risk analysis should be as objective and transparent as possible. Step risk and scenario tree methods should be used in risk assessment whenever appropriate, as they identify the critical steps in the production and use of the products where risks arise and help to characterise those risks.

The same conclusions about risk analysis may be reached by differing methods. Where methods may differ in countries, the concept of equivalence should apply wherever possible and the methods should be validated to ensure they are of comparable sensitivity.

MANUFACTURING PRACTICES

The manufacture of veterinary vaccines has special characteristics which should be taken into consideration when implementing and assessing the quality assurance system. Due to the large number of animal species and related pathogenic agents, the variety of products manufactured is very wide and the volume of manufacture is often low; hence, work on a group basis is common. Moreover, because of the very nature of this manufacture (cultivation steps, lack of terminal sterilisation, etc.), the products must be particularly well protected against contamination and cross contamination. The environment must also be protected especially when the manufacture involves the use of pathogenic or exotic biological agents and the worker must be particularly well-protected when the manufacture involves the use of biological agents pathogenic to man.

These factors, together with the inherent variability of immunological products, means that the role of the quality assurance system is of the utmost importance. It is important that vaccines should be manufactured in accordance with a recognised codified system that includes specifications regarding equipment, premises, qualification of personnel as well as quality assurance and regular inspections.

A commonly agreed system of facility inspection carried out by qualified and specialised inspectors must be in place to assure confidence.

INFORMATION TO BE SUBMITTED WHEN APPLYING FOR RELEVANT REGULATORY APPROVAL IN THE IMPORTING COUNTRY

The manufacturer or Veterinary Authority of the exporting country should make available to the importing country the pharmacopoeia it uses. For the importing country it is necessary to have documented both the quality control methods used and the source of each batch of starting materials. The key steps of the manufacturing process of veterinary vaccines should be described in detail to help risk analysis. Risk analysis has to be focused on the quality and safety parts of the application file. Laboratory safety testing should cover target and non-target organisms to obtain sufficient biological data. All test procedures used should correspond with the state of scientific knowledge at the time and should be validated.

The description of the method of preparation of the finished product should include an adequate characterisation of the substances needed to prepare the working seeds, the description of the treatments applied to starting materials to prevent contamination, and a statement of the stages of manufacture at which sampling is carried out for process control tests.

The results of control tests during production and on finished product, as well as the sensitivity of these tests, have to be available for risk analysis. The stepwise procedures of the control tests should also be available.

CATEGORISATION OF VETERINARY VACCINES

To assist in risk analysis, countries should establish a system of categorisation of veterinary vaccines taking into account criteria such as pathogens used as active ingredients, their inherent characteristics and the risk they pose.

In case of live vectored vaccines, the safety of the vector to the targeted and non-targeted species and to human beings must be assessed. Special attention should be paid to potential tissue tropism or host range modification of the recombinant.

VACCINOVIGILANCE

Exporting countries and importing countries should ensure that a reliable system of vaccinovigilance (post-approval monitoring) is established to identify, at the earliest stage, any serious problems encountered from the use of veterinary vaccines. Vaccinovigilance should be ongoing and an integral part of all regulatory programmes for veterinary vaccines, especially live vaccines.

RISK COMMUNICATION

Reliable data in support of applications submitted in importing countries should be provided by the manufacturer or the Veterinary Authority of the exporting country. Relevant data on risk analysis, changes in animal health situations and vaccinovigilance should be shared by Veterinary Authorities on a continuous basis.

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CHAPTER 1.1.9.

TESTS FOR STERILITY AND FREEDOM FROM CONTAMINATION OF BIOLOGICAL MATERIALS INTENDED FOR VETERINARY USE

INTRODUCTION

The international trade-related movements of biological materials intended for veterinary use are subject to restrictions imposed to minimise the spread of animal and human pathogens. Countries may impose requirements for proof-of-freedom testing before allowing the regulated importation of materials of animal derivation and substances containing such derivatives. Where chemical or physical treatments are inappropriate or inefficient, or where evidence of the effectiveness of the treatment is lacking, there may be general or specific testing requirements imposed by authorities of countries receiving such materials. This chapter provides guidance on the approach to such regulated testing, particularly as might be applied to the movement of vaccine master seed and master cell stocks, and to related biological materials used in manufacturing processes. The term seed stocks is used when testing live products, for killed products the preferred reference is master cell stocks. While the onus for ensuring safety of a product remains with the manufacturer and may be regulated by therapeutic guidelines, this chapter provides procedures that are designed in particular to minimise the risk of undetected contaminants in veterinary therapeutics and biological reagents causing the cross-border spread of agents of concern to particular importing countries. Control of contamination with transmissible spongiform encephalopathy (TSE) agents is not covered in this chapter because testing and physical treatments cannot be used to ensure freedom from these agents.

Sterility is defined as the absence of viable microorganisms, which for the purpose of this chapter, includes viruses. It should be achieved using aseptic techniques and validated sterilisation methods, including heating, filtration, chemical treatments, and irradiation that fits the intended purpose. Freedom from contamination is defined as the absence of specified viable microorganisms. This may be achieved by selecting materials from sources shown to be free from specified microorganisms and by conducting subsequent procedures aseptically. Adequate assurance of sterility and freedom from contaminating microorganisms can only be achieved by proper control of the primary materials used and their subsequent processing. Tests on intermediate products are necessary throughout the production process to check that this control has been achieved.

Biological materials subject to contamination that cannot be sterilised before or during use in vaccine production, such as ingredients of animal origin, e.g. serum and trypsin, primary and continuous cells and cell lines, and viral or bacterial seed stocks, etc., should be tested for viable extraneous agents before use. Assays to detect viral contaminants, if present, can be achieved by culture methods supported by cytopathic effects (CPE) detection, fluorescent antibody techniques and other suitable methods such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). As is explained in more detail in this chapter care must be taken when using PCR and ELISA techniques for detection as such tests do not distinguish viable from non-viable agent detection.

Avian materials and vaccines are required to be inoculated on to primary avian cell cultures or eggs for the detection of avian viruses. A combination of general tests, for example to detect haemadsorbing, haemagglutinating and CPE-causing viruses and specific procedures aimed at the growth and detection of specific viruses is recommended to increase the probability of detection. Assays to detect other contaminants, such as bacteria, fungi, protozoa, rickettsia and mycoplasma are also described.

Procedures applied should be validated and found to be "fit for purpose" following Chapter 1.1.6 Validation of diagnostic assays for infectious diseases of terrestrial animals*, where possible.*

It is the responsibility of the submitter to ensure a representative selection and number of items to be tested. The principles of Appendix 1.1.2.1 Epidemiological approaches to sampling: sample size calculations *of Chapter 1.1.2* Collection, submission and storage of diagnostic specimens apply. *Adequate transportation is described in Chapter 1.1.2 and Chapter 1.1.3* Transport of biological materials*.*

A. AN OVERVIEW OF TESTING APPROACHES

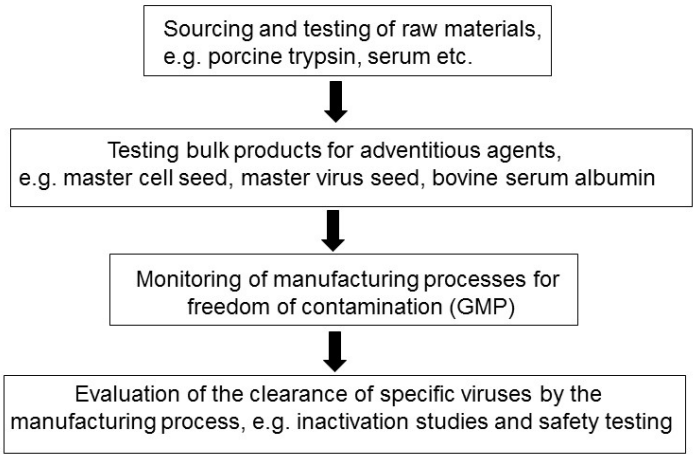
1. Primary materials must be collected from sources shown to be free from contamination and handled in such a way as to minimise contamination and the opportunities for any contaminants to multiply (Figure 1).

2. Materials that are not sterilised and those that are to be processed further after sterilisation must be handled aseptically. Such materials will require further assessment of freedom of contaminants at certain stages of production to assure freedom of adventitious agents.

3. Materials that can be sterilised without their biological activities being affected unduly must be sterilised by a method effective for the pathogens of concern. The method must reduce the level of contamination to be undetectable, as determined by an appropriate sterility test study. (See Section D.1. below). If a sterilisation process is used, it shall be validated to demonstrate that it is fit for purpose. Suitable controls will be included in each sterilisation process to monitor efficiency.

4. The environment in which any aseptic handling is carried out must be maintained in a clean state, protected from external sources of contamination, and controlled to prevent internal contamination. Rules governing aseptic preparation of vaccines are documented in Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*.

Figure 1. Testing algorithm for vaccine production.



Some procedures have been properly validated and found to be "fit for purpose", whilst others may have undergone only limited validation studies. For example, methods for bacterial and fungal sterility have not been formally validated although they have been used for many years. In particular, the *in-vivo* and cell culture methods have essentially unknown sensitivity and specificity (Sheets *et al*., 2012) though there is an accepted theoretical sensitivity of 1 colony-forming unit (CFU). For example, an evaluation of methods to detect bovine and porcine viruses in serum and trypsin based on United States (of America) Code of Federal Regulations, Title 9 (9CFR) revealed gaps in sensitivity, even within virus families (Marcus-Secura *et al*., 2011). It is therefore important to interpret results in the light of specific conditions of cultures employed and considering sensitivity and specificity of detection systems.

Newer, more sensitive methods such as molecular assays may afford the ability to detect contaminants, which may not be successfully amplified in traditional culturing systems. The detection range can be broadened by using

family specific primers and probes if designed appropriately. However, most, if not all such new tests are also able to detect evidence for non-infectious contaminants, such as traces of nucleic acid from inactivated contaminants. Follow-up testing would be required to determine the nature of the contaminant, for example, non-infectious nucleic acid or infectious virus. Attempts at virus isolation or sequencing may remedy this. Note: molecular assays if not designed as fit for purpose may miss detection of contaminating agents or lack sensitivity to do so (Hodinka, 2013).

More recently metagenomic high throughput sequencing (HTS) workflows have shown potential for quality control of biological products (van Borm *et al*., 2013) and vaccines (Baylis *et al.,* 2011; Farsang & Kulcsar, 2012; Neverov & Chumakov, 2010; Onions & Kolman, 2010; Victoria *et al*., 2010) in particular for the identification and characterisation of unexpected highly divergent pathogen variants (Miller *et al*., 2010; Rosseel *et al*., 2011) that may remain undetected using targeted diagnostic tests. Nevertheless, targeted assays, e.g. amplification in cell culture followed by polymerase chain reaction (PCR) may be superior to HTS for specific agent detection (Wang *et al*., 2014) due to lack of sensitivity of HTS at this time. Similarly, recent improvements in protein and peptide separation efficiencies and highly accurate mass spectrometry have promoted the identification and quantification of proteins in a given sample. Most of these new technologies are broad screening tools, limited by the fact that they cannot distinguish between viable and non-viable organisms.

B. LIVING VIRAL VACCINES FOR ADMINISTRATION BY INJECTION

1. Materials of animal origin shall be (a) sterilised, or (b) obtained from healthy animals that, in so far as is possible, should be shown to be free from pathogens that can be transmitted from the species of origin to the species to be vaccinated, or any species in contact with them by means of extraneous agents testing.

2. Seed lots of virus, any continuous cell line and biologicals used for virus growth shall be shown to be free from viable bacteria, fungi, mycoplasmas, protozoa, rickettsia, extraneous viruses and other pathogens that can be transmitted from the species of origin to the species to be vaccinated or any species in contact with them.

   For the production of vaccines in embryonated chicken eggs and the quality control procedures for these vaccines, it is recommended (required in many countries) that eggs from specific pathogen-free birds should be used.

3. Each batch of vaccine shall pass tests for freedom from extraneous agents that are consistent with the country's requirements for accepting the vaccine for use. Published methods that document acceptable testing procedures in various countries include: (US) Code of Federal Regulations (2015); European Pharmacopoeia (2014); European Commission (2006); World Health Organization (WHO) (1998; 2012) and Department of Agriculture (of Australia) (2013).

4. Tests for sterility shall be appropriate to prove that the vaccine is free from extraneous viruses, bacteria including rickettsia and mycoplasmas, fungi, and protozoa. Each country will have particular requirements as to what agents are necessary to exclude for and what procedures are acceptable. Such tests will include amplification of viable extraneous agents using cell culture that is susceptible to particular viruses of the species of concern, tests in embryonated eggs, bacterial, mycoplasma and fungal culturing techniques and, where necessary and possible, tests involving animal inoculation. PCR, fluorescence antibody test (FAT), presence of colonies or cytopathic effects (CPE) and enzyme-linked immunosorbent assay (ELISA) will be used for detection purposes after amplification using culturing techniques. If *in-vitro* or *in-vivo* amplification of the target agent is not possible, direct PCR may be useful if validated for this purpose.

C. LIVING VIRAL VACCINES FOR ADMINISTRATION THROUGH DRINKING WATER, SPRAY, OR SKIN SCARIFICATION

1. Section B applies.

2. A limited number of contaminating, non-pathogenic bacteria and fungi may be permitted (see Section I.2.2 *General Procedure for testing live viral vaccines produced in eggs and administered through drinking water, spray, or skin scarification for the presence of bacteria and fungi*).

D. INACTIVATED VIRAL VACCINES

1. Each batch of vaccine shall pass a test for inactivation of the vaccinal virus and should include inactivation studies on representative extraneous agents if the virus seed has not already been tested and shown to be free from extraneous agents. An example of a simple inactivation study could include assessment of the titre of live vaccine before and after inactivation and assessing the $\log_{10}$ drop in titre during the inactivation process. This would give an indication of the efficacy of the inactivation process. There is evidence that virus titration tests may not have sufficient sensitivity to ensure complete inactivation. In these circumstances, a specific innocuity test would need to be developed and validated to be fit for increased sensitivity. To increase sensitivity more than one passage would be required depending on the virus of concern. An example of this approach can be found at:
https://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_117.pdf.

2. If studies on representative extraneous agents are required, then spiking inactivated vaccine with live representative agents and following the example of an inactivation study as in D.1 above would be useful. The inactivation process and the tests used to detect live virus after inactivation must be validated and shown to be suitable for their intended purpose.

   In addition, each country may have particular requirements for sourcing or tests for sterility as detailed in Section B above.

E. LIVING BACTERIAL VACCINES

1. Section B applies.

2. Seed lots of bacteria shall be shown to be free from other bacteria as well as fungi and mycoplasmas, protozoa, rickettsia, and extraneous viruses. Agents required for exclusion will be dependent on the country accepting the vaccine for use. Use of antibiotics to 'inactivate' the living bacterial seed or vaccine prior to exclusion of viruses and fungi is recommended to ensure testing in culture is sensitive. Interference testing is recommended to ensure that the antibiotics used do not affect the growth of the extraneous virus or fungi that is being excluded.

3. Due to the difficulties and reduced sensitivity in exclusion of extraneous bacteria and some mycoplasma, protozoa, and rickettsia from high-titred seed lots of bacteria, the use of narrow-range antibiotics aimed specifically at reducing seed lot bacteria is recommended if antibiotics do not affect the growth of bacteria being excluded. The optimal concentration of antibiotics can be determined in a dilution experiment such as documented in 9CFR Section 113.25(d). Other methods of exclusion of extraneous bacteria from bacterial seeds may include filtering for size exclusion such as removing bacteria seed to look for mycoplasma contamination and use of selective culturing media. Such processes would require validation to ensure the process does not affect the sensitivity of exclusion of extraneous agents of concern.

4. Direct PCR techniques may be useful when culturing processes fail to be sensitive in detecting extraneous bacteria from live bacterial seeds or vaccines.

F. INACTIVATED BACTERIAL VACCINES

1. Section D applies. It should not be necessary to test for extraneous viruses that would not grow in bacteriological culture media as long as freedom from contamination of all starting materials can be assured. Complete inactivation of the vaccinal bacteria should be demonstrated by means of titration and innocuity tests – in some cases general bacterial sterility testing (Section I.2.1) may suffice.

G. SERA AND DIAGNOSTIC AGENTS FOR ADMINISTRATION TO ANIMALS

1. Section B.1 applies for non-inactivated sera.

2. Some countries require quarantine, health certification, and tests for specific diseases to be completed for all serum donor animals, for example, 9CFR (2015) and Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999).

3. It is recommended that each batch of non-inactivated serum be assessed for viable extraneous agents, including mycoplasma. Each batch of serum shall pass a test for freedom from extraneous agents. Suitable test methods have been published for various countries, for example, European Pharmacopoeia (2014); 9 CFR (2015) and Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999) and Department of Agriculture (of Australia) (2013).

4. Inactivated serum, Section D applies.

5. Section B or D may apply if a virus is used in the production of the diagnostic agent; Section E or F may apply if a bacterium is used.

H. EMBRYOS, OVA, SEMEN

Special precautions must be taken with relation to the use of embryos, ova, semen (Hare, 1985). Most countries will have regulatory guidelines for import of these biologicals for veterinary use. Such guidelines can be found at various websites such as the European Commission, FAO and though many such guidelines give more detail in regard to the food safety aspect.

I. PROTOCOL EXAMPLES

1. General procedures

In principle, proposed testing represents an attempted isolation of viable agents in culturing systems normally considered supportive of the growth of each specified agent or group of general agents. After amplification, potential pathogens can be detected further by sensitive and specific diagnostic tests such as FAT or PCR if required. General detection systems can include haemabsorbance and CPE by immunohistochemistry staining methods. The example procedures for sterility testing and general detection of viable bacteria, mycoplasma, fungi, and viruses described below are derived from standards such as the 9CFR (2015), European Pharmacopoeia (2014), European Commission (2006), WHO (1998; 2012).

Individual countries or regions should adopt a risk-based approach to determine the appropriate testing protocols based on their animal health status. As well as applying general testing procedures documented in national or regional standards as mentioned above, it may be necessary to apply rigorous exclusion testing for specific agents that are exotic to the particular country or region.

General procedures will not necessarily detect all extraneous agents that may be present in biological material; however, they are useful as screening tests. Some examples of agents that may require specific methods for detection in biologicals refer to Table 1 below. Procedures documented in the Review of Published Tests to Detect Pathogens in Veterinary Vaccines Intended for Importation into Australia (2013) available from the Department of Agriculture and Water Resources, Australia are able to address such agents in offering sensitive testing approaches based on reputable publications.

Exclusion of specific agents requires procedures that maximise sensitivity by providing ideal amplification and detection of the pathogen in question. Extraneous agents, such as Maedi Visna virus, bovine immunodeficiency virus, *Trypanosoma evansi* and porcine respiratory coronavirus are difficult to culture even using the most sensitive approaches. In these circumstances, application of molecular assays directly to the biological material in question to assess the presence of nucleic acid from adventitious agents offers an alternative. Refer to Table 1. Consideration must be noted as described in Section A.6 as non-viable agents may also be detected using this procedure.

Table 1 gives some examples of causative infectious agents that may be present in animal biologicals intended for veterinary use. This is not an exhaustive list of agents of concern or by any means required for exclusion by every country, they are just examples of infectious agents that are not culturable using general culturing procedures and require a more specific detection process by means of the indirect fluorescent antibody test, PCR or ELISA, where applicable. Notably, some subtypes of an agent type may be detectable by general methods, and some may require

specialised testing for detection. For example, bovine adenovirus subgroup 1 (serotypes 1, 2, 3 and 9) can be readily isolated using general methods (Vero cells) however bovine adenovirus subgroup 2 (serotypes 4, 5, 6, 7, 8 and 10) are not readily isolated and required specialised methods for isolation.

Table 1. Some examples of infectious agents of veterinary importance that require specialist culturing and detection techniques

Rotaviruses	Pestiviruses (non-CPE)	Turkey rhinotracheitis
Porcine epidemic diarrhoea virus	Bluetongue virus	*Brucella abortus*
Porcine circoviruses (PCV 1, 2)	Swine pox virus	Rickettsias
Swine/equine influenza, some strains	Some adenoviruses	Protozoa
Bovine respiratory syncytial virus	Rabies virus	Some fungi (e.g. *Histoplasma*)

2. Detection of bacteria and fungi

2.1. General procedure for assessing the sterility of viable bacteria and fungi

Standard tests for detecting extraneous bacteria and fungi (sterility testing) in raw materials, master cell stocks, or final product are the membrane filtration test or the direct inoculation sterility test.

For the membrane filtration technique, a filter having a nominal pore size not greater than 0.45 μm and a diameter of at least 47 mm should be used. Cellulose nitrate filters should be used if the material is aqueous or oily; cellulose acetate filters should be used if the material is strongly alcoholic, oily or oil-adjuvanted. Immediately before the contents of the container or containers to be tested are filtered, the filter is moistened with 20–25 ml of Diluent A or B.

2.1.1. Diluent A

Diluent A is for aqueous products or materials. Dissolve 1 g peptic digest of animal tissue in water to make 1 litre, filter, or centrifuge to clarify, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

2.1.2. Diluent B

Diluent B is for oil-adjuvanted products or materials: Add 1 ml polysorbate 80 to 1 litre Diluent A, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

If the biological being tested has antimicrobial properties, the membrane is washed three times after sample application with approximately 100 ml of the appropriate diluent (A or B). The membrane is then transferred whole to culture media, aseptically cut into equal parts and placed in media, or the media is transferred to the membrane in the filter apparatus. If the test sample contains merthiolate as a preservative, fluid thioglycollate medium (FTM) is used and the membranes are incubated at both 30–35°C and 20–25°C. If the test sample is a killed biological without merthiolate preservative, FTM is used at 30–35°C and soybean casein digest medium (SCDM) at 20–25°C. If the sample tested is a live viral biological, SCDM is used at both incubation temperatures. It has been suggested that sulfite-polymyxin-sulfadiazine agar be used to enhance the detection of *Clostridium* spp. when the membrane filtration technique is used (Tellez *et al*., 2005).

If direct inoculation of culture media is chosen, a sterile pipette or syringe and needle are used to aseptically transfer the biological material directly into liquid media. If the biological being tested has antimicrobial properties, the ratio of the inoculum to the volume of culture medium must be determined before the test is started, for example as explained in 9CFR 113.25(d) and detailed testing procedures can be found for example in supplemental assay method (SAM) 903. To determine the correct medium volume to negate antimicrobial activity, 100 CFU of the control microorganisms listed in Table 2 are used. If the test sample contains merthiolate as a preservative, FTM is used in test vessels incubated at both 30–35°C and 20–25°C. Growth should be clearly visible after an appropriate incubation time (see Section I.2.1.3 *Growth promotion and test interference*). If the test sample is a killed biological without merthiolate, or a live bacterial biological, FTM is used at 30–35°C and SCDM at 20–25°C. If the test sample is a live viral biological, SCDM is used at both incubation temperatures. If the inactivated bacterial vaccine is a clostridial biological, or contains a clostridial component, the use of FTM with 0.5% added

beef extract (FTMB) in place of FTM is preferred. It may also be desirable to use both FTM and SCDM for all tests.

Table 2. Some American Type Culture Collection[1] strains with their respective medium and incubation conditions

Medium	Test microorganism	Incubation	
		Temperature (°C)	Conditions
FTM	*Bacillus subtilis* ATCC # 6633	30–35	Aerobic
FTM	*Candida krusei* ATCC # 6258	20–25	Aerobic
SCDM	*Bacillus subtilis* ATCC # 6633	30–35	Aerobic
SCDM	*Candida krusei* ATCC # 6258	20–25	Aerobic
FTMB	*Clostridium sporogenes* ATCC # 11437	30–35	Anaerobic
FTMB	*Staphylococcus aureus* ATCC #6538	30–35	Aerobic

For both membrane filtration and direct inoculation sterility tests, all media are incubated for no fewer than 14 days. At intervals during incubation, and after 14 days' incubation, the test vessels are examined for evidence of microbial growth. Microbial growth should be confirmed by subculture and Gram stain.

2.1.3. Growth promotion and test interference

The sterility of the media should be confirmed by incubating representative containers at the appropriate temperature for the length of time specified for each test.

The ability of the culture media to support growth in the presence and absence of product, product components, cells, seeds, or other test material should be validated for each product to be tested, and for each new batch or lot of culture media for example as outlined in 9CFR 113.25(b). Detailed testing procedures can be found for example in SAMs 900-902.

To test for ability to support growth in the absence of the test material, media should be inoculated with 10–100 viable control organisms of the suggested ATCC strains listed in Table 2 and incubated according to the conditions specified.

To test for ability of the culture media to support growth in the presence of the test material, containers should be inoculated simultaneously with both the test material and 10–100 viable control organisms. The number of containers used should be at least one-half the number used to test the product or product component. The test media are satisfactory if clear evidence of growth of the control organisms appears in all inoculated media containers within 7 days. In the event that growth is evident, the organism should be identified to confirm that it is the organism originally added to the medium. The sterility test is considered invalid if any of the media show inadequate growth response, or if the organism recovered, is not the organism used to inoculate the material.

If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 ml) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

2.2. General procedure for testing live viral vaccines produced in eggs and administered through drinking water, spray, or skin scarification for the presence of bacteria and fungi

Each batch of final container biological should have an average contamination of not more than one bacterial or fungal colony per dose for veterinary vaccines. From each container sample, each of two Petri dishes are inoculated with vaccine equal to ten doses if the vaccine is recommended for poultry, or one dose if recommended for other animals. To each plate 20 ml of brain–heart infusion agar are added

1 American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.

containing 0.007 IU (International Units) of penicillinase per ml. One plate should be incubated at 30–35°C for 7 days and the other at 20–25°C for 14 days. Colony counts are made at the end of each incubation period. An average colony count of all the plates representing a batch should be made for each incubation condition. If the average count at either incubation condition exceeds one colony per dose in the initial test, one retest to rule out faulty technique may be conducted using double the number of unopened final containers. If the average count at either incubation condition of the final test for a batch exceeds one colony per dose, the batch of vaccine should be considered unsatisfactory.

2.3. General procedure for testing seed lots of bacteria and live bacterial biologicals for purity

Each seed lot of bacteria or batch of live bacterial biological should be tested for purity by inoculation of SCDM, which is incubated at 20–25°C for 14 days, and FTM, which is incubated at 30–35°C for 14 days. A sterile pipette or syringe and needle is used to aseptically transfer the quantity of biological directly into the two types of culture medium. The minimum ratio of inoculum to culture medium is 1/15.

If the inoculum or growth of the bacterial vaccine renders the medium turbid so that the absence of atypical microbial growth cannot be determined by visual examination, subcultures should be made from all turbid tubes on day 3 through to day 11. Subculturing is done by transferring 0.1–1.0 ml to differential broths and agar and incubating for the balance of the 14-day period. Microscopic examination by Gram stain should also be done.

If no atypical growth is found in any of the test vessels when compared with a positive control included in the test, the lot of biological may be considered satisfactory for purity. If atypical growth is found but it can be demonstrated by control that the media or technique were faulty, then the first test may be repeated. If atypical growth is found but there is no evidence invalidating the test, then a retest may be conducted. Twice the number of biological containers and test vessels of the first test are used in the retest. If no atypical growth is found in the retest, the biological could be considered to be satisfactory for purity but the results from both the initial and retest should be reported for assessment by the individual countries relevant regulatory agency. If atypical growth is found in any of the retest vessels, the biological is considered to be unsatisfactory for purity. If, however, it can be demonstrated by controls that the media or technique of the retest were faulty, then the retest may be repeated.

2.4. An example of a procedure for exclusion where general testing is not sufficient for detection of *Brucella abortus*

It should be confirmed that each batch of culture medium supports the growth of *B. abortus* by inoculating plates and flasks of biphasic medium with a known number of cells (around 100) of the fastidious *B. abortus* biovar 2. If the media supports the growth of this biotype it will support all other biovars.

Inoculate 1.0 ml of prepared master or working viral or cell seed material (not containing antibiotics) by inoculating 50 µl of the test product into each of 10 flasks containing biphasic medium. At the same time 10 plates of serum dextrose agar (SDA) are inoculated with 50 µl of inoculum and spread with a sterile bent glass Pasteur pipette or hockey stick. An un-inoculated serum dextrose agar plate and a biphasic flask are also set up at the same time as negative controls.

For assessment of inhibitory substances 50 µl of previously prepared master or working viral or cell seed material and 10–100 CFU of *B. abortus* are inoculated on to duplicate SDA plates. Positive controls are prepared by inoculating 10–100 CFU of *B. abortus* on to duplicate SDA plates.

All plates and flasks are incubated at 37°C in a 5–10% CO_2 environment. Plates are incubated with the agar uppermost and flasks with the agar slope vertical. Flasks are incubated with the cap loose.

Plates are checked for growth of colonies at days 4 and 8 of incubation. The biphasic medium is examined every 4 to 7 days for 28 days. After each examination of the flasks, they are tilted so that the liquid phase runs over the solid phase, then righted and returned to the incubator.

During the incubation period, SDA plates with positive control and test material are visually compared with plates with the positive control only and if there is no inhibition of growth of the organism in the

presence of the test material, the interference testing test is successful and testing can be assured to be sensitive.

Any signs of growth of suspicious contaminating microorganisms on SDA plates, cloudiness or colonies in biphasic flasks require follow-up testing by PCR to confirm whether *B. abortus* is present.

2.5. An example of a general procedure for detection of *Salmonella* contamination

Each batch of live virus biological made in eggs should be free from contamination with *Salmonella*. This testing must be done before bacteriostatic or bactericidal agents are added. Five samples of each batch should be tested; 5 ml or one-half of the container contents, whichever is the lesser, of the sample should be used to inoculate 100 ml of tryptose broth and tetrathionate broth. The inoculated broths should be incubated for 18–24 hours at 35–37°C. Transfers from these broths should be made on to MacConkey and *Salmonella–Shigella* agar, incubated for 18–24 hours, and examined. If no growth typical of *Salmonella* is noted, the agar plates should be incubated an additional 18–24 hours and again examined. If colonies typical of *Salmonella* are observed, further subculture on to suitable differential media should be made for positive identification. Sensitive PCR tests are available for the detection of *Salmonella* spp. in cultured material. If *Salmonella* is detected, the batch is determined to be unsatisfactory.

3. Detection of *Mycoplasma* contamination

3.1. An example of a general procedure for detecting *Mycoplasma* contamination

Each batch of live viral vaccine, each lot of master seed virus (MSV), each lot of primary and master cell stock (MCS), and all ingredients of animal origin not steam-sterilised should be tested for the absence of mycoplasmas. Solid and liquid media that will support the growth of small numbers of test organisms, such as typical contaminating organisms *Acholeplasma laidlawii*, *Mycoplasma arginini*, *M. fermentans*, *M. hyorhinis*, *M. orale*, and *M. synoviae* should be used. The nutritive properties of the solid medium should be such that no fewer than 100 CFU should occur with each test organism when approximately 100–200 CFUs are inoculated per plate. An appropriate colour change should occur in the liquid media when approximately 20–40 CFUs of each test organism are inoculated. The ability of the culture media to support growth in the presence of product should be validated for each product to be tested, and for each new batch or lot of culture media.

One sample of each lot of vaccine, e.g. MSV or MCS, should be tested. Four plates of solid medium are inoculated with 0.25 ml of the sample being tested, and 10 ml of the sample inoculated into 100 ml of the liquid medium. An alternative is to inoculate each of the plates with 0.1 ml and to inoculate 100 ml of liquid medium with 1 ml of the sample being tested. Two plates are incubated at 35–37°C aerobically (an atmosphere of air containing 5–10% CO_2 and adequate humidity) and two plates are incubated anaerobically (an atmosphere of nitrogen containing 5–10% CO_2 and adequate humidity) for 14 days. On day 3 or day 4 after inoculation, 0.25 ml from the liquid media are subcultured on to two plates of solid media. One plate is incubated aerobically and the second anaerobically at 35–37°C for 14 days. The subculture procedure is repeated on day 6, 7, or 8 and again on day 13 or 14. An alternative method is to subculture on days 3, 5, 10, and 14 on to a plate of solid medium. All the subculture plates are incubated for 10 days except for the 14-day subculture, which is incubated for 14 days. Liquid media is observed every 2–3 days and, if any colour change occurs, has to be subcultured immediately.

3.2. Interpretation of *Mycoplasma* test results

At the end of the incubation period (total 28 days), examine all the inoculated solid media microscopically for the presence of mycoplasma colonies. The test sample passes the test if the growth of mycoplasma colonies has occurred on the positive controls, and if growth has not occurred on any of the solid media inoculated with the test material. If at any stage of the test, more than one plate is contaminated with bacteria or fungi, or is broken, the test is invalid and should be repeated. If mycoplasma colonies are found on any agar plate, a suitable confirmatory test on the colonies should be conducted, such as PCR. Some mycoplasmas cannot be cultivated, in which case the MSV and MCS have to be tested using an indicator cell line such as Vero cells, DNA staining, or PCR methods.

Further detailed procedures can be found in Veterinary Medicinal Products, VICH GL34: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf

and USDA SAM 910: https://www.aphis.usda.gov/animal_health/vet_biologics/publications/910.pdf, (both accessed 4 July 2022.)

3.3. An example of a specific procedure for exclusion of *Mycoplasma mycoides* subsp. *mycoides* type from biologicals used in production of veterinary vaccines

Prior to beginning testing it is necessary to determine that each batch of media promotes the growth of *M. mycoides* subsp. *mycoides* (*Mmm*) type strain PG1. General mycoplasma broth and agar are used but contain porcine serum as a supplement. Each batch of broth and agar is inoculated with 10–100 CFU of *Mmm*. The solid medium is suitable if adequate growth of *Mmm* is found after 3–7 days' incubation at 37°C in 5–10% CO_2. The liquid medium is suitable if the growth on the agar plates subcultured from the broth is found by at least the first subculture. If reduced growth occurs another batch of media should be obtained and retested.

1 ml of cell or virus seed to be tested is inoculated into 9 ml of the liquid medium and 100 µl on to solid mycoplasma agar. The volume of the product is inoculated so that it is not more than 10% of the volume of the medium. The liquid medium is incubated at 37°C in 5–10% CO_2 and 100 µl of broth is subcultured on to agar at days 7, 14 and 21. The agar plates are incubated at 37°C in 5–10% CO_2 for no fewer than 14 days, except those corresponding to day 21 subculture, which are incubated for 7 days. An un-inoculated mycoplasma broth and agar plate are incubated as negative controls. For assessment of inhibitory substances, inoculate 1 ml of sample to be tested into 9 ml of the liquid medium and 100 µl on to solid medium and add 10–100 CFU of *Mmm* to each. Prepare positive control by inoculating 9 ml of mycoplasma broth and a mycoplasma agar plate with 10–100 CFU of *Mmm*. Incubate as for samples and negative controls.

During incubation time, visually compare the broth of the positive control with sample present with the positive control broth and, if there is no inhibition of the organism either the product possesses no antimicrobial activity under the conditions of the test, or such activity has been satisfactorily eliminated by dilution. If no growth or reduced growth of *Mmm* is seen in the liquid and solid medium with test sample when compared with the positive control, the product possesses antimicrobial activity, and the test is not satisfactory. Modifications of the conditions to eliminate the antimicrobial activity and repeat test are required.

If antimicrobial activity is present it is necessary to dilute the test product further. Repeat the test above using 1.0 ml of sample in 39 ml of mycoplasma broth and then inoculate with 10–100 CFU of *Mmm* and incubate as above. All broths and plates are examined for obvious evidence of growth. Evidence of growth can be determined by comparing the test culture with the negative control, the positive control, and the inhibition control.

If evidence of microbial growth is found in the test samples the contaminating bacterium will be identified and confirmed as *Mmm* by PCR assay.

4. Detection of rickettsia and protozoa

There are no general test procedures for exclusion of rickettsia or protozoa. Procedures to exclude specific agents of concern such as *Coxiella burnetti* (Q fever), *Ehrlichia canis, Trypanosoma evansi* and *Babesia caballi* can be found for example, in the Review of Published Tests to detect pathogens in veterinary vaccines Intended for Importation into Australia (Department of Agriculture [of Australia] [2013]). The review is based on the reading and interpretation of applicable published papers from reputable journals and are regarded as examples of sensitive methods for detection of specified agents.

4.1. An example of protocol based on published methods for exclusion of *Babesia caballi* and *Theileria equi*

Babesia caballi and *Theileria equi* can be cultured *in vitro* in 10% equine red blood cells (RBC) in supportive medium supplemented with 40% horse serum and in a micro-aerophilic environment. Culture isolation of *T. equi* is more sensitive than for *B. caballi*. Giemsa-stained blood smears are prepared from cultures daily for 7 days (Avarzed *et al*., 1997; Ikadai *et al*., 2001). *Babesia caballi* is characterised by paired merozoites connected at one end. *Theileria equi* is characterised by a tetrad formation of merozoites or 'Maltese cross'. Confirmation of the diagnosis is by PCR (see Chapter 3.6.8 *Equine piroplasmosis*).

Molecular diagnosis is recommended for the testing of biological products that do not contain whole blood or organs. Molecular diagnosis by PCR or loop-mediated isothermal amplification (LAMP) assay are the most sensitive and specific testing methods for detection of the pathogens of equine *piroplasmosis* (Alhassan *et al*., 2007).

5. Detection of viruses in biological materials

In brief, general testing usually includes the use of continuous and primary cell lines of the source species, e.g.; cells of known susceptibility to the likely viral contaminants, which are inoculated for a period of up to 4 weeks with weekly subcultures. Virus seeds also require testing on a primary cell line of the species in which the final product is intended. At Day 21 or 28, assessment of the monolayers is done using H&E staining to assess CPE, and haemadsorption with guinea-pig and chicken RBC to assess the presence of haemadsorbing agents. Note that general testing is useful as a screening tool though not sufficiently sensitive enough to detect all viruses of concern.

Specific testing requires test material to be inoculated on to sensitive, susceptible cells lines for the virus to be excluded; the amplification process in cell culture is usually up to 28 days but depending on the virus, may require longer culturing times. Detection of specific viral contaminants is by recognition of CPE in conjunction with more sensitive antigen detection or molecular tests such as FAT and PCR and ELISA after the amplification process in cell culture.

All testing using cell lines to amplify for target viruses is contingent on the sensitivity of the cells for the target agent and the ability to recognise the presence of the agent in the cells. The quality, characteristics, and virus permissibility profile of cell lines in use should be determined as fit for purpose and appropriately maintained. Positive and negative controls should be used at all passages of cell culture to determine sensitivity and specificity. Interference testing should be performed at first pass to ensure that the test sample does not inhibit the growth of the virus being excluded for.

5.1. An example of general testing for the exclusion of viruses from virus and cell seed stocks used in production of veterinary vaccines

If the test virus inoculum is cytopathogenic, the effect must be specifically neutralised without affecting the likelihood of isolation of the target agent. For affected cell type, 1 ml of the test master (or working) virus seed (MVS) is thawed or reconstituted and neutralised with the addition of 1 ml mono-specific antiserum. The serum must be shown to be free from antibodies against any agents for which the test is intended to detect. Antiserum must be tested for nonspecific inhibiting affects. Serum should be of sufficiently high titre to neutralise the seed virus effectively with the use of an approximately equal volume or less of serum. A microplate titration is used to determine the titre of the antiserum required to neutralise the MVS of concern. The antiserum is allowed to neutralise the MVS at 37°C for 1 hour. The MVS and antiserum mixture is then inoculated on to a 75 cm^2 flask with appropriate cells. If the MVS is known to be high-titred or difficult to neutralise, the blocking antiserum can be added to the growth medium at a final concentration of 1–2%.

Master cell stocks do not require a neutralisation process.

The cells should be passaged weekly up to a 28-day period. Certain relevant viruses may be selected as indicators for sensitivity and interference (positive controls) but these will not provide validation for the broader range of agents targeted in general testing. The final culture is examined for cytopathology and haemadsorption.

May–Grünwald–Giemsa or H&E staining procedures are used to assess for cytopathological changes associated with virus growth. Monolayers must have a surface area of at least 6 cm^2 and can be prepared on chambered tissue culture slides and incubated for 7 days. The plastic wells of the slides are removed leaving the rubber gasket attached to the slide. The slides are rinsed in Dulbecco's phosphate buffered saline (PBS), fixed in acetone, methanol or formalin depending on the stain used and placed on a staining rack. For May–Grünwald–Giemsa staining: the slides are stained for 15 minutes at room temperature with May–Grünwald stain diluted 1/5 with absolute methanol. The May–Grünwald stain is removed by inverting the slides. The slides are then stained for 20 minutes with Giemsa stain diluted 1/15 in deionised water. The Giemsa stain is removed by inverting the slides and rinsing them in deionised water for 10–20 seconds. The slides are air-dried and mounted with a coverslip using paraffin oil. The May–Grünwald–Giemsa stain differentially stains ribonucleoprotein (RNP); DNA RNP stains red-purple, while RNA RNP

stains blue. The monolayers are examined with a conventional microscope for the presence of inclusion bodies, an abnormal number of giant cells, or other cytopathology attributable to a viral contaminant of the test product. The inoculated monolayers are compared with suitable control non-inoculated monolayers. If specific cytopathology attributable to an extraneous virus is found, results are reported, and additional specific testing may be conducted.

Testing for haemadsorption uses 75 cm^2 area monolayers established in tissue culture flasks after the 28-day passage period described above. Guinea-pig, chicken, and any other blood for use in this assay is collected in an equal volume of Alsever's solution and may be stored at 4°C for up to 7 days. Immediately prior to use, the stored erythrocytes are again washed by adding 5 ml of blood in Alsever's solution to 45 ml of calcium and magnesium-free PBS (PBSA) and centrifuging in a 50 ml centrifuge tube at 500 ***g*** for 10 minutes. The supernatant is aspirated, and the erythrocytes are suspended in PBSA and re-centrifuged. This washing procedure is repeated at least twice until the supernatant is clear. Erythrocytes from each species are combined by adding 0.1 ml of each type of packed blood cells to 100 ml of PBSA. The erythrocytes from different species may be kept separate or combined, as desired. To each flask, add 5 ml of the erythrocyte suspension, and incubate the flasks at 4°C for 30 minutes. The monolayers are washed twice with PBSA and examined for haemadsorption. If no haemadsorption is apparent, 5 ml of the erythrocyte suspension is added to each flask; the flasks are incubated at 20–25°C (room temperature) for 30 minutes, rinsed as before, and examined for haemadsorption. Separate flasks may be used for each incubation temperature if desired. Monolayers are examined for the presence of haemadsorption using an illuminated light box and microscopically. Non-inoculated monolayers are used as negative controls. The PBSA and fresh erythrocytes should prevent most nonspecific haemadsorption from occurring. If specific haemadsorption attributable to an extraneous agent is found, results are reported, and additional specific testing may be conducted.

Specific testing requires specialised test procedures that are sensitive to amplifying a particular agent in culture and then detection of that agent by means of fluorescence, antigen-capture ELISA or PCR; whichever is more sensitive. Specific testing is usually required when general procedures are not adequate for effective exclusion of more fastidious, viruses Some examples are listed in Table 1.

5.2. An example of specific virus exclusion testing of biologicals used in the production of veterinary vaccines

5.2.1. Porcine epidemic diarrhoea virus (PEDV)

Trypsin is required at inoculation and in the culture medium for isolation of porcine epidemic diarrhoea virus (PEDV) in Vero cells (CCL81, ATCC). Just confluent monolayers (100%) are required; under confluent monolayers are more sensitive to the presence of trypsin and will be destroyed well before the 7 days required for each passage in culture. An over confluent or aging monolayer will not be sensitive for growth of PEDV. Maintenance media (MM) formulation consists of Earle's MEM (minimal essential medium) (with 5.6 M HEPES [N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid] and glutamine) + 0.3% Tryptose phosphate broth, 0.02% yeast extract and 4 μg/ml TPCK treated trypsin. The addition of the trypsin into the MM should occur on the day the media is to be used.

Prior to inoculation, confluent 75 cm^2 monolayers are washed twice with the MM (with trypsin added) to remove FCS. Virus or cell seed (1 ml) is added with 1 ml of MM to each monolayer; incubate at 37°C for 2 hours, then add 30 ml/flask of MM. Negative control monolayers of the same size are set up prior to inoculation of test material. Positive and interference controls are set up last in a separate laboratory area. Assessment for sensitivity and interfering substances requires assessment of PEDV of known titre. A control for interference using co-inoculation of test sample and PEDV needs only to be set up on the first pass. Positive controls must be set up at every pass to ensure each monolayer used gives expected sensitivity. PEDV virus is titrated in log dilutions starting at 10^{-1} to 10^{-6} in MM (depending on the endpoint titre of reference virus) in duplicate rows of 6 wells of a 24-well tissue culture plate. For the interference test, PEDV is titrated in the same dilution series but using MM spiked with a 10% volume of test material. Decant off the growth media and discard. Wash plates to ensure no FCS is present. Two washes using approximately 400 μl/well MM (with trypsin added) are sufficient.

Add 100 µl of diluted virus on to each of two duplicate wells. Rock inoculated plates to distribute the inoculum evenly over the surface of the monolayer. Incubate at 37°C with 5% CO_2 for 2 hours then add 1 ml volumes/well of MM.

After 7 days, 75 cm^2 monolayers have cells disrupted using two freeze–thaw cycles at –80°C. Positive control plates are read for end-point titres, and these are compared with virus in the presence of test material to ensure titres are comparable and interference has not occurred. Freeze–thaw lysates are clarified at 2000 ***g*** for 5 minutes and re-passed on to newly formed monolayers as for the first passage. Passages are repeated until a total of four passages are completed at which point cell lysates are assessed by PCR for detection of PEDV and day 7 monolayers in 24-well plates are fixed and stained by IFA. If seed virus is to be tested and requires neutralisation using antiserum, extra care in the isolation of PEDV needs to be considered. Trypsin is rendered inactive in the presence of serum proteins and without trypsin present, PEDV is unable to grow in cell culture. Washing off the inoculum with two MM washes is required after an extended adsorption time of up to 4 hours to ensure acceptable sensitivity.

J. INFORMATION TO BE SUBMITTED WHEN APPLYING FOR AN IMPORT LICENCE

When undertaking risk analysis for biologicals, Veterinary Authorities should follow the *Terrestrial Manual*. The manufacturer or the Veterinary Authority of the exporting country should make available detailed information, in confidence as necessary, on the source of the materials used in the manufacture of the product (e.g. substrates). They should make available details of the method of manufacture (and where appropriate inactivation) of the substrates and component materials, the quality assurance procedures for each step in the process, final product testing regimes, and the pharmacopoeia with which the product must conform in the country of origin. They should also make available challenge organisms, their biotypes and reference sera, and other means of appropriate product testing.

K. RISK ANALYSIS PROCESS

Risk analysis should be as objective and transparent as possible and should be performed in accordance with Section 2 of the *Terrestrial Code*, and certification in line with Section 5 of the *Terrestrial Code*. Of necessity, assessment of the country and commodity factors and risk reduction measures will be based largely on manufacturers' data. These data depend on quality assurance at all stages of manufacture, rather than on testing of the final product alone.

Domestic exposure may be influenced by the approved usage of the product. Veterinary Authorities may place limits on usage of some products (e.g. restricting usage to institutions of appropriate biosecurity).

L. BIOCONTAINMENT

Suitable biocontainment may be necessary for many forms of biologicals. In particular, the importation of exotic micro-organisms should be carried out in accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

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FURTHER READING

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NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 1.1.10.

VACCINE BANKS

SUMMARY

Vaccine banks provide antigen or vaccine reserves, either of ready-to-use vaccines or of antigenic components that can be quickly formulated into the final product for emergency use or other vaccination campaigns. They may be established for national or international use. For some international banks in particular, it is important to define the drawing rights of the members of the controlling consortium, and to establish clear governance mechanisms. For banks managed by intergovernmental organisations such as WOAH, appropriate financing should be in place, together with eligibility criteria for access to the bank. Vaccine banks may be deployed by Veterinary Authorities for different purposes ranging from systematic mass vaccinations, to emergency vaccinations, or to strategic interventions.

The major advantage of storing antigen in banks over storing formulated vaccines is the ability to store bulk vaccine antigen as concentrated stocks with prolonged retention of potency in low temperature storage. Appropriate serotypes and strains, alone or in combination, can also be selected from the bank according to the needs at the time of deployment. The disadvantage of antigen banks is the delay between the decision to deploy and the availability of the final formulated vaccine. This delay can, to some extent, be reduced by pre-testing trial batches of vaccine as part of acceptance into the bank, provided that agreement has been reached with the Competent Authority to provide early release certification based on reduced testing in the event of need.

Ready-to-use formulated vaccines can be deployed rapidly and are available for immediate use for the full duration of the shelf life of the vaccine. When not used, a disadvantage is the defined shelf life as this is usually much shorter than for banked antigens stored at low temperature. Also, for pathogenic agents that have several serotypes and/or show extensive strain variation in their antigenic characteristics, the fixed formulation may not sufficiently protect against infection with the strain involved in a given outbreak.

Whether storing antigen or ready-to-use formulated vaccines, plans should be in place to replenish stocks before the end of the shelf life or, where possible, to rotate and replace stock in a timely manner. Vaccines and antigens beyond their approved shelf life are not acceptable for use and must be discarded and destroyed.

Planning for the components of a vaccine bank, and for the quantity of material to be stored, should involve all relevant stakeholders including the Competent Authorities, vaccine bank administrators, vaccine manufacturers and reference laboratories. When combined with recommendations from reference laboratories on matching of field and vaccine strains, information technology (IT) tools involving modelling and epidemiological risk assessment can help in deciding the amounts and strains to include in a bank. Relevant information to inform the planning should include the epidemiology of the disease in question, its geographical occurrence, the nature of the pathogen, patterns of animal production, movement and trade, pre-existing vaccine coverage of the population and the logistics of deployment of the banked vaccine.

The regulatory principles of vaccine production apply equally to vaccine banks and vaccines produced should comply with the requirements of Chapter 1.1.8 Principles of veterinary vaccine production *of this* Terrestrial Manual *and the disease-specific WOAH standards in Part 3 of this* Terrestrial Manual.

A. DEFINITION OF A VACCINE BANK

Vaccine banks are defined as strategic antigen or vaccine reserves. Banks may consist of physical stocks of antigen or vaccine stored in a made-for-purpose facility or may be based on contractual arrangements with manufacturers to supply agreed amounts of one or more defined vaccines within a specific period of time. Hybrid approaches are also possible. Banks may hold the antigen component, or a ready-to-use formulated vaccine, or both. The vaccines may be deployed for different purposes ranging from systematic mass vaccination to emergency vaccinations, or to strategic interventions.

B. TYPES OF BANKS

Vaccine banks can be classified by their geographical coverage as well as by the nature of product stored.

A country may hold its own national bank, or it may be part of a larger group of countries that share a bank, which either have predefined drawing rights, or an *ad hoc* mechanism to determine these drawing rights. Such international consortia are based on shared risk and may share a common geographical region, or have similar disease status and approach to preventing and controlling a given disease. The bank may be held on the territory of one or several of the group members or be retained by the manufacturer(s). Intergovernmental organisations have also recognised the need to set up banks to support and prioritise immediate access to high quality vaccines, in particular by low- to middle-income countries, and thereby avoid the delay associated with conventional procurement procedures. An adequate system of governance should be established for all vaccine banks.

Certain vaccine antigens can be stored as concentrated stocks and retain potency for several years at temperatures below –70°C, depending on the stability of the antigen. Such antigens have to be formulated into vaccines before deployment. Using this approach, the vaccine composition, including choice of strains and potency, can be adjusted according to need, provided that such flexibility has been built into the approval process of the vaccine concerned (usually termed multi-strain vaccine approval). Formulation of antigens for use may be done either by the manufacturer of the antigen or in a dedicated facility maintained by the bank members. In the latter case, the facility needs an appropriate license to formulate the final product in compliance with the principles of Good Manufacturing Practice (GMP) or other manufacturing standards that apply in the country concerned. In these situations, the antigen bank itself assumes the responsibilities of the holder of the product licence for the vaccine released (also termed marketing authorisation or product registration). Vaccine manufacture and release need to be approved by the national regulatory authority where the vaccine is produced. If used in a different country, import and use of the vaccine need to be approved by the country in which it is deployed. Depending on the country concerned, licensing authorities may require that vaccines released from vaccine banks meet the same standards as the commercial vaccines used in food-producing animals or may apply less demanding standards, in particular where vaccinated animals are not intended to enter the food chain (i.e. vaccination and subsequent stamping out). Approval from the national Competent Authority will also cover aspects such as product labelling and literature, such as the product leaflet, in line with the approved conditions for use as detailed in a Summary of Product Characteristics (SPC), or equivalent documents.

In addition to ensuring appropriate approvals for manufacture, formulation and storage, the arrangements for vaccine banks need to clearly define all the essential requirements including: time between receiving an order and delivery, import permits, customs clearance, transportation, and maintenance of the cold chain. In the case of an antigen bank, specific arrangements are recommended to ensure that the contract for the supply of the antigen(s) includes a requirement for the associated supply of control samples (see Sections D. *Quantities of vaccine required in a bank* and F. *Acquisition of antigens or vaccines for a bank*). Contractual arrangements with manufacturers should include storage, formulation, availability of reagents and suitably sized vials, and the supply of formulated vaccines.

The main advantages of vaccine banks holding antigens are the speed that antigens can be turned into the final licensed vaccine when compared with vaccine production beginning from the working seed, the long shelf life of the antigen, and the option for a flexible combination of antigens to address different vaccination strategies. However, there is always a necessary time delay between giving the order for formulation and the availability of the ready-to-use formulated vaccine, which may not be suitable for rapid vaccination in emergency situations. Where appropriate approvals have been received from the Competent Authority responsible for batch release, frequently referred to as early release certification, this delay can be reduced. Early release is possible in an emergency situation (for example an outbreak of foot and mouth disease [FMD] in a country previously free of that disease as

foreseen in Chapter 3.1.8. *Foot and mouth disease* [*infection with foot and mouth disease virus*]), in a quality controlled manufacturing system in accordance with WOAH standards (WOAH *Terrestrial Manual* Chapter 1.1.8 *Principles of veterinary vaccine production*). Reduced testing might be acceptable at the time of release provided that full finished product testing has previously been performed on a batch of vaccine that is representative of the product to be released, particularly in terms of the method of production and the type and amount of antigen.

Ready-to-use formulated vaccines can be deployed rapidly and have a pre-determined formulation with a finite shelf life that is indicated in the product registration and has been validated by appropriate testing. The approved shelf life will depend on the characteristics of the vaccines when stored in appropriate temperature-controlled facilities as described in the product literature.

The main advantage of ready-to-use formulated vaccines is their immediate availability for use, though this should be balanced with what remains of the ready-to-use formulated vaccines' approved shelf-life when accessed, as it could lead to a very restrictive period of application compared with vaccines formulated on request from banked antigen stocks. Another inconvenience of ready-to-use formulated vaccines for pathogenic agents that have several serotypes and/or exhibit extensive strain variation in their antigenic characteristics is that the fixed formulation may not sufficiently protect against the strain involved in a given outbreak.

For all banks, whether storing antigen or ready-to-use formulated vaccines, there is the need to renew the stocks at the end of the approved shelf life of the antigen or formulated vaccine, respectively. Antigens or formulated vaccines that are beyond their approved shelf life should not be used and must be discarded and destroyed in appropriate specialised facilities (Lombard & Füssel, 2007) unless suitable studies have been carried out and regulatory approval obtained for extending the shelf life. Renewal orders and managing the period between the expiry date of the current stock and the arrival of new stock should be considered in a timely manner. Alternatively, stocks of antigen or ready-to-use formulated vaccines could be rotated and replenished to ensure there is a continuous supply of product within shelf life. Rotation of stock is relatively easily achieved by manufacturers where a market exists for the vaccine concerned. Rotation requires more forethought in the case of banks intended for emergency use, but some banks make antigen or vaccine available to non-member countries in cases of emergency or to low- to middle-income countries in a timely manner to assure its use before the scheduled expiry date.

Authorities responsible for disease control may set up contractual arrangements for long-term supply with one or more manufacturers rather than setting up physical vaccine banks. This approach avoids the substantial capital costs of setting up and maintaining the complex facilities that are needed for storage and production of vaccine. Furthermore, the responsibility for gaining and maintaining regulatory approvals rests with the manufacturer rather than the vaccine bank holder. The costs of maintaining facilities, and of carrying out the trials necessary to demonstrate quality, safety and efficacy in line with at least the minimum standards defined in the WOAH *Terrestrial Manual* have increased substantially as requirements have developed over the years. Commercial manufacturers have the necessary facilities and operate to these standards routinely whilst it can be challenging for non-commercial vaccine manufacturers to meet the required standards. This is particularly the case when countries require full regulatory approval before permitting use of the vaccine in animals of food-producing species, even under exceptional circumstances. In such long-term arrangements, a contract is concluded whereby manufacturers agree to supply defined amounts of one or more vaccines that meet agreed technical specifications within a specified time period of receiving an order. In this way the risks and benefits are shared between the contractor and the manufacturer. Contractors benefit by assuring the availability of an appropriate vaccine in the event of need and manufacturers benefit from a predictable source of funding to maintain their facilities, licenses and stocks even when vaccines are not used to control an outbreak.

C. SELECTION OF VACCINES FOR A BANK

Depending on the disease targeted and the likely contingency requirements, a range of vaccine strains may be required. Competent Authorities in consultation with the vaccine bank administrators and relevant reference laboratories must decide which vaccine strains should be held and whether they should be stored as a separate antigen component for subsequent formulation, or as a ready-to-use formulated vaccine. The value of any vaccine bank is dependent upon the appropriateness of what it holds for field application, particularly in respect of pathogenic agents that have several serotypes and/or exhibit extensive strain variation in their antigenic characteristics.

The potential for an outbreak not adequately covered by a banked vaccine must be alleviated by continual monitoring of the global disease situation, taking into account animal health information systems such as WOAH's WAHIS[1] and disease-specific updates in the periodic reports by international reference laboratory networks, and supported by laboratory genetic and antigenic characterisation facilitated by reference and other suitable laboratories. Additional vaccine strains may need to be included in the banks' portfolio or, where no suitable vaccine strain is available, developed as quickly as possible for subsequent inclusion in the bank. Close cooperation is therefore required between reference laboratories, manufacturers, international animal health organisations and bank managers to ensure that newly emerging strains are rapidly identified and made available to manufacturers for evaluation as potential new vaccine seed strains.

The world is an interdependent community that encompasses rapid and extensive movement of people, animals and animal products, and the increasing awareness of deliberate release of a pathogen through bioterrorism, heightens the risk of disease incursion and makes prediction of a specific threat difficult. Increased cooperation and collaboration between different international, regional and national reference laboratories, vaccine banks, and national, regional, international authorities or organisations should be encouraged as well as mechanisms for consultation with vaccine manufacturers. Structured risk assessment, preferably conducted at a country or regional level, should be used to determine the antigen or vaccine to be stored with a suitable priority level. In the case of FMD, a specific tool (Pragmatist) has been made available for this purpose. Effective risk management of FMD is only possible on the basis of knowledge of what strains are actively circulating and the extent of antigenic match between these strains and the virus antigens stored in the vaccine bank. This, in turn, relies on the continual submission of outbreak strains to reference laboratories for vaccine matching studies. Authorities are encouraged to share as much information as possible between themselves for the purpose of effective risk assessment and risk management whilst recognising that there may be a need to restrict the amount of information relating to the storage of antigens or vaccines that is made publicly available on the basis of both national security and, in some cases, commercial confidentiality.

D. QUANTITIES OF VACCINE REQUIRED IN A BANK

The decision as to how many doses of vaccine are required is complex, embracing questions of epidemiology, vaccinology, logistics and resources (human, technical and financial).

Factors bearing on the decision include:

i) the nature of the disease in question (serotypes, strains, pathogenesis, routes and rapidity of spread, presence and competence of vectors, etc.);

ii) the characteristics of the available vaccines (serotypes, strains, monovalent or polyvalent types of formulation), compatibility with DIVA strategies (DIVA: differentiation of infected from vaccinated animals), potency of the vaccines and the antigenic match between field and vaccine strains;

iii) the number, species, location and density of the animals to be protected;

iv) the types of emergency vaccination likely to be applied, and the number of doses in the vaccination regime;

v) logistical requirements (the availability of trained personnel, storage facilities, maintenance of the cold chain, transport, vaccination equipment, consumables, etc.);

vi) the current and predicted global, regional and national epidemiology of the disease;

vii) patterns of animal production, movement and trade;

viii) analysis of the risk of the introduction and spread of the disease in question (which may include epidemiological modelling);

ix) application of contingency planning (including risk–benefit and cost–benefit analysis and the construction of decision trees, awareness and acceptance by stakeholders).

Decisions on the quantity of the product inevitably involve a compromise between the potential economic impact of the disease, fixed cost of the maintenance of the vaccine bank, cost of purchase, storage and replacement, cold

1 https://wahis.woah.org/#/home

chain capacities of the beneficiaries and the likely number of doses required. The post-vaccination disease control and surveillance strategy further impacts the decision on the number of vaccine doses required.

The minimum vaccine requirement should be based on a country's planned vaccination strategy as detailed in its contingency plan. In the case of FMD, a modelling tool (VADEMOS) has been developed to estimate current and future vaccine dose demand at national, regional and global levels. The procurement of additional supplies of vaccine, either from other banks or from commercial sources, is likely to take considerable time. It would be beneficial in terms of cost, time and volume, for different national or regional vaccine banks to cooperate, or to consider setting up regional or international vaccine banks.

When relevant, vaccine banks may adopt a balanced approach combining a proportion of stored antigens (core strains and optional strains), with a proportion of ready-to-use formulated vaccines (for rapid deliveries).

E. REGULATORY CONSIDERATIONS

The manufacture of a vaccine, including vaccines intended to be exported for inclusion in a vaccine bank in a third country, requires approval by the national Competent Authority of the country in which the vaccine is produced, usually in the form of a manufacturing licence. Likewise, the deployment and use of a vaccine requires approval by the national Competent Authority of the country in which it is used. National Competent Authorities in WOAH Members should ensure that vaccines comply with at least the minimum standards defined in the relevant general chapters (e.g. chapter 1.1.8 and the relevant chapters in Section 2.3 *Veterinary vaccines*) and disease-specific chapters of this WOAH *Terrestrial Manual* (such as 3.1.8 *Foot and mouth disease).* National Competent Authorities may apply WOAH standards or may apply more stringent requirements as specified in national legislation. In addition to licensing products in compliance with full regulatory requirements in inter-epidemic periods, most countries also have systems in place to approve products more rapidly and with reduced data requirements in emergency situations or where there is no prospect of generating a full data package. In the event of disease emergencies, countries may also approve the use of unauthorised products or products authorised in another country, relying on existing data for the product concerned and placing specific controls on its import and use. In view of the different approaches adopted by different countries and for different diseases, those intending to use antigen and/or ready-to-use vaccines procured from a vaccine bank need to ensure that they are aware of the regulatory requirements for the production, regulatory approval, and use of vaccines produced from the bank and how these requirements will be met.

For vaccine banks the following additional considerations apply, with more detail developed throughout this chapter, as relevant:

i) storage conditions:
    a) facilities,
    b) containment of stored antigen or vaccine,
    c) labelling of stored antigen or vaccine;
ii) monitoring of stored concentrated antigen;
iii) transport to storage facilities;
iv) transport for deployment:
    a) transport of antigen for reformulation,
    b) transport of vaccines for use.

In setting up and gaining regulatory approval for manufacture, the vaccine bank will need to demonstrate that these aspects are conducted in line with the relevant chapter in the WOAH *Terrestrial Manual* and with national requirements.

Countries requesting vaccines from a vaccine bank need to ensure that their contingency plans include arrangements for import permits, customs clearance and maintenance of the cold chain as required.

F. ACQUISITION OF ANTIGENS OR VACCINES FOR A BANK

According to the type of bank and the disease concerned, the acquisition of the appropriate vaccine(s) or antigen(s) will depend on whether they are available from the commercial sector, government institutions or other production facilities. Irrespective of the source, antigens and vaccines need to be produced in facilities approved in line with regulatory requirements for manufacture and need to meet the quality requirement specified in the relevant regulatory approval.

Vaccine banks may use direct procurement or may use international calls for tender to acquire antigens or vaccines. They may wish to seek advice from appropriate official regulatory authorities to ensure that the standards specified in the technical specifications for the procurement are fully aligned with the terms of regulatory approval. Requests for tenders can then ensure not only a competitive price, but also a veterinary medicinal product manufactured to an acceptable level of quality, the standards being at least those set out in this WOAH *Terrestrial Manual*. For diseases where there is official WOAH recognition of that disease status, vaccines used in the Members concerned must comply with the standards in the WOAH *Terrestrial Manual*. It is recommended that the process of selecting suppliers should not focus solely on lowest cost but should also take into consideration technical and quality criteria as well as delivery capacities. This could be achieved by a stepwise process, first assessing the technical proposal responding to the technical specifications, followed by an evaluation of the commercial bid of the eligible candidate suppliers. The technical specification should establish that suppliers can produce the desired vaccines or antigens in the necessary amounts within a specified time period that includes the time required for mandatory tests of compliance such as sterility, safety, and potency.

G. STORAGE OF VACCINES OR ANTIGENS IN A BANK

It is important that the areas of storage for vaccine banks comply with recognised quality standards (e.g. chapter 1.1.8), which also address security of the premises (e.g. restricted access to premises, logbooks, continuity of access to electric power). Storage areas for vaccine banks should be regularly inspected by the Competent Authorities to ensure continual compliance. In the case of a vaccine that has regulatory approval, the storage conditions for both the antigen and for the formulated vaccine that is stored before release form part of the approval. Likewise, the location and operation of storage areas fall within the scope of the certificate of GMP (or other manufacturing standard) approved by the national Competent Authority. Any deviation from the approved conditions or areas for storage therefore requires approval from the relevant regulatory authority. If antigens are stored in one location but formulated and finished in another, both areas need to be covered by the 'GMP envelope' and agreed arrangements must be in place for transfer of material between the two.

If the vaccine bank is co-located with a laboratory or other facility where pathogens are handled, the bank storage facilities should be completely independent and be protected by positive air pressure with high efficiency particulate air (HEPA) filtration at the air inlets. Maintenance and monitoring personnel should, where they have had a possible exposure to relevant infection, obey a quarantine procedure before entering the bank.

Storage of antigens or vaccines in a bank should be appropriate to the product. The antigen may be a chemically inactivated or killed organism, a subunit derived from recombinant technology, or it may be a live vaccine based on an attenuated or recombinant strain. Antigens may be concentrated in frozen liquid form held at temperatures below –70°C in suitable containers that are appropriately labelled (see e.g. Chapter 3.1.8, Section C.6 *Storage and monitoring of concentrated antigens*). Freeze-dried vaccines and their diluents should be stored in accordance with the manufacturer's specification, typically at +4°C or –20°C, or as appropriate. Backup storage, equipment and contingency power supplies should be in place. For all methods of storage it is vitally important that vaccines or antigens are optimally maintained and routinely monitored, and that their storage is properly documented, in order to have assurance that they will be fit for use when needed. Managers of vaccine banks should therefore ensure that the necessary arrangements are in place to monitor their reserves on a routine basis as specified in the product licence and to include, where necessary and at appropriate time intervals, a testing regime to ensure integrity of the antigen component or acceptable potency of the final product. For example, storage facilities should be equipped with continuous temperature recording and alarm systems to detect divergence outside the required range; periodic inspection should also be carried out of the antigen containers for cracks or leakage. In this context, managers may wish to also consider the possibility of independent testing, or of greater reliance on overseeing or auditing of the manufacturer's test procedures.

In the case of antigen-containing banks, there is a need for routine stability testing of stocks. Sufficient smaller volume samples that are representative of the bulk antigen stock are necessary for such purposes and both bulk and samples should be stored side by side.

Where the requirement is to hold antigens or vaccines at a site other than at the principal site of manufacture, Competent Authorities should ensure that appropriate testing is carried out before the antigens are formulated into finished vaccine. Any new antigens should be held in a defined quarantine area until the necessary tests have been performed to formally accept them into the bank.

H. DEPLOYMENT PLANNING

Deployment planning addresses all aspects of the release of antigen for formulation into the final product, and the release and delivery of the ready-to-use formulated vaccine to the destination.

The request to deploy should be made by the Competent Authority(ies) of the requesting country or region, the decision for deployment being taken in agreement with the governance and management of the vaccine bank. The approval issued by the Competent Authority will include the conditions that must be met for release of the vaccine from the control of the manufacturer into the distribution chain. This can take a number of forms, from a simple import license for specific batches of vaccine through to official control authority batch release, depending on the terms of the license and the countries in which the vaccine is to be released and used. Early release may be possible if approved for emergency situations or release may only be possible once all tests are completed and verified. Members of vaccine banks need to ensure that the arrangements for batch release are fully understood and agreed with their respective Competent Authority to avoid delay in the event of need.

For the optimal use of a vaccine bank, the order for deployment should be informed, where appropriate, by results from a diagnostic laboratory (preferably a reference laboratory) with the ability to characterise the agent causing the disease and match the field strain with the available stored antigens or the ready-to-use formulated vaccines. In recent years a greater focus has been placed on the fate of vaccinated animals and the implications of vaccination as set out in the *Terrestrial Code* Chapter 4.18 *Vaccination*. For many diseases, DIVA vaccines have been developed together with companion diagnostic tests to substantiate that vaccinated animals and their products are free from infection. Successful deployment of DIVA vaccines requires the use of an accompanying diagnostic test. An important aspect of licensing vaccines with DIVA claims is to verify that the claims made for the combination of the vaccine and the accompanying test have been verified by the manufacturer to an acceptable standard and in line with general and specific guidance in the WOAH *Terrestrial Manual* (e.g. Chapter 3.1.8. Section C.5.4 *Purity: testing for antibody against NSP* for FMD vaccines). Using vaccines and accompanying tests that have been independently verified as complying with WOAH standards is very helpful to countries.

Competent Authorities should have emergency plans to ensure that the stored vaccine is distributed and administered to meet disease control goals. In an outbreak situation, the speed of the implementation of the vaccination programme is critical in reducing the number of infected premises, the duration of the epidemic and, for certain diseases, the number of animals that need to be culled. The Competent Authorities should ensure that the necessary cold-chain facilities for vaccines and diluents, if appropriate, are available, that vaccination protocols are defined in advance, that vaccination teams are established and trained appropriately, that all the other necessary documentation, equipment, reagents and clothing are stored at sufficient levels to support any potential vaccination campaign, and that stakeholders are aware of the need for such a campaign (see WOAH *Terrestrial Code* Chapter 3.5. *Communication*). Performing periodic exercises and simulations should be considered.

I. CONSIDERATIONS FOR VACCINE BANKS MANAGED BY INTERGOVERNMENTAL ORGANISATIONS

Vaccine banks managed by intergovernmental organisations, such as WOAH, may rely on specific funds for financing, to achieve prevention and control of specific diseases. Such mechanisms have been used to establish vaccine banks for FMD, peste des petits ruminants, avian influenza, and rabies for dog vaccination, and may be considered for other animal diseases in the future.

With the financial support of donors, in the context of international aid or with the use of other financial mechanisms such as trust funds or complementary funding, an intergovernmental organisation (regional or global) may manage

regional or global vaccine banks that are licensed and retained by the manufacturers selected through specific international calls for tender. Multiple donor funding mechanisms allow for cost sharing (establishment or replenishment), and for the management of donor-specific requirements.

Eligibility criteria are defined for countries that have access to such vaccine banks as well as guidelines on the use of regional and global vaccine banks. Depending on the disease, these banks may include vaccines produced and delivered on demand (planned deliveries), or specific replenishment mechanisms for rolling stocks.

The benefits of regional (or global) vaccine banks are numerous and seek to:

i) save costs (economies of scale);

ii) facilitate the delivery of determined quantities of high quality vaccines complying with WOAH standards;

iii) deliver more doses at a lower cost with access to more vaccine strains;

iv) reduce the risks associated with the storage of vaccines;

v) facilitate the harmonised implementation of regional or global disease control strategies;

vi) create incentives for the implementation of disease control programmes;

vii) reduce the number of procurement procedures;

viii) promote the use of vaccines that comply with WOAH quality standards for national and international disease control programmes.

Specific financial mechanisms can also allow countries or intergovernmental organisations to purchase directly from such banks.

Collaboration between vaccine banks (including those managed by intergovernmental organisations) and regional organisations is an economical way of increasing the amount of emergency vaccines available. Care is required to ensure that collaborating vaccine banks and regional organisations operate to the same or equivalent standards. Drawing rights should be clearly defined, and regular contact should be maintained between vaccine banks and regional organisations to confirm the quality of the vaccines. In the case of shared banks, regulatory compliance needs to be addressed at an early stage to ensure that vaccine produced from the bank is manufactured to WOAH quality standards and will therefore be readily accepted for use in recipient countries.

Some vaccine banks also rely on long-term supply arrangements with selected providers that may include replenishment mechanisms, production on demand for non-urgent or planned deliveries, and buy-back schemes. While vaccine banks often hold physical stocks of antigens or vaccines, it is also possible to establish virtual vaccine banks with reduced physical stock. Specific service contracts between the bank holder and the manufacturers operating any of the above solutions should set out clear obligations, pricing specifications, maximum delays for delivery and contractual penalties in case of failure to meet the conditions of the service contract.

FURTHER READING

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NB: FIRST ADOPTED IN 2008 AS *GUIDELINES FOR INTERNATIONAL STANDARDS FOR VACCINE BANKS.*
MOST RECENT UPDATES ADOPTED IN 2023.

PART 2

SPECIFIC RECOMMENDATIONS
DISEASES

SECTION 2.1.

LABORATORY DIAGNOSTICS

CHAPTER 2.1.1.

LABORATORY METHODOLOGIES FOR BACTERIAL ANTIMICROBIAL SUSCEPTIBILITY TESTING

SUMMARY

With the increase in bacterial resistance to traditionally used antimicrobials, it has become more difficult for clinicians to empirically select an appropriate antimicrobial agent. As a result, in-vitro *antimicrobial susceptibility testing* (*AST*) *of the relevant bacterial pathogens, from properly collected specimens, should be performed using validated methods. Thus, AST is an important component of prudent antimicrobial use guidelines in animal husbandry worldwide and veterinarians in all countries should have these data available for informed decision-making.*

Although a variety of methods exist, the goals of in-vitro *antimicrobial susceptibility testing are either to provide a reliable predictor of how an organism is likely to respond to antimicrobial therapy in the infected host or to assess for surveillance purposes whether there has been development of resistance. This type of information aids the clinician in selecting the appropriate antimicrobial agent, aids in developing antimicrobial use policy, and provides data for epidemiological surveillance. Such epidemiological surveillance data provide a base to choose the appropriate empirical treatment* (*first-line therapy*) *and to detect the emergence and/or the dissemination of resistant bacterial strains or resistance determinants in different bacterial species. The selection of a particular AST method is based on many factors such as validation data, practicality, flexibility, automation, cost, reproducibility, accuracy, standardisation and harmonisation.*

The use of genotypic approaches for detection of antimicrobial resistance genes has also been promoted as a way to increase the speed and accuracy of susceptibility testing. Numerous DNA-based assays are being developed to detect bacterial antimicrobial resistance at the genetic level. These methods, when used in conjunction with phenotypic analysis, offer the promise of increased sensitivity, specificity, and speed in the detection of specific known resistance genes and can be used in tandem with traditional laboratory AST methods.

INTRODUCTION

The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognised by the World Organisation for Animal Health (WOAH, founded as OIE), the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as a serious global human and animal health problem. The development of bacterial antimicrobial resistance is neither an unexpected nor a new phenomenon. It is, however, of increasing concern due to the frequency with which new emerging resistance phenotypes are occurring among many bacterial pathogens and commensal organisms, such as resistance to carbapenems, colistin, linezolid, macrolids, etc.

Historically, many infections could be treated successfully according to the clinician's past clinical experience or because susceptibility could be reliably predicted (i.e. empirical therapy); however, this is becoming more the exception than the rule (Walker, 2007). Resistance has been observed to essentially all of the antimicrobial agents currently approved for use in human and veterinary clinical medicine. This, combined with the variety of

antimicrobial agents currently available, makes the selection of an appropriate agent an increasingly challenging task. This situation has made clinicians more dependent on data from *in-vitro* antimicrobial susceptibility testing, and highlights the importance of the diagnostic laboratory in clinical practice.

A number of antimicrobial susceptibility testing (AST) methods are available to determine bacterial susceptibility to antimicrobials. The selection of a method is based on many factors such as practicality, flexibility, automation, cost, reproducibility, accuracy, accessibility and individual preference. Standardisation and harmonisation of AST methodologies, used in epidemiological surveillance of antimicrobial drug resistance, are critical if data are to be compared among national or international surveillance/monitoring programmes of WOAH Members. It is essential that AST methods provide reproducible results in day-to-day routine laboratory use and that the data be comparable with those results obtained by an acknowledged 'gold standard' reference method. Currently the reference AST method is the broth micro-dilution method that determines minimum inhibitory concentration (MIC) as described by the ISO (International Organization for Standardization, 2006). In the absence of standardised methods or reference procedures, susceptibility results from different laboratories cannot be reliably compared. The method used to select samples for inclusion in antimicrobial resistance surveillance programmes, as well as the methods used for primary bacterial isolation, are also important factors that should be standardised or harmonised to allow direct comparison of data between different regions; consideration of these issues is addressed in a WOAH document (Dehaumont, 2004).

As the science of AST has progressed, a greater understanding of the multiple factors that could affect the overall outcome of susceptibility testing has become clearer (WHO, 2017). This document provides guidelines and standardisation for AST methodologies, and interpretation of antimicrobial susceptibility test results.

1. Test requirements

The following requirements should be applied to achieve standardisation of AST methods and comparability of AST results:

i) the use of standardised AST methods is essential, including the harmonisation of AST test parameters such as media, inoculum, incubation time, quality controls, choice of antimicrobial agents and subsequent interpretive criteria,

ii) standardised AST methods, including all critical specifications and interpretive criteria, should be clearly defined, documented in detail and used by all participating laboratories,

iii) all AST methods should generate accurate and reproducible data,

iv) quantitative susceptibility data (MIC) should be reported,

v) establishment of national or regional reference laboratories is essential for the coordination of AST methodologies, interpretations and appropriate operational techniques used to ensure accuracy and reproducibility (e.g. quality controls),

vi) microbiological laboratories should implement and maintain a formal quality management programme (see Chapter 1.1.5 *Quality management in veterinary testing laboratories*),

vii) laboratories should have acquired a third party accreditation that includes the AST methodologies to be used within the scope of that accreditation. The accreditation body should meet accepted international Laboratory Accreditation Cooperation [ILAC]) standards and guidelines regarding the standards used for the accreditation process. The accreditation standards used should include the requirement for participation in proficiency testing programmes,

viii) specific bacterial reference/quality control strains are essential for determining intra- and inter-laboratory quality control, quality assurance and proficiency testing.

2. Selection of antimicrobials for testing and reporting

Selecting the appropriate antimicrobials for susceptibility testing can be difficult given the vast numbers of agents available. The following guidelines should be noted:

i) the FAO/WOAH/WHO expert workshop on non-human antimicrobial usage and antimicrobial resistance recommends creating a list of veterinary and human critically important antimicrobials for susceptibility testing and reporting,

ii) selection of the most appropriate antimicrobials is a decision best made by each WOAH Member in consultation with the appropriate bodies and organisations,

iii) antimicrobials in the same class may have similar *in-vitro* activities against select bacterial pathogens. In these cases, a representative antimicrobial should be selected that predicts susceptibility to other members of the same class,

iv) certain microorganisms can be intrinsically resistant to particular antimicrobial classes; therefore it is unnecessary and misleading to test certain agents for activity *in vitro.* The type of intrinsic resistance has to be determined for these organisms from either the scientific literature or through testing,

v) the number of antimicrobials to be tested should comply with the guideline used (CLSI/EUCAST/ISO) and at least contain class representatives to ensure the relevance and practicality of AST (see also WHO, 2017).

Periodic review of microorganisms that are currently predictably susceptible to certain antimicrobial agents is recommended to ensure that emergent, unexpected resistance is detected. Emerging resistance may also be suspected following poor response or treatment failure to a standard antimicrobial treatment regime.

3. Antimicrobial susceptibility testing methodologies

The following requirements should be respected:

i) bacteria subjected to AST must be isolated in pure culture from the submitted sample,

ii) standard reference methods should be used for identification so that the subject bacteria are consistently and correctly identified to the genus and/or species level,

iii) bacterial isolates considered to be the most important and other selected isolates, should be stored for future analysis (either lyophilisation or cryogenic preservation at –70°C to –80°C).

The following factors influencing AST methods should be determined, optimised, and documented in a detailed standard operating procedure:

i) once the bacterium has been isolated in pure culture, a standardised concentration of the inoculum must be prepared using a nephelometer or spectrophotometer to ensure a defined number of colony forming units to obtain accurate and repeatable susceptibility results. Bacteria or other organisms used in AST testing should be from a fresh 24-hour culture,

ii) the composition and preparation of the agar and broth media used (e.g. pH, cations, thymidine or thymine, use of supplemented media) should comply with guidelines (CLSI/EUCAST/ISO). Performance and sterility testing of media lots should also be determined and documented as well as the procedures used,

iii) the content, range/interval and concentration of the antimicrobials used (microtitre plates, disk, strip, tablet) should follow guidelines (CLSI/EUCAST/ISO) and be relevant to the species tested,

iv) composition of solvents and diluents for preparation of antimicrobial stock solutions,

v) growth and incubation conditions (time, temperature, atmosphere e.g. CO_2),

vi) agar depth,

vii) the test controls to be used, including the reference organisms used,

viii) the subsequent interpretive criteria (clinical breakpoints, epidemiological cut-off values – ECOFFs).

For these reasons, special emphasis has to be placed on the use of documented procedures and validated, well documented methods, as sufficient reproducibility can be attained only through the use of such methodology.

4. Selection of antimicrobial susceptibility testing methodology

The selection of an AST methodology may be influenced by the following factors:

i) ease of performance,

ii) flexibility,

iii) adaptability to automated or semi-automated systems,

iv) cost,

v) reproducibility,

vi) reliability,

vii) accuracy,

viii) the organisms and the antimicrobials of interest in that particular WOAH Member,

ix) availability of suitable validation data for the range of organisms to be susceptibility tested.

5. Antimicrobial susceptibility testing methods

The following three methods have been shown to consistently provide reproducible and repeatable results when followed correctly (Clinical and Laboratory Standards Institute [CLSI], 2008; Walker, 2007):

i) disk diffusion,

ii) broth dilution,

iii) agar dilution,

5.1. Disk diffusion method

Disk diffusion refers to the diffusion of an antimicrobial agent from a disk or tablet containing a specified concentration of the agent tablets into a solid culture medium (normally Müller–Hinton agar) that has been inoculated with a pure culture (see Section 3). The disk diffusion result is determined by measurement of the diameter of the inhibition zone around the disk, the diameter being proportional to the bacterial susceptibility to the antimicrobial present in the disk.

The diffusion of the antimicrobial agent into the culture media results in a gradient of the antimicrobial. When the concentration of the antimicrobial becomes so diluted that it can no longer inhibit the growth of the test bacterium, the zone of inhibition is demarcated. The diameter of this zone of inhibition around the antimicrobial disk is related to MIC for that particular bacterium/antimicrobial combination; the zone of inhibition correlates inversely with the MIC of the test bacterium. Generally, the larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms. However, this depends on the concentration of antimicrobial agent in the disk and its diffusibility. Antimicrobial agents that are very large molecules diffuse poorly in agar making disk diffusion methods unreliable for these compounds. For this reason disk diffusion methods are not recommended for example for the susceptibility testing of colistin/polymyxin (Matuschek *et al*., 2018).

Note: Disk diffusion tests based solely on the presence or absence of a zone of inhibition without regard to the size of the zone of inhibition are not acceptable AST methodology.

5.1.1. Considerations for the use of the disk diffusion methodology

Disk diffusion is easy to perform, reproducible if standardised, and does not require expensive equipment. Its main advantages are:

i) low cost,

ii) ease in modifying test by changing antimicrobial disks when required,

iii) can be used as a screening test against large numbers of isolates,

iv) can identify a subset of isolates for further testing by other methods, such as determination of MICs.

v) the procedure is controlled by inclusion of appropriate control organisms for which a target zone size range is available (or has been derived) for each of the relevant antimicrobial agents being tested in the disk diffusion test procedure.

Manual measurement of zones of inhibition may be time-consuming. Automated zone-reading devices are available that can be integrated with laboratory reporting and data-handling systems. The disks should be distributed evenly on the agar surface so that the zones of inhibition around

antimicrobial discs in the disc diffusion test do not overlap to such a degree that the zone of inhibition cannot be determined. Generally, this can be accomplished if the discs are no closer than 24 mm from centre to centre, though this is dependent on disk concentration and the ability of the antimicrobial to diffuse in agar. Contamination of culture plates may be harder to detect using automated readers.

The diameter of the zone of inhibition obtained in disk diffusion tests is strongly influenced by the density of the bacterial inoculum applied, underlining the requirement to standardise the inoculum in accordance with guidelines (CLSI, EUCAST, ISO). A denser inoculum than intended will result in reduced zones of inhibition and a sparse inoculum will result in increased zones of inhibition (BSAC [British Society for Antimicrobial Chemotherapy], 2015).

5.2. Broth and agar dilution methods

The aim of the broth and agar dilution methods is to determine the lowest concentration of the antimicrobial that inhibits the visible growth of the bacterium being tested in either broth or on agar (MIC, usually expressed in μg/ml or mg/litre). The range of concentrations tested in broth and agar dilution methods generally includes the breakpoint (clinical or microbiological) with doubling dilutions either side of that value as considered appropriate. However, the MIC does not always represent exactly the concentration which was tested. The 'true' MIC is a point between the lowest test concentration that inhibits the growth of the bacterium and the next lower test concentration. Therefore, MIC determinations performed using a dilution series may be considered to have an inherent variation of ±1 dilution.

Antimicrobial ranges should encompass both the interpretive criteria (susceptible, intermediate and resistant) for a specific bacterium/antibiotic combination and appropriate quality control reference organisms. Target MIC ranges should be available for each antimicrobial agent being tested.

Antimicrobial susceptibility dilution methods are more reproducible than agar disk diffusion which is why broth microdilution is the current reference test method. However, antibiotics are usually tested in doubling dilutions, which can produce inexact MIC data. The continuous range of zone diameter values obtained with disk diffusion can therefore be advantageous in certain circumstances, such as screening large numbers of susceptible isolates.

Any laboratory that intends to use a dilution method and set up its own reagents and antibiotic dilutions should have the ability to obtain, prepare and appropriately maintain stock solutions of reagent-grade antimicrobials, to account for the potency of the antimicrobial (supplied by the manufacturer) and to generate complex working dilutions on a regular basis. Published methods should be consulted. It is then essential that such laboratories use quality control organisms (see below) to assure accuracy and standardisation of their procedures.

5.2.1. Broth dilution

Broth dilution is a technique in which a suspension of a bacterium of a predetermined optimal concentration is tested against varying concentrations of an antimicrobial agent (usually serial twofold dilutions) in a liquid medium of predetermined, documented formulation. The broth dilution method can be performed either in tubes containing a minimum volume of 2 ml (macrodilution) or in smaller volumes using microtitration plates (microdilution). Numerous microtitre plates containing lyophilised or dried prediluted antibiotics within the wells are commercially available. The use of the same batches of microdilution plates may assist in the minimisation of variation that may arise due to the preparation and dilution of the antimicrobials at different laboratories. The use of these plates, with a documented test protocol, including specification of appropriate reference organisms, will facilitate the comparability of results among laboratories.

Due to the fact that most broth microdilution antimicrobial test panels are prepared commercially, this method is less flexible than agar dilution or disk diffusion in adjusting to the changing needs of the surveillance/monitoring programme.

Because the purchase of antimicrobial plates and associated equipment may be costly, this methodology may not be feasible for some laboratories.

5.2.2. Agar dilution

Agar dilution involves the incorporation of varying concentrations of antimicrobial agent into an agar medium, usually using serial twofold dilutions, followed by the application of a defined bacterial inoculum to the agar surface of the plate. This method may be considered the most reliable for MIC determination for some antimicrobials (fosfomycin, mecillinam) and for certain bacteria where broth dilution methods are not well established.

The advantages of agar dilution methods include:

i) the ability to test multiple bacteria, except bacteria that swarm, on the same set of agar plates at the same time,

ii) the potential to improve the identification of MIC endpoints and extend the antibiotic concentration range,

iii) the possibility to semi-automate the method using an inoculum-replicating apparatus. Commercially produced inoculum replicators are available and these can transfer between 32 and 60 different bacterial inocula to each agar plate.

Agar dilution methods also have certain disadvantages, for example:

i) if not automated, they are very laborious and require substantial economic and technical resources,

ii) once the plates have been prepared, they normally should be used within 1–3 weeks depending in quality control (or less, depending on the stability of the antimicrobials tested),

iii) the endpoints are not always easy to read.

Agar dilution is often recommended as a standardised AST method for fastidious organisms (CLSI, 2015), such as anaerobes and *Helicobacter* species.

5.3. Other bacterial AST and specific antimicrobial resistance tests

Bacterial antimicrobial MICs can also be obtained using commercially available gradient strips that diffuse a predetermined antibiotic concentration. However, the use of gradient strips can be expensive and MIC discrepancies can be found when testing certain bacteria/antimicrobial combinations compared with results of other methods (Ge *et al.*, 2002; Rathe *et al.*, 2009). Gradient strip methods are not recommended for testing the susceptibility of the antimicrobial agent colistin because of the large size of this molecule and its poor diffusion in agar (Matuschek *et al.,* 2018).

Regardless of the AST method used, the procedures should be documented in detail to ensure accurate and reproducible results, and appropriate reference and control organisms should always be tested every time AST is performed in order to ensure accuracy and validity of the data.

The appropriate AST choice can be dependent on the growth characteristics of the bacterium in question, as well as the objective of testing. In special circumstances, novel test methods and assays may be more appropriate for detection of particular resistance phenotypes. For example, chromogenic cephalosporin-based tests (CLSI, 2018) (e.g. nitrocefin) may provide more reliable and rapid results for beta-lactamase determination in certain bacteria, whereas inducible clindamycin resistance in *Staphylococcus* spp. may be detected using a disk diffusion method employing standard erythromycin and clindamycin disks in adjacent positions and measuring the resultant zones of inhibition (e.g. D-zone or D-test) (Zelazny *et al.*, 2005).

Similarly, extended-spectrum beta-lactamase (ESBL) (CLSI, 2018) activity in certain bacteria can also be detected by using standard disk diffusion susceptibility test methods incorporating specific cephalosporins (cefotaxime and ceftazidime) separately and in combination with a beta-lactamase inhibitor (clavulanic acid) and measuring the resulting zones of inhibition. Penicillin-binding protein 2a (PBP 2a) can also be detected in methicillin resistant staphylococci with a latex agglutination test (Stepanovic *et al.*, 2006). It is essential that testing of known positive and negative control strains occurs alongside clinical isolates to ensure accurate results.

Susceptibility testing may also be performed using breakpoint values specifically intended to detect particular mechanisms of bacterial resistance of clinical or public health importance, for example resistance to the carbapenems, which are used prudently to treat highly-resistant bacterial in humans (EUCAST [European Committee on Antimicrobial Susceptibility Testing], 2017).

5.4. Future directions in antimicrobial susceptibility/resistance detection

The use of genotypic approaches for detection of antimicrobial resistance genes has been promoted as a way to increase the rapidity and accuracy of susceptibility testing (Cai *et al.*, 2003; Chen *et al.*, 2005). Numerous DNA-based assays are being developed to detect bacterial antimicrobian resistance at the genetic level. The newest and perhaps most state-of-the-art approach is to use genome sequencing to predict antimicrobial resistance phenotypes via identification and characterisation of the known genes that encode specific resistance mechanisms.

Methods that employ the use of comparative genomics, genetic probes, microarrays, nucleic acid amplification techniques (e.g. polymerase chain reaction [PCR]), and DNA sequencing offer the promise of increased sensitivity, specificity, and speed in the detection of specific known resistance genes (Cai *et al.*, 2003; Chen *et al.*, 2005; Perreten *et al.*, 2005). Genotypic methods have been successfully applied to supplement traditional AST phenotypic methods for other organisms including methicillin-resistant staphylococci, vancomycin-resistant enterococci, and detection of fluoroquinolone resistance mutations (Cai *et al.*, 2003; Chen *et al.*, 2005; Perreten *et al.*, 2005). PCR methods have also been described for beta-lactamases, aminoglycoside inactivating enzymes, and tetracycline efflux genes (Cai *et al.*, 2003; Chen *et al.*, 2005; Frye *et al.*, 2010; Perreten *et al.*, 2005).

Technological innovations in DNA-based diagnostics should allow for the detection of multiple resistance genes and/or variants during the same test. The development of rapid diagnostic identification methods and genotypic resistance testing should help reduce the emergence of antimicrobial resistance, by enabling the use of the most appropriate antimicrobial when therapy is initiated. However, DNA techniques have to be demonstrated to be complementary to AST methods and results.

Additionally, new technological advances may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data for surveillance and monitoring programmes (Frye *et al.*, 2010). However, despite the new influx of genotypic tests, documented and agreed upon phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens and to detect and characterise newly discovered mechanisms of resistance for the development and validation of genetic testing. A literature review (Ellington *et al.,* 2017) considered the role of whole genome sequencing (WGS) in antimicrobial susceptibility testing of bacteria and concluded there was insufficient published evidence to support the use of AST via WGS to replace phenotypic AST in clinical settings for all bacterial species, although certain bacteria (e.g. *Salmonella, Staphylococcus aureus*) had been well characterised for that purpose. Subsequently several publications have added support to the use of genetic AST (e.g. McDermott *et al.,* 2016, Zhao *et al.,* 2016). The future of genetic testing in the detection of antimicrobial resistance is promising, but phenotypic testing will remain an important mainstay.

6. Antimicrobial susceptibility breakpoints and zone of inhibition criteria

The primary objective of *in-vitro* AST is to predict how a bacterial pathogen may respond to an antimicrobial agent *in vivo.* The results generated by bacterial *in-vitro* antimicrobial susceptibility tests, regardless of whether disk diffusion or dilution methods are used, are generally interpreted and reported as resistant, susceptible or intermediate to the action of a particular antimicrobial by applying clinical breakpoints. No single formula for selection of optimal breakpoints has been established. The process involves a review of existing data and is influenced by the methods used to select appropriate breakpoints.

Generally, antimicrobial susceptibility breakpoints are established by national standards organisations, professional societies or regulatory agencies. The relevant documents should be consulted. However, there can be notable differences in breakpoints for the same antimicrobial agent within and among countries due to differences between standards setting organisations and regulatory agencies and because of regional or national differences in dosing regimens (Brown & MacGowan, 2010; de Jong *et al.*, 2009; Kahlmeter *et al.*, 2006).

As mentioned previously, antimicrobial susceptibility testing results should be recorded quantitatively:

i) as distribution of MICs in mg/litre or μg/ml,

ii) or as inhibition zone diameters in millimetres.

The following two primary factors enable a bacterial isolate to be interpreted as susceptible or resistant to an antimicrobial agent:

i) The development and establishment of quality control ranges (CLSI, 2015), for disk diffusion or dilution testing, for quality control reference microorganisms.

   Establishment of quality control ranges for control organisms is essential for validating test results obtained using a specific AST method. The allowable interpretive category ranges for reference control organisms should be established in addition to determining breakpoints for susceptibility or resistance. The use of reference organisms is a quality control and quality assurance activity.

ii) The determination of the appropriate interpretive criteria regarding establishment of breakpoints (CLSI, 2015).

   This involves the generation of three distinct types of data:

   a) MIC population distributions of the relevant microorganisms,

   b) pharmacokinetic parameters and pharmacodynamic indices of the antimicrobial agent,

   c) results of clinical trials and the outcome of treatment of clinical cases of disease.

The development of a concept known as 'microbiological breakpoints', or 'epidemiological cut-off values' (the highest MIC value for the bacterium and antimicrobial agent under consideration, where the bacterium is devoid of any phenotypically expressed resistance to that antimicrobial agent), may be more appropriate for some antimicrobial surveillance programmes. Epidemiological cut-off values are derived by examining MIC population distributions for specific bacterial species and antimicrobials performed at several laboratories according to a standardised broth microdilution method. Bacterial isolates that possess any acquired phenotypic resistance (that is, have an MIC above the epidemiological cut-off value) and therefore deviate from the normal wild-type fully-susceptible population are designated as non-wild type (also termed microbiologically resistant) and shifts in susceptibility to the specific antimicrobial/bacterium combination can thus be monitored (Kahlmeter, 2015; Kahlmeter *et al.*, 2006; Turnidge *et al.*, 2006). There is a great advantage in the recording of quantitative susceptibility data in that data may be analysed according to clinical breakpoints as well as by using epidemiological cut-off values.

The development of breakpoint criteria for disk diffusion tests usually involves comparing disk diffusion data against dilution data by creating a scattergram of the bacterial population distribution (representative bacterial isolates), by plotting the zone of inhibition against the logarithm to the base 2 of the MIC for each bacterial isolate for an individual bacterial species. The selection of breakpoints is then based on multiple factors, including regression line analysis that correlates MICs and zone diameters of inhibition, bacterial population distributions, error rate bounding, pharmacokinetics, and ultimately, clinical verification.

7. Antimicrobial susceptibility testing guidelines

A number of national standards and guidelines are currently available. International standards and guidelines for antimicrobial susceptibility testing and subsequent interpretive criteria throughout the world are:

Clinical Laboratory and Standards Institute (CLSI, 2018),
European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017).

At this time, only the CLSI has developed protocols for susceptibility testing of bacteria of animal origin and determination of interpretive criteria (CLSI, 2018). A veterinary sub-committee (VETCAST) has also been set up under the umbrella of EUCAST. However, protocols and guidelines are available from a number of standards organisations and professional societies, including those listed above for susceptibility testing for similar bacterial species that cause infections in humans. It is possible that such guidelines can be adopted for susceptibility testing for bacteria of animal origin, but each country must evaluate its own AST standards and guidelines. Additionally, efforts focusing on both standardisation and harmonisation of susceptibility/resistance breakpoints on an international scale are progressing. These efforts have primarily focused on the adoption of the standards and

guidelines of CLSI and EUCAST, which provide laboratories with methods and quality control values enabling comparisons of AST methods and generated data (CLSI, 2018; Kahlmeter *et al.*, 2006). For those WOAH Members that do not have standardised AST methods in place, the adoption of either set of standards would be an appropriate initial step towards acceptable methods and harmonisation.

Many bacteria that cause disease in aquatic animals require growth conditions (e.g. lower temperatures, supplemented or semisolid media) that may vary considerably as compared to terrestrial bacterial pathogens. This necessitated the need for the development of antimicrobial testing methods for bacteria isolated from aquatic species. Further information with regards to methods for disk diffusion or broth dilution antimicrobial susceptibility testing for bacteria isolated from aquatic animals can be referenced in two CLSI documents (CLSI, 2006; 2014b). Further information with regards to methods for disk diffusion or broth dilution antimicrobial susceptibility testing for infrequently isolated or certain fastidious bacteria (e.g. *Campylobacter*, *Pasteurella*) can also be referenced in the CLSI M45-A document (CLSI, 2015).

As a first step towards comparability of monitoring and surveillance data, Members should be encouraged to strive for harmonised and standardised programme design (Brown & MacGowan, 2010; Kahlmeter *et al.*, 2006; White *et al.*, 2001). Data from countries using different methods and programme design may otherwise not be directly comparable (Brown & MacGowan, 2010). Notwithstanding this, data collected over time in a given country may at least allow the detection of emergence of antimicrobial resistance or trends in prevalence of susceptibility/resistance in that particular country (Petersen *et al.*, 2003). However, if results achieved with different AST methods are to be compared, then comparability of results must be demonstrated and consensus on interpretation achieved. This will be best accomplished by the use of accurate and reliable documented AST methods used in conjunction with monitoring of AST performance while using well characterised reference microorganisms among participating laboratories.

Table 1. Phenotypic susceptibility testing methods available and their features

Susceptibility testing method	International standard available	Published methods available	Use in national surveillance programmes	Use in susceptibility testing for therapeutic purposes	Breakpoints that may be applied	Test output	Comparability of outputs	Features
Broth (micro) dilution MIC determination	Yes (ISO 20776-1), CLSI, EUCAST	Yes (CLSI, EUCAST)	Yes, broth microdilution MIC determination is preferred	Yes	Clinical breakpoints or epidemiological cut-off values (ECOFFs)	MIC	High	Current reference method. Recording MIC values allows interpretation of the test outputs using different breakpoints (e.g. clinical breakpoint or ECOFF), as well as re-evaluation of historical data if changes occur to breakpoints and evaluation of shifts in MIC. Numerous national surveillance programmes adopt this method. The MIC value can sometimes indicate the likely mechanism of resistance (e.g. high-level amikacin resistance and rRNA methylases) or provide an epidemiological marker. Currently, this is the only method suitable for determining susceptibility to colistin.
Agar dilution MIC determination	No	Yes (CLSI, EUCAST)	Not widely used	Yes	Clinical breakpoints or ECOFFs	MIC	Dependent on congruity of methods used	Reference method. The breakpoints appropriate for broth dilution may not be directly applicable to agar dilution. Currently used in particular for testing certain fastidious organisms.
Breakpoint method	No	Yes (scientific literature)	Not widely used	Yes	The test is performed at a set breakpoint	Resistant or susceptible at selected breakpoint	Dependent on congruity of methods used	Changes to breakpoints in this method result in the inability to interpret historical data. Shifts in susceptibility within the S or R categories cannot be detected. The breakpoint method relies on the growth or absence of growth of bacteria in broth or on agar containing an antimicrobial at a single (breakpoint) dilution.
Gradient strip method	No	Yes (manufacturer)	Not widely used	Yes	Clinical breakpoints or ECOFFs	MIC	High	Provide a convenient alternative method of determining MIC with minimal additional equipment required.
Disc diffusion test	No	Yes (CLSI, EUCAST) A number of different methods are available. These are not in general equivalent.	May be used, but broth microdilution MIC determination is preferred	Yes	Clinical breakpoints (ECOFFs are also available for the EUCAST disc diffusion method).	Diameter of zone of inhibition, interpreted as resistant or susceptible according to test guidelines	Dependent on congruity of methods used	Frequently used to provide an indication of susceptibility for therapeutic purposes. Versatile in that different discs can be used, according to the antimicrobials authorised for treatment. Different methods are not usually equivalent (zone sizes obtained using one method cannot be interpreted using criteria from another, different method). The collection of zone size data can allow shifts in susceptibility to be detected. Disc diffusion methods may be harmonised to a degree with other methods, by using the same breakpoint.

The susceptibility testing method selected should provide details of the method, appropriate controls and quality control ranges and breakpoints. The comparability of outputs obtained in surveillance programmes is not only dependent on the laboratory methodology used but is also dependent on the target population of livestock included in the study and method of sampling.

8. Comparability of results

To determine the comparability of results originating from different surveillance systems, results should be reported quantitatively including information on the performance of the methods, the reference organisms and breakpoints used and the antimicrobial.

AST data, consisting of cumulative and ongoing summary of susceptibility patterns (antibiograms) among clinically important and surveillance microorganisms should be created, recorded and analysed periodically at regular intervals (CLSI, 2014a). Data must also be presented in a clear and consistent manner so that both new patterns of resistance can be identified and atypical findings confirmed or refuted. This data should be available on a central data bank and published yearly.

Cumulative AST data will be useful in monitoring susceptibility/resistance trends in a region over time and assessing the effects of interventions to reduce antimicrobial resistance.

9. Quality control (QC) and quality assurance (QA)

Quality control/quality assurance systems should be established in accordance with chapter 1.1.5 in laboratories performing AST:

i) quality control refers to the operational techniques that are used to ensure accuracy and reproducibility of AST,

ii) quality assurance includes, but is not limited to, monitoring, record keeping, evaluating, taking potential corrective actions if necessary, calibration, and maintenance of equipment, proficiency testing, training and QC. A QA programme helps ensure that testing materials and processes provide consistent quality results.

The following components should be determined and monitored:

i) precision of the AST procedure,

ii) accuracy of the AST procedure,

iii) qualifications, competence, and proficiency of the laboratory personnel, as well as the personnel that interpret the results and those that are involved in monitoring of antimicrobial resistance,

iv) performance of the appropriate reagents.

The following requirements should be respected:

i) Strict adherence to specified and documented techniques in conjunction with quality control (i.e. assurance of performance and other critical criteria) of media and reagents.

ii) Record keeping of:

   a) lot numbers of all appropriate materials and reagents,

   b) expiration dates of all appropriate materials and reagents,

   c) equipment calibration and monitoring,

   d) critical specifications for AST performance (reference results, time, temperature etc.).

iii) The appropriate reference microorganism(s) should always be used regardless of the AST method employed.

iv) Reference microorganisms are to be obtained from a reliable source for example, from the American Type Culture Collection (ATCC®), reliable commercial sources, or institutions with demonstrated reliability to store and use the organisms correctly.

v) Reference microorganisms should be catalogued and well characterised, including stable defined antimicrobial susceptibility phenotypes. Records regarding these reference organisms should include the

established resistant and susceptible ranges of the antimicrobials to be assayed, and the reference to the method(s) by which these were determined.

vi) Laboratories involved in AST should use the appropriate reference microorganisms in all AST testing.

vii) Reference strains should be kept as stock cultures from which working cultures are derived and should be obtained from national or international culture collections. Reference bacterial strains should be stored at designated centralised or regional laboratories. Working cultures should not be subcultured from day to day as this introduces contamination and the method of producing working cultures should ensure that stock cultures are rarely used. This may be accomplished with the production of an intermediate stock of cultures derived from the original cultures that are used to crate day-to-day working cultures.

viii) The preferred method for analysing the overall performance of each laboratory should test the working stock of the appropriate reference microorganisms on each day that susceptibility tests are performed.

Because this may not always be practical or economical, the frequency of such tests may be reduced if the laboratory can demonstrate that the results of testing reference microorganisms using the selected method are reproducible. If a laboratory can document the reproducibility of the susceptibility testing methods used, testing may be performed on a weekly basis. If concerns regarding accuracy, reproducibility, or method validity emerge, the laboratory has a responsibility to determine the cause(s) and repeat the tests using the reference materials. Depending on the cause(s), daily reference material use and any other corrective action may be re-initiated.

ix) Reference microorganisms should be tested each time a new batch of medium or plate lot or batch of disks is used and on a regular basis in parallel with the microorganisms to be assayed.

x) Appropriate biosecurity issues should be addressed in obtaining and dispersing microorganisms to participating laboratories.

10. External proficiency testing

Laboratories should participate in external quality assurance and/or proficiency testing programmes in accordance with chapter 1.1.5. Laboratories are also encouraged to participate in international inter-laboratory comparisons (e.g. WHO External Quality Assurance System) (Hendriksen *et al.*, 2009). All bacterial species subjected to AST should be included.

National reference laboratories should be designated with responsibility for:

i) monitoring the quality assurance programmes of laboratories participating in surveillance and monitoring of antimicrobial resistance,

ii) characterising and supplying to those laboratories a set of reference microorganisms,

iii) creating, managing, and distributing samples to be used in external proficiency testing,

iv) creating a central database available on the internet (e.g. European Antimicrobial Resistance Surveillance System [EARSS]) that contains the different susceptibility/resistance profiles for each bacterial species under surveillance.

11. Conclusion

Although a variety of methods exist, the goal of *in-vitro* antimicrobial susceptibility testing for clinical veterinary purposes, surveillance and monitoring is the same: to provide a reliable predictor of how a microorganism is likely to respond to antimicrobial therapy in the infected host. This type of information aids the clinician in selecting the appropriate antimicrobial agent, provides data for surveillance, and aids in developing antimicrobial judicious use policies (WOAH, 2018).

In-vitro antimicrobial susceptibility testing can be performed using a variety of formats, the most common being disk diffusion, agar dilution, broth macrodilution, broth microdilution, and a concentration gradient test. Each of these procedures requires the use of specific testing conditions and methods, including media, incubation conditions and times, and the identification of appropriate quality control organisms along with their specific QC ranges. It is essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable with those results obtained by an acknowledged 'gold standard' reference method. In the absence

of standardised methods or reference procedures, antimicrobial susceptibility/resistance results from different laboratories cannot be reliably compared.

The use of genotypic approaches for detection of antimicrobial resistance genes has also been promoted as a way to increase the rapidity and accuracy of susceptibility testing. New technological advances in molecular techniques (e.g. microarray) may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data into surveillance and monitoring programs (Ojha & Kostrzynska, 2008; Poxton, 2005). Standardised phenotypic AST methods will still be required to detect novel and emerging resistance mechanisms among bacterial pathogens and to validate their detection via genetic techniques (Ellington *et al.,* 2017).

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NB: There is a WOAH Reference Laboratory for antimicrobial resistance (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratory for any further information on antimicrobial resistance

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2019.

CHAPTER 2.1.2.

BIOTECHNOLOGY ADVANCES IN THE DIAGNOSIS OF INFECTIOUS DISEASES

INTRODUCTION

Modern methods of livestock production and increased global trade demand the development of early warning diagnostic platforms, global capacity, effective assays and diagnostic harmonisation to enhance livestock productivity and health and to facilitate trade in healthy animals as the basis of the global One Health initiative.

The risk of pathogen-transfer between countries or continents has increased markedly in recent years, due to several factors, such as increased national and international movement of people, animals, food and feed products, and various goods, and climatic changes, causing new epidemiological scenarios and threats. This new situation highlights the requirement for the development and application of a wide range of powerful novel diagnostic methods that can detect spreading pathogens very rapidly, with high diagnostic specificity and sensitivity.

The purpose of this chapter is to provide general information and updates on the most important biotechnology-based methods, currently used in our diagnostic laboratories. For further information, two issues of the WOAH Scientific and Technical Review *concerned with biotechnology and the diagnosis of animal diseases are available on the WOAH website (https://www.woah.org/en/what-we-do/publications/scientific-and-technical-review/; Potential applications of pathogen genomics: Volume 35, Issue 1, 2016; Biotechnology applications in animal health and production: Volume 24, Issue 1, 2005).*

A. TECHNIQUES FOR THE DETECTION AND ANALYSIS OF NUCLEIC ACIDS

The direct detection and amplification of specific nucleic acid sequences in the genomes of pathogens have been shown to have considerable advantages. Polymerase chain reaction (PCR)-based technologies have revolutionised the diagnosis of infectious diseases. DNA amplification platforms are highly sensitive, specific, rapid and robust. The platforms are also cost-effective and adaptable to automation and therefore ideal for high-throughput screening. The equipment required is highly adaptable to the detection of many different targets, however the quality of the specimens is key to successful use of these technologies.

1. Nucleic acid extraction

An important initial step in most molecular diagnostic procedures is the extraction of 'clean' nucleic acid mixtures from the clinical sample. While it is relatively easy to extract DNA from bacterial cultures or blood, it is technically more challenging to prepare suitable material from field specimens, such as faeces, internal organs or aborted fetuses. Care during sampling in the field and in laboratory processing during the extraction of nucleic acids is important to avoid cross contamination of specimens, leading to false positive results. For this reason, nucleic acid extraction should be performed using strict working procedures, in an isolated room separated from nucleic acid amplification procedures.

Nucleic acid isolation and purification consists of three main steps: disruption of cells, removal of proteins and contaminants, and recovery of nucleic acid suitable for analysis. There are numerous methods of nucleic acid extraction, including manual extraction using phenol-chloroform and guanidinium thiocyanate to concentrate DNA in a hydrophilic phase, centrifugal spin column-based elution where nucleic acid binds to a solid phase such as silica and proteins are removed with the aid of centrifugation and magnetic bead extraction where the nucleic acid binds to magnetic particles while the proteins are washed away. While extraction methods may vary in the relative yield

and purity of the targeted nucleic acids, effective and reproducible extraction is an essential part of nucleic acid detection and thus pathogen detection. PCR, or other methods described below, can amplify and detect the specific target, but the quality of the starting material is an inherent part of the detection process.

Several specialised extraction methods for particular types of samples and tissues are now commercially available either as manual or automated systems for robotic workstations. The development and accessibility of the robotic extraction platforms not only minimises the risk of contamination, but also enables processing of large numbers of samples under constant reaction conditions with minimal operator manipulation. Consequently, these platforms have contributed to the establishment of high-throughput, robust diagnostic assays, shortening the processing time required per sample from hours to minutes (Belák *et al.,* 2009; Oberacker *et al.,* 2019). These are destined to improve the reliability of nucleic acid extraction from different specimens, but it remains a challenging area.

As an alternative to nucleic acid extraction, biotechnologists are increasingly focusing on PCR-inhibitor resistant polymerases and novel amplification assays, which are now available to directly amplify nucleic acids from pathological specimens without the requirement for extraction steps (Pavsic *et al.,* 2016).

2. Conventional polymerase chain reaction (PCR) and real-time PCR

PCR is a widely used, highly sensitive diagnostic tool for detecting pathogen-specific nucleic acid sequences in host tissues and vectors. Over the past 30 years, PCR has revolutionised the diagnostic process, allowing rapid, highly sensitive and specific detection of a wide range of pathogens.

PCR can identify and amplify a specific, selected target region of nucleic acid from a complex mixture of heterogenous sequences (Saiki *et al.,* 1988). PCR achieves *in-vitro* amplification by exploiting natural DNA replication mechanisms, using a succession of cyclic incubation steps at different temperatures. Amplification facilitates the detection of target nucleic acid sequences within a sample, even when only a small number of targets were present originally.

Diagnostic PCR is performed on nucleic acids extracted from clinical samples. The heterogenous sample DNA is first heat-denatured to separate the two complementary strands, thereby creating a single-stranded template. Specific primers are then used to identify the target regions for amplification. Primers are short, synthetic, single-stranded DNA molecules complementary to the target sequences. If the target sequence is present in the sample, the primers will anneal to these regions as the temperature cycle cools. Following binding, the cycle enters an intermediate temperature phase in which bound primers enable extension of the target sequence by DNA polymerase. Once the polymerase has synthesised a new complementary strand of DNA, the product is separated from the template by heating to a higher temperature and the cycle repeated.

These cycles are repeated, usually between 35–50 times, resulting in the amplification of target DNA sequences. Each cycle has the potential to double the number of targets from the previous cycle leading to the exponential increase in target DNA that gives the method its high sensitivity. The key to the amplification of target DNA sequences by the PCR is the selection of paired primers that, when extended, will create additional reciprocal primer-annealing sites for primer extension in subsequent cycles. To detect RNA (e.g. RNA viruses), a complementary DNA (cDNA) copy of the RNA must first be made using reverse transcription (RT). The cDNA then acts as a template for amplification by PCR. This technique is referred to as reverse transcription PCR (RT-PCR).

Once amplified, the target sequence can be detected. Amplified products are detected most frequently using methods that also help to confirm the identity of the PCR products, including:

i) Methods that define their characteristic size (such as gel electrophoresis)

ii) DNA probes (see below)

iii) High resolution melting (see Section A.2.1)

iv) Fluorescence-based detection systems (real-time technology)

v) Nucleotide sequencing technologies

Increasingly, since the advent of automated cycle sequencing techniques, identification is made more and more frequently via PCR techniques and agent characterisation by direct sequencing of the PCR products.

To provide robust, reproducible results, the PCR protocols should be carefully designed, optimised and fully validated. Chapter 2.2.3 *Development and optimisation of nucleic acid detection methods* provides comprehensive information and guidance on such assays.

To expand its utility in veterinary diagnostics and pathogen identification, PCR has been modified extensively over the years. Traditional PCR requiring detection at the end-point of the reaction, typically by agar gel electrophoresis, has been largely superseded by detection while the reaction is occurring i.e. real-time PCR. The most common variations; the main advantages and the limitations of PCR technologies are summarised in Table 1.

Table 1. Summary of commonly used variations in PCR techniques, their advantages and disadvantages

PCR: amplifies a selected region of target nucleic acid in a sample			
Variations	**Details**	**Advantages**	**Disadvantages**
Nested PCR	Two rounds of PCR, with the second round further amplifying a pre-amplified genomic fragment from the first round. Generally superseded by the real-time PCR	High sensitivity	High risk of contamination as it is not a closed system and cross-contamination or carry-over may occur via handling of amplified products
Consensus PCR	Targets a conserved genomic region in a group of pathogens	Can identify new but closely related pathogens	Not specific, may require further characterisation
Real-time PCR	Results of amplification are detected in real time, through fluorescence of a region-specific probe. Quantification is possible, if standards with known copy numbers are used in the run (hence the alternative name of quantitative PCR)	Highly sensitive and highly specific; decreased risk of contamination as the system is closed and there is no post-amplification manipulation	Cost (probes; platform). Risk of false negatives with highly mutable agents: primer probe sets can be designed to be highly target specific or universal to detect sequences conserved across lineages and strains
Multiplex real-time PCR	Detects multiple targets at once	Allows for screening numerous pathogens in one assay	Sensitivity can be compromised. Limited by the number of different fluorescent dyes available

2.1. High resolution melting

For DNA organisms such as parasites and bacteria, and for DNA viruses, the use of high-resolution melting (HRM) allows the genotyping of subtypes of a pathogen based on nucleotide differences. HRM is a post-PCR analysis method for analysing the melting (dissociation) of the PCR products in the presence of improved double-stranded DNA-binding dyes. As most real-time PCR machines have integrated this feature, HRM represents a cost-effective method of choice as no labelled probe is required. It is possible to increase the specificity and genotyping accuracy of HRM methods by using unlabelled probes to interrogate a short segment of the PCR amplicon, where the targeted mutation is present. The unlabelled probes must be blocked at the 3'-end to prevent polymerase extension. HRM techniques can be used in singleplex or multiplex format for the simultaneous detection and differentiation of species within the same genus (Banowary *et al.,* 2015; Gelaye *et al*., 2013), drug-resistance screening (Loiacono *et al.,* 2017) or differential diagnosis (Gelaye *et al.,* 2017).

2.2. Mass-tag PCR

Until recently, the detection of amplified target sequences in a real-time PCR assay relied on the use of various chemistries, such as intercalating dyes, hydrolysis probes, molecular beacons, primer probe energy transfer (PriProET), scorpion primers, dual hybridisation probes and dye-labelled oligonucleotide ligation (Belák *et al.,* 2009). The availability of detection methods limited the number of different targets (and thus, different pathogens) that could be detected simultaneously in a multiplex PCR. Mass-tag PCR assays have been developed to overcome this limitation.

In a mass-tag, multiplex PCR assay, the various primers are marked with tags of known, but different, molecular weights. After amplification of the targeted DNA fragments, the tags are released using UV light and subsequently measured using mass spectrometry. This approach enables detection of a far larger range of amplified DNA targets than previously possible, as the assay is not limited to the number of dyes available. This allows a single assay to simultaneously test for a large panel of diseases. Application of the mass-tag PCR assay has been proven already in the differential diagnosis of syndromic diseases (respiratory, haemorrhagic, enteric pathogens, meningitis/encephalitis syndrome) and in the detection of new clades of pathogens (Lipkin, 2010). A modification of this method uses matrix-assisted laser desorption-ionisation (MALDI), which directly measures the molecular weights of the PCR products and compares them with known databases (Angeletti, 2017; Jang & Kim, 2018).

2.3. Digital PCR (dPCR)

In recent years there has been renewed interest in the application of digital PCR (dPCR), where single copies of target DNA and RNA are isolated and individually amplified by PCR. The technique was first described in the 1990s and has since been revitalised due to progress in chemistry and equipment development. The distinctive feature of dPCR is the separation of the reaction mixture into thousands to millions of partitions, which is followed by real-time or end-point detection (conventional PCR) of the amplification. Target sequences are distributed into partitions according to the Poisson distribution model, thus allowing accurate and absolute quantification of the target from the ratio of positive partitions against all partitions at the end of the reaction. This omits the need to use reference materials with known target concentrations and increases the accuracy of quantification at low target concentrations compared with real-time PCR. dPCR has also shown higher resilience to inhibitors in many different types of specimens (Gutierrez-Aguirre *et al.,* 2015). Several research papers have been published on the application of dPCR to the detection and quantification of animal pathogens such as African swine fever virus and pseudorabies virus, demonstrating increased sensitivity and linearity (Ren *et al*, 2018; Wu *et al,* 2018).

2.4. Portable real-time PCR machines

The development of portable real-time PCR machines and assays raises the prospect of these techniques being used for rapid (less than 2 hours) diagnosis of disease outbreaks in the field. However, the isothermal amplification techniques described below may be better, as these require less complex and costly equipment.

3. Isothermal amplification

Isothermal amplification technologies offer the advantage of omitting thermocycling, enabling DNA amplification at a constant temperature. Consequently, the required equipment is less complex and therefore cheaper. The basic principle of this technology has been developed, resulting in several distinct methodologies (Gill & Ghaemi, 2008).

The most widely used method is the loop-mediated isothermal amplification (LAMP) method, which deploys four or more primers forming a stem-loop DNA by self-primed DNA synthesis and a DNA polymerase with strand displacement activity. The result of the amplification process is the production of loops at the ends of the complementary strands, which are continually extended. The amplification process is indicated by either the occurrence of turbidity in the reaction mixture, the generation of a fluorescent signal using fluorescent dyes (Gill & Ghaemi, 2008) or, if primers are biotinylated, the reaction products can be observed using lateral flow devices (see Section B.3 Immunochromatography). The process is also less prone to inhibition from contaminants compared with PCR; however, protocols based on this technology might be difficult and complex to develop. Isothermal amplification is less suitable for multiplex assays than PCR, as the potential for primer dimer formation and/or competitive reactions is higher and the assay design is more difficult in certain cases.

4. Analysis by restriction fragment length polymorphisms (RFLP) and related DNA-based approaches

The restriction fragment length polymorphisms (RFLP) method allows individual genomes to be identified based on unique patterns caused by restriction enzymes cutting in specific regions of DNA. The RFLP approach is based on the fact that the genomes of even closely related pathogens are defined by variation in sequences. For example, the linear order of adjacent nucleotides comprising the recognition sequence of a specific restriction enzyme in one

genome may be absent in the genome of a closely related strain or isolate. The RFLP procedure consists of digesting the pathogens' nucleic acid (DNA or PCR products) with one or a panel of restriction enzymes and then separating the DNA fragments by agarose gel electrophoresis to determine the number of fragments and their relative sizes. A restriction enzyme is an endonuclease that recognises and cleaves double-stranded DNA at specific nucleotide sequences called restriction sites (Loza-Rubio *et al.,* 1999).

Pulsed-field gel electrophoresis (PFGE) can be used for the separation of large (up to megabase size) fragments of DNA and can be a useful adjunct to the basic RFLP analysis. These technologies are extensively used in the official programmes for detection and discrimination of food-borne pathogens (*Escherichia coli* O157:H7, *Salmonella, Shigella, Listeria*, or *Campylobacter*) worldwide (https://www.cdc.gov/pulsenet/).

RFLPs have a clear value for use in epidemiological studies, however, an RFLP difference may not be functionally significant. Although the RFLP and PCR-RFLP are much less powerful compared with the modern sequencing technologies, they are relatively inexpensive, easy to perform and sufficiently descriptive for epidemiological investigations of outbreaks and identification of individual strains of pathogens.

5. Genome sequencing

In recent times, the cost of sequencing has reduced and genome sequencing has become increasingly important in the detection and characterisation of pathogen nucleic acid in diagnostic samples. Genome sequencing involves determining the order in which nucleic acid bases occur in a gene. Sequencing of PCR products targeting an informative region of a gene is particularly useful for pathotyping various organisms. For example, the sequence analysis of influenza virus A haemagglutinin gene is commonly used to locate the cleavage site and thereby classify the virus as highly pathogenic or low pathogenic influenza. Complete genome sequencing is currently the ultimate discriminatory procedure for pathogen identification. Since 1977, the Sanger method (Sanger *et al.,* 1977) has dominated DNA sequencing, although recent advances in high-throughput, next generation sequencing are revolutionising this approach.

Genome sequencing is a complex process. The basic principle is cycle sequencing of targeted DNA fragments with labelled di-deoxy nucleotides, which halt the elongation on binding. Each of the four di-deoxy nucleotides is labelled with a different fluorescent dye, enabling distinction between individual di-deoxy nucleotides. During the PCR process, these labelled di-deoxy nucleotides bind to the DNA fragments wherever their complementary base pairs exist. Once bound, they block further elongation of the DNA fragment. This process creates a mixture of DNA fragments, each ending with a defined nucleotide. The reaction mixture is then analysed using capillary electrophoresis, which separates the fragments by length and reads the fluorescence of each fragment. Analytical software is then used to convert the specific fluorescence signals into a nucleotide sequence.

Genome sequencing allows the analysis of pathogens detected during the diagnostic process. Such analyses include:

i) Identifying pathogenic strains to facilitate the implementation of appropriate control efforts and policies and where indicated, the update of vaccines and development of specific diagnostic tests (Beerens *et al.,* 2017; Busquets *et al.,* 2019).

ii) Evaluating the genetic similarity of isolates to other pathogens of the same species (subtype), to determine their phylogenetic properties or to determine the origin of an outbreak/infection. This can be valuable in the epidemiological analysis of outbreaks.

Importantly, conventional sequencing methods can be used to detect and characterise newly emerging pathogen strains. This overcomes one of the major limitations of PCR-based methods, which require known primers for pathogen detection. For example, sequencing is commonly used to identify a new virus within a family by using degenerate primers (a mix of similar but not identical primers) to amplify a gene common to that virus family, followed by sequencing of the PCR amplicon. Similarly, universal primers that anneal to a conserved sequence can be used to amplify and sequence genes in all bacterial species; the most common application is the use of 16S rRNA gene for bacterial identification (Cai *et al.,* 2014).

Advantages and limitations of the various sequencing techniques commonly applied in diagnostics are summarised in Table 2.

Table 2. Summary of commonly used variations of nucleic acid sequencing techniques, their advantages and disadvantages

Sequencing: determines the nucleotide composition of a selected region of target nucleic acid in a sample			
Variations	**Details**	**Advantages**	**Disadvantages**
Sanger sequencing (also known as di-deoxy sequencing)	DNA fragments from a PCR product are labelled with di-deoxy nucleotide, which can be detected through fluorescence	Highly specific Cost effective for short lengths of DNA Quick and simple work flow and analysis	Sequence length is limited and has no depth. For one sequencing reaction, only one sequence read is generated
High throughput sequencing (HTS) also known as next generation sequencing (NGS) and massive parallel sequencing (MPS)	Genomes are fragmented, and universal adapters and primers are used to generate sequence. HTS is enabling the following types of studies: • Whole genome sequencing • Pathogen discovery (*de novo* sequencing) • Metagenomics • Microbiome detection • Transcriptome detection	No prior knowledge of target is required as the reaction is primer independent Can analyse multiplex samples, using barcodes, which brings the cost down	Cost Requires large data storage and deep bioinformatic analysis, as the amount of data generated is enormous Can take several days to get results however this is offset by the large amount of data generated (e.g. whole genomes) Access to bioinformatics skills and data libraries can be problematic

5.1. High throughput sequencing (HTS) and mobile technologies

High throughput sequencing (HTS) is the term used to describe highly parallel sequencing methods. These methods produce data at the genome level and provide greater diagnostic opportunities compared with conventional sequencing (Belák *et al.,* 2009; Lipkin, 2010). Although various instruments are available, each with its own sequencing methods, the so-called second-generation sequencing technologies share the same general strategy, consisting of clonal amplification of DNA templates followed by sequencing in massively parallel sequencing reactions to produce thousands or millions of reads, ranging in length from 50 to 700 base pairs. These reads are subsequently assembled into longer sequences, known as contigs, using bioinformatics tools (see Chapter 1.1.7 *Standards for high throughput sequencing, bioinformatics and computational genomics*). The availability of bench-top versions of these instruments with reduced costs has made HTS accessible to average-sized laboratories for pathogen identification and characterisation. These new technologies have not only increased the speed of sequencing but also reduced its cost radically. As a consequence, these sequencing methods are becoming ever more significant tools in pathogen detection and definition.

Another application of HTS is whole genome sequencing of infectious agents. HTS allows the assembly of the complete genome or genome segment and reveals reassortment or recombination events and virulence markers. HTS can be used to perform deep re-sequencing of viral sequences within single specimens to assess the evolutionary dynamic (Granberg *et al.,* 2016). Phylogenetic studies and tracing the source of infections can also be undertaken using HTS. However, most HTS platforms produce short reads and so have widely accepted limitations in the *de novo* assembly of new genomes. In this context, a third generation of sequencing methodology producing longer read lengths but lower throughput, provides a means to increase the resolution of the genome assembly, thus enabling more efficient *de novo* sequencing of microbial genomes (Levy & Myers, 2016). At present, portable devices in a flash-drive format are available and can produce reads of 5–50 kb (Granberg *et al.,* 2016). Although the error rate of these instruments, 5–20%, looks very high, the hybrid assembly of short reads from second generation sequencing platforms facilitates the *de novo* assembly of novel microbial genomes. Today, a common technology applied by the portable sequencing devices relies on the principles of nanopore sequencing, which is based on measuring changes in electrical properties as the DNA molecule traverses a pore. These electrical changes are then used to identify the exact DNA base going through the pore.

5.2. Multilocus sequence typing

Multilocus sequence typing (MLST) is a method of sequencing used in pathogen typing and epidemiological studies. Using this approach, fragments of a number of gene targets are amplified by PCR and sequenced. For each locus, unique alleles are assigned arbitrary numbers and, based on the allelic profile, the sequence type is determined. Most MLST methods give highly reproducible results due to the international harmonisation of the nomenclatures; allele sequences and profiles are available in large central databases with companion on-line analysis tools.

6. Metagenomics

Metagenomics refers to culture-independent applications of HTS for investigating the complete viral and microbial genetic composition (microbiome) of samples (Bexfield & Kellam, 2011). Significantly, it is an unbiased approach as organisms are not selected by favourable culture methods (or deselected by unfavourable conditions) and does not require prior knowledge. It is important to consider that without targeted amplification, the metagenomic technologies are less sensitive for pathogen-detection compared with PCR-based methods. However, they are being used more frequently in diagnostic laboratories, because, by detecting a wide range of infectious agents simultaneously, they are powerful diagnostic tools. These technologies have also made it possible to identify pathogens more rapidly, including pathogens that can be difficult to identify by cultivation *in vitro* (Hoper *et al.,* 2017).

From a diagnostic viewpoint, the capacity to detect a wide range of infectious agents simultaneously (Belák *et al*. 2013) is a significant advantage of metagenomic technologies. Some of the agents identified may be novel and metagenomic approaches, accompanied by bioinformatics, have led to the discovery of a high number of infectious agents, including novel bocaviruses, Torque Teno viruses, astroviruses, rotaviruses and kobuviruses in porcine disease syndromes and new virus variants in honey bee populations. It should be noted that the power of metagenomic technologies to identify all organisms present in a sample in an unbiased manner, can present the diagnostic laboratory with the challenge of identifying which of the many organisms detected in a sample might be the causative agent of the disease.

These results, and the recent experience gained in several diagnostic centres worldwide, indicate that the metagenomic detection of infectious agents is becoming a powerful, cultivation-independent and useful diagnostic tool, both in veterinary and human medicine (Granberg *et al.,* 2016; Wylezich *et al.,* 2018).

B. IMMUNODIAGNOSTIC TECHNIQUES

All immunoassays exploit the innate ability of antibodies to bind to an antigen (or epitope(s) within the antigen) with high specificity and affinity. Antibodies are often referred to as being monoclonal or polyclonal. Monoclonal antibodies (MAbs) are derived from a single B-lymphocyte, meaning that each antibody produced is identical to the next and will therefore bind only to a single specific epitope on an antigen. Polyclonal antibodies (PAbs) are essentially a collection of different MAbs as they are derived from a population of B-lymphocytes, so that each antibody is different to the next and may therefore bind specifically to different epitopes within the antigen.

MAbs are produced by immortalized B-lymphocyte clones called hybridomas, which are formed by the fusion of a myeloma cell with an individual B-lymphocyte taken from the spleen of an immunised animal. PAbs are produced in animals that have been exposed to a foreign antigen such as a pathogen or a vaccine and represent the full antibody response to that antigen. Thus, sera being tested will contain polyclonal antibodies whereas antibodies used as reagents in a test may be polyclonal or monoclonal.

Immunoassays are designed to detect pathogen-specific antigen or antibody in test samples, as indicators of active or prior infection. Antigens may be the pathogen itself, one or more of its subunits or proteins, a recombinant protein or a synthetic polypeptide. Antibodies will be those of host origin that the assay targets as a marker of infection and/or the reagent antibodies used to bind specific antibody or antigenic markers of disease. Reagent antibodies are commonly described according to their function. As examples, a primary antibody is used to bind directly to an antigen; a secondary antibody will bind to the primary antibody and be used to carry a label for detection; and a capture antibody will be used to immobilise an antigen.

Depending on the immunoassay format, antibody or antigen is first bound to a solid phase – typically the surface of micro-beads, a membrane, or the wells of an enzyme-linked immunosorbent assay (ELISA) plate. This provides a stationary point to which a specific target of interest binds or is captured. Thus, an immunoassay designed to detect an antibody response in a serum sample from an infected animal might use an antigen bound to the solid phase that is bound by pathogen-specific antibody in a test serum and this in turn is detected using a labelled secondary antibody. Where the target in a test sample is an antigen, an antibody on a solid phase is used to capture the antigen via a specific epitope and a second (often labelled) antibody directed towards an alternative epitope sandwiches the antigen and is used for detection. There are many variations on these assay formats but in all cases the specificity of antibodies for a given antigen forms the basis of the immunoassay. A wide range of useful reports is available in the literature, where various diagnostic systems were compared and recommendations were given for diagnostic laboratories concerning the specificity, sensitivity and usefulness of the various immunodiagnostic assays (Kittelberger *et al.*, 2011).

1. Enzyme-linked immunosorbent assay (ELISA)

ELISAs are the predominant immunodiagnostic tool used in veterinary diagnostic laboratories around the world. ELISAs exploit specific antigen-antibody interactions to detect specific antibodies to a pathogen, or a specific antigen of the pathogen. The conventional design of the ELISA has been extensively modified to meet a range of diagnostic requirements.

1.1. Principles of operation

The basic principle that underpins all ELISAs is the specific binding of antigen to antibody. Each type of ELISA exploits this interaction in a different way.

1.1.1. Indirect ELISA

Indirect ELISAs (I-ELISA) are used for the detection of antibodies in a serum sample to an antigen. In I-ELISA, the target antigen (whole or purified) is bound to the solid surface of the wells of an ELISA plate (solid phase). To perform the test, the following steps are performed:

i) Serum samples at an appropriate specified dilution are added to the wells. If specific antibodies against the coated antigen are present in the serum, they will bind to the antigen.

ii) The ELISA plates are then washed to remove any unbound antibodies.

iii) Antibodies raised against the immunoglobulins of the animal species being examined, are then added to the wells. These anti-species antibodies (or sometimes Protein A or G) are conjugated with an enzyme (usually a peroxidase).

iv) As all unbound antibodies from the serum sample are removed during the initial washing step, conjugated antibodies added at step (iii) can only bind to those antibodies already bound to the solid phase of the ELISA during step (i) (i.e. antibodies that are specific to the antigen of interest).

v) A second washing step is performed to remove any unbound conjugated antibodies.

vi) The enzyme substrate with a suitable chromogen, in substrate buffer, are then added. If any bound conjugated antibodies are present in the well (i.e. wells containing the antibodies of interest), the presence of the conjugated enzyme will cause the colour of the substrate buffer to change.

vii) This colour development is measured at a defined wavelength using a spectrophotometer. Higher quantities of antibodies in the sample result in increases in optical density.

I-ELISA is very sensitive for antibody detection, because reagents are added in excess (excess reagent is washed away) and this promotes rapid reactions and the amplification of signal due to the continual action of the enzyme that generates the colour change in the final step of the assay. The format can be made even more sensitive by adding more enzyme per molecule of conjugate. This may be done by biotinylating antibodies and then adding streptavidin-conjugated enzymes, or by using detection reagents that have scaffolds to which many more molecules of enzyme are attached, e.g. poly-HRP (horseradish peroxidase) streptavidin.

1.1.2. Antigen-capture ELISA

Antigen-capture ELISAs (Ag-ELISA) are used to detect the presence of pathogen-specific antigens in a sample and are useful for diagnosis prior to or during clinical disease.

The Ag-ELISA commonly follows a sandwich assay format. MAb or PAb specific to the antigens of interest are bound to the solid surface of the wells of an ELISA plate. The sample is then added to the well and any target antigens are captured by the bound antibodies. Captured antigens are subsequently detected through the addition of a second, enzyme-labelled MAb or PAb. The wells are then washed to remove any unbound conjugated antibodies before enzyme substrates and buffers are added. As for I-ELISAs, this will result in a detectable colour change in positive wells. Note that if the detecting antibody is not labelled, then an enzyme-conjugated antibody that targets the detecting antibody is used.

The desired characteristics of the capture antibodies are strong binding to the pathogen, recognition of a conserved epitope highly specific for the target agent, and the ability to attach to an ELISA plate without loss of reactivity. The detection antibody should recognise an epitope other than that recognised by the capture antibody that is bound to the ELISA plate. It may be difficult to identify MAbs of comprehensive intra-type reactivity and polyclonal antisera may be preferred to increase the likelihood of reaction against all antigenic variants.

Ag-ELISAs have been developed to detect many infectious agents, e.g. bovine viral diarrhoea virus (BVDV, see Mignon *et al.,* 1991), rinderpest and peste des petits ruminants virus (PPRV) (Libeau *et al.,* 1994). Related antigen-capture methods using antibody-coated immunomagnetic beads are now important and well accepted methods for detecting certain bacterial infections, including *Listeria, Salmonella* and *E. coli.* The principle of this technology relies on immunomagnetic separation, i.e. using small super-paramagnetic particles or beads coated with antibodies against bacterial surface antigens. The magnetic properties of the beads allow them to be retained whilst the beads are washed and moved to different buffers in a manner analogous to the methods described above for ELISA. Antigens such as intact bacteria or their soluble antigenic determinants can be detected after magnetic extraction from the test sample using a second antibody in a sandwich format. Antigen-capture assays using immunomagnetic beads can enhance the kinetics of the antigen–antibody reaction as the beads mix within the sample reducing the incubation time.

1.1.3. Blocking ELISA

Blocking ELISA (B-ELISA), used for detection of serum antibodies, shares some similarities with Ag-ELISA. Antibodies specific to the target antigen are bound to the solid phase of the ELISA plate. The antigen, prior to adding to the antibody-coated plate, is incubated with the samples. If the samples contain antibodies against that antigen, they will bind (block) to the antigen. When added to the wells, the blocked antigen will be unable to bind to the coating antibodies. Consequently, when the detecting antibody is added to the wells it will not recognise any bound antigen (no colour development). In contrast, if the sample is derived from a negative case, binding will occur and there will be colour development.

1.1.4. Competitive ELISA

Competitive ELISA (C-ELISA) is a variant of ELISA used to detect or quantify antibody or antigen using a competitive method. The principle of a competitive assay for the detection of antibodies is competition between antibodies that may be present in the test serum and the detecting antibody (which in this case binds directly to the antigen). Specific binding of the detecting antibody is detected using an appropriate anti-species conjugate or the antibody itself may be labelled. A reduction in the expected colour obtained is caused by binding of antibodies present in the test serum that are specific to the antigen, which therefore competes with the specific detecting antibody for antigen-binding sites. The C-ELISA offers significant advantages over I-ELISA; for example, samples from many species may be tested without the need for species-specific enzyme-labelled conjugates for each species under test, assays may be performed using less purified antigens, and may be faster to perform as they require less incubation time. In some cases, C-ELISAs may have lower analytical sensitivity than indirect ELISAs. Where the C-ELISA relies on competition between antibodies in the test serum and an MAb (detecting antibody) for binding to a single epitope on the antigen, false negatives may occur where the humoral response

of the host to the pathogen has targeted distinct epitopes on the antigen that differ from the MAb target epitope.

2. Immunoblotting

Immunoblotting is performed in diagnostic laboratories to identify and/or characterise infectious agents based on antigen specificity or using known antigens to detect a specific serological response. Immunoblotting combines the high resolution of gel electrophoresis with the specificity of immuno-detection and offers a means of identifying immunodominant proteins recognised by antibodies from infected animals or antibodies directed against the target agent. As an example of antigen detection, immunoblotting has been used on a large scale as a major screening method for bovine spongiform encephalopathy (BSE) and scrapie; it has been used on millions of brain stem samples in Europe and elsewhere for the detection of prion protein (Schaller *et al.,* 1999). It has now largely been replaced as a screening test by Ag-ELISA or lateral flow device-based methods but is still an important confirmatory test and is integral to the differentiation of transmissible spongiform encephalopathies strains into typical and atypical BSE and scrapie.

False-positive and false-negative results in other diagnostic assays can often be resolved by immunoblotting (Molina Caballero *et al.*,1993). Immunoblotting can also be used to determine the specificity of individual MAbs. Individual purified polypeptides (or recombinant proteins) may also be transferred to nitrocellulose or polyvinylidene difluoride membranes by immunoblotting to examine the reactivity of test sera to individual proteins. This characteristic profile of reactivity may be used to help distinguish between animals that have been vaccinated or infected, such as the enzyme-linked immunoelectrotransfer blot (EITB), a western blot for foot and mouth disease (FMD) that is widely used in South America (Bergmann *et al.,*1993).

The major factor affecting the success of an immunoblotting technique is the nature of the epitopes recognised by the antibodies. Most high-resolution gel techniques involve some form of denaturation of the antigen, which destroys conformational determinants and allows only the detection of linear or non-conformational epitopes. Most polyclonal antisera contain antibodies to both linear and conformational epitopes, but MAbs recognise single epitopes; thus, if they target conformational epitopes, they will not react with denatured protein.

3. Immunochromatography (lateral flow devices)

Immunochromatography provides a convenient method to detect antigens, antibodies (or any labelled molecule for which a ligand has been coated onto the chromatographic membrane, e.g. biotinylated LAMP products [see Section A.3 Isothermal amplification]) in several minutes without special apparatus (Fowler *et al.,* 2014; Hanon *et al.,* 2016; Waters *et al.,* 2014).

The sample is applied (usually with buffer) to one end of a membrane where there is a pad containing antibody- or antigen-conjugated microbeads (beads such as latex or colloidal gold). The antigen or antibody (dependent on format) in the sample forms an immunocomplex with the labelled microbeads. This complex moves along the membrane due to capillary action, until it makes contact with a corresponding antibody, antigen or ligand immobilised on the membrane, where it forms an immuno-complex and generates a coloured product that can be visualised by eye or read with a portable hand-held reader. Lateral flow devices (LFDs) show great potential for development into portable, pen-side diagnostic tests. Microbeads incorporating fluorophores can be used to produce LFDs with greater analytical sensitivity when applied with specific portable detectors (Liang *et al.,* 2017). Low cost, paper-based fluidics, may also have a greater role in the future, especially in the pen-side context (e.g. Yang *et al.,* 2018).

4. Reporter virus neutralisation tests

Reporter viruses may be derived from genetically manipulated viruses that are fully attenuated and that bear a marker gene encoding, for example, green fluorescent protein, or the *Renilla* or firefly luciferases. Alternatively, the envelope glycoproteins of the virus to be studied are expressed on a replication-deficient particle of a heterologous viral species that carries a marker gene, for example lyssaviral glycoproteins expressed on retroviral particles (viral pseudotypes). These innovative approaches, which reduce the bio-risk for the veterinary diagnostician and increase the sensitivity of virus neutralisation tests (VNT), have been developed for a range of viral pathogens, including rabies virus (Wright *et al*., 2008), influenza A virus (Carnell *et al.,* 2015), PPR, rinderpest and canine distemper viruses (Logan *et al.*, 2016a; 2016b), and Rift Valley fever virus (Schreur *et al.,* 2017). These technologies allow rapid quantification of neutralising antibodies as they obviate the need to wait for the development of plaques or other

cytopathic effects. They can potentially be multiplexed to develop VNT assays capable of detecting neutralising antibodies against distinct antigens (Molesti *et al.,* 2014).

5. Luciferase immunoprecipitation system

The luciferase immunoprecipitation system (LIPS) can also be used for pathogen-specific antibody detection. A recent application of this approach was described for the detection of PPRV-specific antibodies (Berguido *et al.,* 2016). In this assay, a reporter enzyme, *Renilla* luciferase (Ruc), is fused to an antigen of interest and expressed in mammalian cells. Subsequently, the Ruc-antigen fusion protein is recognised by antigen-specific antibodies, and antigen–antibody complexes are captured by protein A/G beads, which recognise the Fc region of the IgG antibody. The relative amount of antibody bound to the Ruc-tagged antigen is determined using a luminometer to measure the light produced when adding coelenterazine, the substrate for Ruc. This approach is species independent, provides a very specific and sensitive detection and requires very low volume of test serum.

6. Rapid homogeneous assays

Rapid homogeneous assays are those where the reagents are mixed together to form a homogeneous solution or suspension within which the antibody and antigen reactions rapidly take place. The assays require no further separations or washing and can be read without further liquid-handling steps. Examples of such assays include agglutination assays whereby a serum sample is mixed with a suspended particulate antigen and where the presence of serum antibody against the antigen causes the antigen to agglutinate (due to the multivalent nature of antibodies). More sophisticated methods include the fluorescence polarisation assay (FPA). This measures how the rate of spin of a molecule, for example an antigen, changes as it becomes larger due to the binding of antibody. An example of this approach is the *Brucella* FPA using fluorescein conjugated O-polysaccharide (Nielsen *et al.,*1996). A limitation of this approach is the need for highly purified antigen and an antigen that is small enough to spin rapidly when not bound to antibody. Two readings are required, one to measure the background fluorescence of the sample and the other to measure the fluorescence of the reaction after antigen addition. Other methods that are used in the pharmaceutical industry for high-throughput compound screening include competitive methods where complementary ligands, analogous to antigens and antibodies, are labelled with tags that measurably interact with each other on a proximity basis, with signals increasing as they get closer (indicating binding has taken place). The introduction of competing reagents, for example unlabelled serum antibody, impedes this reaction leading to the consequent measurable reduction of signal (McGiven *et al*., 2009). When compared with other diagnostic methods, FPA assays exhibited high sensitivity, e.g. for the diagnosis of bovine brucellosis (Praud *et al*., 2016).

C. BIOTECHNOLOGY APPROACHES FOR THE PRODUCTION OF DIAGNOSTIC REAGENTS

1. Recombinant DNA technology to produce diagnostic reagents

These technologies are now also being used to produce protein-conjugated polysaccharides (Cuccui & Wren, 2015) as the genetic code for the protein carrier, the proteins required for the synthesis of the glycans and the code for the proteins required to conjugate the glycans to the carrier can all be inserted into host bacterial cells (such as *E. coli*). Although directed primarily towards the generation of vaccines, such recombinant glycoproteins can also be used for serodiagnosis, for example the detection of anti-*Brucella* antibodies in cattle and pigs. (Ciocchini *et al*., 2014; Cortina *et al.,* 2016). These antigens offer the traditional advantages of recombinant proteins, such as purity of product and avoiding the need to culture the native organism (in this case a highly infectious zoonotic agent) but also enable the production of highly important non-protein diagnostic antigens such as glycans in a form (conjugated) that facilitates their use as diagnostics and vaccines.

Recombinant DNA technology enables the production of antigens for various diagnostic applications such as ELISA, agglutination, haemagglutination inhibition (HI) tests, agar gel immunodiffusion (AGID) tests, complement fixation tests (CFT), as well as microarray and bead-based technologies.

For antibody production, recombinant antibodies are used as an alternative to the traditional hybridoma-based technology in generating high quality antibodies. Phage display, yeast display and ribosomal display are some of the approaches used to produce antibodies in prokaryotic, eukaryotic and *in-vitro* systems, respectively (Sutandy *et al.,* 2013).

2. Synthetic antigen biology

By circumventing biological systems completely, the recreation of antigen structure via synthesis also offers many advantages over native antigen extraction. This includes high levels of purity and reproducibility, simple scale up capability and bespoke antigen design.

An upcoming trend in the production of antigens for use in assays is the development of synthetic peptide antigens. This allows antigens to be tested as diagnostic reagents based on the gene sequence; expression of the whole protein is unnecessary, thus shortening the process. Glycosylated peptides can also be synthesised (Bednarska *et al.,* 2017). The cost and benefits are such that recombinant production systems still dominate for the production of large proteins. However, for non-protein antigens where the underlying genetic basis for production is unclear and thus recombinant production is not possible, synthesis is a very viable and successful option for diagnostic antigen production. This has been shown for brucellosis where the epitopes that exist in the native structure of the O-polysaccharide from *B. abortus* S99 and *B. melitensis* 16M have been synthesised and the synthetic antigens have been shown to be highly effective for serodiagnosis (Guiard *et al.,* 2013; McGiven *et al.,* 2015).

Synthetic DNA can be generated from sequence data and then used with recombinant technology to generate proteins and even complete viruses for the rapid production of vaccines, such as influenza (Dormitzer *et al.,* 2013).

D. TECHNOLOGIES APPLICABLE TO DETECTION AND ANALYSIS OF ANTIGENS, ANTIBODIES AND NUCLEIC ACIDS

1. Microarray technologies

A microarray is a two-dimensional arrangement of specific biological probes (e.g. DNA, proteins, peptides, glycans) immobilised on solid substrates such as a glass slide, polymer-coated glass, plastics, nitrocellulose, etc. (Barbulovic-Nad *et al.,* 2006). A microarray provides high multiplexing capability, i.e. hundreds or thousands of detections can be performed at a time. Microarray chips can be created to identify the cause of syndromic disease, for example, a chip that targets panels of encephalitic pathogens or to target species-related diseases, e.g. swine disease chips. Similarly, chips of very high specificity may be designed to detect multiple targets for a given pathogen. Using array-based approaches can present some challenges in handling and analysis of the very large data sets that are generated. Instrumentation and consumables may also be cost-prohibitive for some laboratories.

1.1. DNA microarrays

DNA microarrays exploit the ability of complementary strands of nucleic acids to hybridise, and involves the following steps:

i) Nucleic acids isolated from biological sources are amplified and labelled with a fluorescent dye.

ii) The single-stranded, labelled DNA products are then added to the surface of the DNA microarray. This results in hybridisation of the sample nucleic acids with the complementary biological probes in the microarray, creating a labelled double-stranded molecular structure on the surface of the array.

iii) The microarray is then rinsed to remove non-specifically bound target molecules and evaluated using a laser scanner.

DNA microarray technology is a useful tool with a variety of diagnostic applications. This technology offers the possibility of detecting and identifying pathogens of veterinary and public health importance in the target sample (Ojha & Kostrynska, 2008). It can also be used for epidemiological investigations and genotyping of pathogens. With technological advances, DNA microarrays have also progressed toward the discovery of new emerging pathogens. For instance, an array consisting of highly conserved 70-mer probes from all sequenced reference viral genomes was used to demonstrate that a coronavirus was responsible for severe acute respiratory syndrome (SARS).

DNA microarrays are particularly useful for multiplex assays and have been used to detect and determine all possible HA and NA subtypes of avian influenza virus (Belák *et al.,* 2009). The most recent generation

of microarrays enables DNA sequence analysis, often providing complete genome sequences in a single experiment.

1.2. Protein microarrays

Protein microarrays are produced similarly to DNA arrays by immobilising proteins at high density on a solid surface (Sutandy *et al.,* 2013). The arrays contain specific probing molecules such as antigens or antibodies that can be recognised via fluorescent labels or detected by mass spectrometry (MS) (SELDI-TOF [surface-enhanced laser desorption/ionization-time of flight] or MALDI-TOF [matrix-assisted laser desorption/ionisation-time of flight]) (Sutandy *et al.,* 2013; Yu *et al*., 2006). The principle of the reaction between an immobilised capture molecule and a protein target analyte present in the sample relies on antibody/antigen recognition or protein/protein interaction. Protein arrays are used for antigen or antibody detection in blood samples, the discovery of disease biomarkers and the discovery of the mechanism of pathogenesis and immune response to a pathogen by different host. For example, a protein microarray containing 1406 predicted *Brucella melitensis* proteins was used to screen sera from experimentally infected goats and naturally infected humans and to demonstrate the differences in the immune response in goat and humans following *B. melitensis* infection (Liang *et al*., 2010). In addition, protein microarrays can be used for detection of antibodies directed against specific pathogens and monitoring the changes in cellular protein expression.

Surface-enhanced laser desorption/ionisation-time of flight mass spectrometry

Technological advances have enabled the microarray platform to achieve sufficient standardisation and method validation as well as efficient probe printing, liquid handling and signal visualisation. Some major challenges from using protein microarrays in routine diagnostics are the need for highly specific antibodies to prevent false-positive results and the need to produce large numbers of antibodies in a high throughput fashion (Sutandy *et al*., 2013).

2. Bead-based arrays

Bead-based arrays and cytometric bead arrays are variants of probe-based assays that provide the opportunities for multianalyte profiling targeting nucleic acids, antigens or antibodies (Christopher-Hennings *et al.,* 2013). In the technique, pathogen-specific nucleic acid, antigen or antibody is covalently linked to microsphere beads. An advantage of the technology is its multiplex capability that results from the beads themselves having a fluorescent signature (such that each bead can carry a specific probe). Bead-based assays are increasingly being used in multiple pathogen detection tests to look for the nucleic acids of several pathogens (Boyd *et al.,* 2015) or antibodies to different pathogens in a single sample (Sánchez-Matamoros *et al.,* 2016). If there is a requirement for testing many serum samples for a panel of diseases (e.g. several different antigens), including the possibility of multiple antigens per disease, then multiplex bead-based arrays offer an efficient means for this. Recent examples exploiting the multiplex capability of this technology are the application to genotyping of African swine fever virus (LeBlanc *et al*., 2013) and DIVA (differentiation between vaccinated and infected animals) test for FMD (Chen *et al.,* 2016).

3. Biosensors

Biosensors use an immobilised biosensing element (DNA, RNA, antigen/antibodies or glycans), also known as a bioreceptor, to recognise a characteristic biomarker of the pathogen. The resulting biochemical interaction between the biomarker and the bioreceptor is converted into a measurable signal by the transductor and displayed (Vidic *et al.,* 2017). Biosensing is based on optical electrochemical and mass-based transduction methods (Alahi & Mukhopadhyay, 2017). This approach has been used for influenza A antigen detection (Hideshima *et al.,* 2013; Lee *et al.,* 2013), and the detection of antibodies to *Mycoplasma bovis* (Fu *et al.,* 2014).

4. Mass spectrometry

Mass spectrometry (MS) can detect biomarkers present in a sample, based on their mass. Infection with a microorganism can be detected by comparing the profile of biomarkers present in a sample to a database of samples known to be positive for the organism of interest. This is the premise of diagnostic modalities including MALDI-TOF MS. This approach was used for *Staphylococcus intermedius* and for the direct identification of bacteria in blood cultures (Guardabassi *et al.,* 2017). Some approaches have also been developed to identify protein biomarkers as a means of virus identification. MALDI-TOF MS can also be used for sequencing short DNA

fragments, as an alternative to conventional sequencing when high throughput automated screening for mutations is needed.

One electrospray ionisation (ESI)-based platform, combining the accuracy and the sensitivity of MLST with the speed and throughput of MS, has been developed for rapid identification of pathogens (Kailasa *et al*., 2019). Similarly, several applications using the MALDI-TOF MS are available for genotyping or single nucleotide polymorphisms (SNP) typing and resequencing.

4.1. Proteomics

MS is also used in proteomics. The proteome is the total complement of proteins expressed within a cell, a tissue or an organism. Proteomics is the study of proteins, including their expression level, post-translational modification and interaction with other proteins. As not all proteins are expressed at all times, but are dependent on physiological and environmental factors, proteomics can provide an excellent overall view of disease processes at the protein level. For example, definitive diagnosis of chronic hepatitis B virus (HBV) infection still relies on liver biopsy, but proteomic analysis of serum samples shows that the expression of at least seven serum proteins is changed significantly in chronic HBV patients. Similarly, the ante-mortem differential diagnosis of Creutzfeldt-Jakob disease (CJD) may be aided by proteomics as preliminary data show that seven proteins in cerebro-spinal fluid (CSF) are differentially expressed between patients with variant or sporadic CJD (Choe *et al.,* 2002). Within the veterinary field, proteomic studies have been developed for a variety of applications for animal and zoonotic diseases (Katsafadou *et al*., 2015; Patramool *et al*., 2012; Torre-Escudero *et al*., 2017).

5. *In-situ* detection of antigens and nucleic acids

Different techniques are available for the direct detection of pathogen proteins, antigens or nucleic acids in animal tissues or body fluids. In some cases, the same technology can be applied to the detection of antibodies in serum.

5.1. Immunofluorescence

The fluorescent antibody test (FAT) is used for the detection of pathogens in animal tissues or fluids, using specific antibodies against the targeted antigens. As the method is based on direct binding of the labelled antibody to the antigens of the infectious agent present in the sample, it is commonly called direct immunofluorescence. The method is commonly used in diagnostic laboratories, e.g. for the detection of the rabies virus in the brains of dead animals and classical swine fever virus in tissues of suspected pigs.

A modification of the FAT can be used for the detection of specific antibodies, produced by the immune system against various pathogens during infections. Essentially, the modification is in the use of secondary antibody specific for the antibodies of the species examined. This procedure is commonly referred to as indirect immunofluorescence. The method is commonly used in diagnostic laboratories for the detection of antibodies raised against a wide range of pathogens, e.g., African swine fever virus (Cubillos *et al*., 2013), *Coxiella bernetii*, causative bacteria of Q fever (Roest *et al.,* 2013) and many other infectious agents in veterinary medicine.

Another modification of direct immunofluorescence uses an unlabelled primary antibody derived from a specific species to recognise pathogen-specific antigen, and this antibody is subsequently bound by a secondary anti-species immunoglobulin antibody that is conjugated to a fluorophore. This procedure is also commonly referred to as indirect immunofluorescence.

5.2. Immunohistochemistry

Immunohistochemistry involves the *in-situ* detection of antigens in fixed tissues using labelled antibodies. As an adjunct to the isolation of causative organisms from tissue, immunohistochemistry has become a standard tool to identify pathogens in tissue, and for confirmation of the results obtained using other diagnostic technologies. Immunohistochemistry is commonly used for the detection of abnormal prion protein (PrP^{Sc}) in brain tissue to confirm scrapie, BSE and other transmissible spongiform encephalopathies, and is more sensitive than standard histopathological examination (Thorgeirsdottir *et al*., 2002). In recent years, diagnostic tests based on immunohistochemistry have been successfully

developed and applied to rabies virus detection in clinical specimens (Rahmadane *et al*., 2017). As the number of antibodies to defined antigens increases, the use of immunohistochemistry for the identification of organisms and other specific markers for autoimmune diseases and neoplasia is increasing.

Due to the use of fixatives to prepare samples, IHC offers several advantages over pathogen isolation methods, including:

i) convenience of sample submission;

ii) safe handling of zoonotic pathogens;

iii) retrospective studies of stored specimens;

iv) rapidity; and

v) the detection of nonviable organisms (Haines & Clark, 1991).

As formalin fixation can denature the antigenic epitopes (i.e. the three-dimensional structure recognised by some antibodies) the limiting step in the application of immunohistochemistry is to identify an appropriate antibody/antigen combination that will bind antigen in formalin-fixed tissues. This may be overcome by using frozen sections or employing antigen retrieval techniques (e.g. proteolytic enzyme digestion, microwaving) before immunostaining.

5.3. *In-situ* hybridisation (ISH)

This technology exploits two natural phenomena: the unique genetic code or signature a pathogen contains in its genome and the ability of single-stranded nucleic acid sequences (DNA or RNA) to anneal or bind with complementary single-stranded nucleic acid sequences to form double-stranded hybrids. In its simplest form, *in-situ* hybridisation (ISH) uses single-stranded DNA or RNA probes that are produced synthetically and are designed to complement a short specific region of a pathogen's genome. Probes are coupled to labels that are readily detectable using microscopy and may include fluorescent dyes, fluorescent nanoparticles or enzymes that produce a chromogenic product when treated with substrate.

Recent commercial supply of ISH reagents has made the technique more accessible and is likely to see greater uptake by diagnostic laboratories in the future. A noteworthy application of ISH is in the rapid development of tests for detection of newly emerging pathogens. As soon as sequence data generated during identification of the novel pathogen by high throughput sequencing (see Section D.vi) becomes available, probes may be designed and assays developed. In contrast, antibody/antigen-based reagents required for the development of immunoassays take considerably longer to generate.

E. IMPLICATIONS OF THE NEW TECHNOLOGIES

There are several trends in diagnostic technology development, e.g. multiplexing of assays, evaluation of complexity in the biology of infection, that will have an impact on the way disease diagnoses will be approached in the future, affecting the laboratory environment, data analysis and disease control:

i) The global development of chip technologies has led to a strong trend towards miniaturisation of the test format in both molecular and protein detection assays. The test formats range from several millimetres to several centimetres. In parallel, a wide range of simple diagnostic tools, such as lateral flow assay devices were developed for the improved, on site (point-of care) diagnosis of many diseases. These changes enable on-site testing, facilitating the rapid and affordable diagnosis of the targeted infectious diseases. Appropriate infrastructure and preparedness must be an integral component of technology update, including training in the use and interpretation of such methods.

ii) Simplification of technologies, development of smaller, more simple and affordable devices is also seen in other fields of veterinary diagnostic microbiology, for example in rapid and high throughput sequencing technologies. The development of simple devices and improved sample preparation technologies will facilitate the direct sequence analysis of clinical samples not only in central institutes, but also on site in the field or in simply equipped field laboratories. During this work it will be very important to focus not only on the

rapid technical development of sequencing equipment, but in parallel, to strengthen the knowledge of bioinformatics and to maintain general knowledge in veterinary medicine and epidemiology.

iii) The development of alternative sources of signal generation/amplification, replacing light with mass measurement, piezoelectric effect or concentration of the ligand will lead to the development of a whole new platform of technologies.

iv) Although the development of new technologies can often mean faster results and improved capability, consideration should always be paid to the actual value and the role of the confirmatory test in diagnosis and of the modes of reaction of appropriate Competent Authorities. In this context, the expertise present in laboratories remains as important as ever in explaining the significance and limitations of diagnostic results.

v) Increasingly, traditional, well understood methods that use low cost equipment are being replaced with more complex methods requiring increased investment. These newer methods often require higher diversity of expertise in laboratory personnel and are associated with increased turnover in platforms as relatively recent systems become redundant. This has implications for laboratory resourcing and the structure of laboratory networks within and between countries as laboratories become more specialised.

vi) In-depth analysis of pathogens, for example through high throughput sequencing, is increasingly becoming an expected part of reference laboratory operations. As the diagnostic platforms are continuously changing in the ways described above, several components of the disease control chain will be affected. Appropriate communication technologies/information systems will need to be developed to systematically collect, store and analyse the large datasets produced by the new technologies in a relatively short time. For example, there is likely to be an increasing trend towards real-time inputs of results via mobile platforms. These trends will require ongoing development of bioinformatics, information technology and data-handling systems.

vii) Methods with high levels of analytical sensitivity and specificity are increasingly available to laboratories and are enabling the rapid identification and response to infectious veterinary disease, improving the effectiveness of control and eradication methods. These developments provide new opportunities for the identification and characterisation of the infectious agents and for the improved control of infectious diseases in veterinary medicine.

viii) Novel, biotechnology-based methodologies open a wide range of new possibilities and challenges in diagnostic microbiology. Thus, it is strongly advised to consider powerful new methods and to introduce these new technologies at our diagnostic laboratories. However, it is important to determine what is the exact diagnostic capacity and value of the new technologies and to what extent can these methods replace the classical diagnostic approaches. In general, the safest way is to maintain a multidisciplinary complex of diagnostic approaches and capacities at our laboratories, providing a practical combination of powerful new and classical technologies and a properly balanced knowledge of veterinary diagnostic medicine, infection biology and epidemiology.

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NB: FIRST ADOPTED IN 1996 AS BIOTECHNOLOGY IN THE DIAGNOSIS OF INFECTIOUS DISEASES AND VACCINE DEVELOPMENT. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 2.1.3.

MANAGING BIORISK: EXAMPLES OF ALIGNING RISK MANAGEMENT STRATEGIES WITH ASSESSED BIORISKS

A. INTRODUCTION

Chapter 1.1.4 Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities *describes the use of biorisk analysis for effectively identifying and managing laboratory biosafety and biosecurity risks for individual veterinary laboratories and animal facilities based on their unique facility, infrastructure, and the surrounding environment in which the biological agent or toxin is to be handled, including relevant national policies and legislation. The process of biorisk analysis includes biohazard identification, biorisk assessment, biorisk management, and biorisk communication.*

This Guideline provides, in a table format, two working ***examples*** *of the risk assessment component of the process. The risk assessments include review of laboratory control measures that a fictional veterinary laboratory evaluated in order to protect laboratory workers from inadvertent exposures and infection, and also importantly to protect as appropriate the local and regional animal populations, human populations, and environment from accidental or intentional release and spread of a biological agent or toxin from their laboratory or animal facility. As such, the risk analysis includes consideration of the likelihood of an event occurring and the consequences of such an event.*

The first example uses Bacillus anthracis*, a zoonotic agent, as an example of a biosafety risk managed by veterinary laboratories that is also considered a biosecurity risk based on its potential as a biothreat agent. The second example uses foot and mouth disease (FMD) virus as an example of a veterinary pathogen of variable economic significance depending on the endemic disease status and the location of the laboratory in relation to geopolitical borders. The FMD example represents an assessment completed by a laboratory located in an FMD-endemic area, and importantly notes in the assessment where laboratory control measures will be re-evaluated and modified as FMDV is eradicated in the specific region. Disease control and eradication programmes increase the economic impact of the disease and associated biorisks of handling the agent in the laboratory.*

NOTE: *It must be stressed that the two examples provided here are just that – examples invented for the purpose of showing what an actual laboratory may need to evaluate or consider as part of its biorisk assessment. The text in bold in the tables indicates matters that should be included in any agent-specific risk assessment. The text not in bold is fictitious data included to help demonstrate the process. It is equally important to note that the examples are short, concise summaries of biorisk analyses. Dependent on the complexity of the specific-agent, the laboratory facility, the proposed laboratory procedures, and the regulatory environment, actual biorisk analyses may vary from a similar short summery to a multiple-page dossier.*

Example Risk Analysis 1: Anthrax

Hazard: *Bacillus anthracis*

Location: Country where anthrax is not usually considered endemic but disease freedom not claimed – laboratory located in site where there are no domestic animals and no habitations (20 km around the facility).

Pathogen and disease: Anthrax is an acute bacterial disease primarily of herbivores and is transmissible to humans. It is caused by *B. anthracis*, a Gram-positive spore-forming rod-shaped bacterium.

Animals become infected by ingesting spores or possibly by being bitten by flies that have fed on an infected animal or carcass. Infected animals are usually found dead as death can occur within 24 hours. A careful post-mortem examination of recently dead animals may show any number of lesions, none of which is pathognomonic or entirely consistent. To avoid environmental contamination, post-mortem examinations conducted in the field (outside of laboratory containment) of carcasses of animals suspected to have died of anthrax is discouraged. Lesions most commonly seen are those of a generalised septicaemia often accompanied by an enlarged spleen having a 'blackberry jam' consistency and poorly clotted blood. Haemorrhage from the nose, mouth, vagina and/or anus at death is not a common sign.

More than 95% of human anthrax cases take the cutaneous form and result from handling infected carcasses or hides, hair, meat or bones from such carcasses. *Bacillus anthracis* is not invasive and requires a lesion to infect. Protection for veterinarians and other animal handlers involves wearing gloves, and other protective clothing when handling specimens from suspected anthrax carcasses and never rubbing the face or eyes. The risk of gastrointestinal anthrax may arise if individuals eat meat from animals infected with anthrax.

NOTE: The Table that follows is an example. The cells have been filled in in response to an imaginary scenario. The data are fictitious and included to help demonstrate the process. The requirements in the hypothetical examples are based on the country's standards and guidelines, as would be used for an actual biorisk analysis.

RISK ASSESSMENT (Likelihood and Severity)	RISK MANAGEMENT Administrative Controls	RISK MANAGEMENT Operational Controls	RISK MANAGEMENT Engineering Controls	RISK MANAGEMENT Personal Protective Equipment
Health Risk **Humans** – YES, treatment available. **Animals** – YES, treatment not usually attempted or recommended, case fatality rate high, herd morbidity variable. **Transmissible by** contact with body fluids of infected animals or carcasses, excretions of infected animals, or contaminated environment.	**Requirements:** **1. Communication plan**: reportable disease list and reporting requirements for both animal and public health authorities. **2. Qualified/suitable staff**: training and competency requirements for work with infectious diseases in place and current for all laboratory staff. **3. Health and safety programme**: prophylaxis and treatment for anthrax exposures reviewed. **4. Accident/incident reporting programme** **5. Emergency response plan**: actions including notification list for accidental exposure, spill/ contamination in lab, release from lab, and theft of agent. **6. Agent inventory management programme** **7. Waste management policy** **8. Security programme**: laboratory biosecurity for preventing unauthorised access to *B. anthracis*.	**Requirements:** **1.** Standard operating procedures (SOPs) and training materials comprehensively covering **laboratory safety and laboratory biosecurity** comprehensively that address the biorisks posed by the handling and storage of suspect diagnostic case materials and *B. anthracis* cultures. Good Laboratory Practices included in all working SOPs. **2. Disinfection/decontamination** SOPs are written for disinfecting/decontaminating necropsy and laboratory areas and equipment, for carcasses and tissues, for packaging and shipping materials, and for laboratory wastes. **3. Transport** SOPs, regulatory approvals, and certifications for packaging and shipping to the reference laboratory are current. Sample (specimen) containment SOPs are in place for movement within the laboratory facility. **4. Response plan** drill/exercise specific to *B. anthracis* identification from clinical materials and to associated laboratory responses, including incident response to spills and release from the laboratory. **5. Accident/incident reporting** and response SOPs and forms, audit schedule for reviewing accident reporting. **6. Audit and review schedule** for *B. anthracis* biorisk assessment and management	**Requirements:** **1. Biocontainment** addresses prevention of accidental and/or deliberate release of infectious material. **2. Separation of incompatible activities** **3. Equipment maintenance, calibration, certification** current **4. Facility security**	**Requirements:** **Personal protective equipment** (PPE): respiratory protection and dedicated laboratory clothing.
In the Laboratory **Necropsy:** body fluids released, environment contaminated, high risk of human	**Assessment findings:** 1. Reporting plan for *Bacillus anthracis* and contact list (names	**Assessment findings:** 1. Good microbiology practices followed by all lab staff; review included in	**Assessment findings:** 1. Biosafety cabinets located in sample receiving area and microbiology area. Centrifuge equipped with closed carriers	**Assessment findings:** 1. Respiratory protection available on-site for receiving, necropsy, microbiology staff.

RISK ASSESSMENT (Likelihood and Severity)	RISK MANAGEMENT Administrative Controls	RISK MANAGEMENT Operational Controls	RISK MANAGEMENT Engineering Controls	RISK MANAGEMENT Personal Protective Equipment
exposure by contact with body fluids and by inhalation. **Handling of specimens:** unknowns via diagnostic submissions including tissues, carcasses. (High risk for exposure to spores), swabs of blood, other body fluids or swabs taken from incisions in tissues or organs can be source of exposure (low exposure risk) **Volumes and concentrations:** Anticipate two diagnostic cases per year (moderate risk). Amplification with short and long-term storage on solid media, <1 mg agent (moderate risk) **Diagnostic test methods:** Identification and characterisation of the agent: Culture, immunological detection, characterisation and differentiation from vaccine strain by polymerase chain reaction (PCR). **Research:** No experimental work to be conducted **Bacterial isolate archives:** Short and long term storage of isolates. (High risk for intentional misuse – see Security risks.)	and numbers) is current. Includes public health, animal health. 2. Training and competency in good microbiology practices current for all staff. Anthrax-specific training for clinical disease recognition and handling competency documented for pathology staff. Anthrax-specific training for handling of high risk specimens for receiving and testing lab staff. 3 and 4. Employee health and safety programme in place. - All employees enrolled in the health and safety programme, trained in accident reporting policy and procedures. - Policy and procedure for providing health information and health surveillance related to Anthrax exposure for non-employee trainees and students present in the laboratory is in place. - Anthrax prophylaxis and treatment options reviewed with occupational health officer: decision made not to vaccinate lab staff. 5. Response plan addresses accidental and intentional release of *Bacillus anthracis*. All employees trained on plan. 6. Agent inventory management plan addresses access, secure storage, transfer, and destruction. Mandates annual audit and reconciliation of inventory records. Laboratory storage and archive system and procedures comply with	annual audit of laboratory practices to insure compliance. Current SOPs are in place for receiving, processing, testing, storage, and disposal of *B. anthracis* suspect case materials. Microbiology culture SOP includes steps to minimise potential for spore formation and subsequent exposure of staff to spores (reduction in anthrax inhalation biorisk) 2. General disinfection and decontamination protocols in place for all laboratory areas. Gap: The general disinfection/decontamination plan for the laboratory does not include specific references for disinfectants, concentrations, and contact times for *B. anthracis.* SOPs and training materials for recognition, handling, and destruction of high risk *B. anthracis* specimens in place. 3. Certification current for staff member responsible for packaging and shipping to reference laboratory. SOP for movement of *B. anthracis* within the laboratory includes requirement for transport in a secondary leak-proof non-breakable container marked with a biohazard label. 4. Emergency response plan was exercised within the prior 12 months; exercise specific to anthrax identified a gap: *Actions needed: Restrict potential for inadvertent contamination from necropsy area by (1) restricting visitors, (2) developing a policy for visitors (no street clothing or shoes in necropsy area), (3) review SOP with staff on rendering of carcasses from necropsy area to address*	(aerosol containment). Additional documents needed: Effluent treatment verification records, certifications for directional airflow. 2. Clerical areas separate from testing areas. Dedicated secure freezer used for agent inventory. 3. Biosafety cabinets in receiving and microbiology sections have certification of correct operation (air-flow) verified annually per pre-existing preventive maintenance schedule. 4. Doors to testing areas can be locked. Entry to building controlled by key-card access and alarmed off-hours. Inventory storage location is secured by lock and key, with key access available only to authorised personnel. The necropsy area used for short term storage of the carcass prior to incineration and disposal is secure and signage is posted to prevent unauthorised access.	Dedicated coveralls and boots used in necropsy area. Laboratory coats, gloves used for all specimen handling. SOPs for putting on and removing PPE available and systematically followed, PPE regularly collected and laundered in appropriate facility *Gap: Insure availability of coveralls and boots on-site for authorised visitors (students/trainees) to necropsy area.*

RISK ASSESSMENT (Likelihood and Severity)	RISK MANAGEMENT Administrative Controls	RISK MANAGEMENT Operational Controls	RISK MANAGEMENT Engineering Controls	RISK MANAGEMENT Personal Protective Equipment
	relevant national regulations for *B. anthracis.* 7. Waste management policy addresses carcasses, tissues, and all laboratory wastes containing potentially infectious material. All anthrax-suspect material to be diverted from laboratory rendering. 8. Laboratory biosecurity includes restricted access to testing areas. Biohazard signage available to post at entrance doors. Key-controlled access to incubator and freezer used for storage of *B. anthracis* cultures. *Gap: Laboratory policy needed to define the boundaries regarding types of research to be allowed, and* B. anthracis *to be included in risk profiles of organisms with which research will not be conducted.*	*holding, decontaminating or destroying potentially contaminated hides/ carcasses, and (4) develop a laboratory labelling system to clearly identify high-risk and/or potential zoonotic agent containing carcasses or specimens during short and long-term storage.* 5. Accident/incident reporting protocols cover *B. anthracis*. Laboratory director and safety officer review of all accident reports. Accident/Incident reporting is a component of the laboratory quality system and continual improvement plan and is audited annually. 6. Audit of *B. anthracis* control measures is scheduled for 30 days following first case presented. Review of risk assessment and management plan is scheduled to be completed annually and immediately after any significant change to the laboratory facility or management.		
ECONOMIC RISKS				
Endemic in region: 1. Sporadic cases possible.				
Trade barrier: A possibility to be managed. (High risk for restriction against movement of wool and hides.)	*In progress: Veterinary Authority advised that* B. anthracis *may be worked with in the laboratory, currently reviewing economic risks to region, working with trade partners to assess impact on trade related to laboratory handling and storing* B. anthracis.			
ENVIRONMENTAL RISKS				
Stabile in environment: YES. (High risk for soil contamination; spores stabile for years.)		SOP on handling of anthrax suspect carcasses, includes movement from necropsy area to holding area, decontamination and destruction of potentially	Microbiology laboratory liquid waste and solid waste inactivated before release.	

RISK ASSESSMENT (Likelihood and Severity)	RISK MANAGEMENT Administrative Controls	RISK MANAGEMENT Operational Controls	RISK MANAGEMENT Engineering Controls	RISK MANAGEMENT Personal Protective Equipment
		contaminated hides/carcasses to insure no release to landfill or rendering facilities.		
SECURITY RISKS				
Short- and long-term storage of isolates. (High risk for intentional misuse.)	Security policy and procedures in place: laboratory security programme includes visitor restrictions. Biothreat: Authority/agency has reviewed laboratory security policy, chain of custody and investigative protocol required if laboratory becomes involved in diagnosis/investigation of intentional Anthrax exposure incident.	Access to inventory records restricted by password protection. Inventory reconciled with inventory records annually, and immediately if unauthorised access is suspected.	Inventory access controlled by lock on storage freezers, and alarm to inventory area.	

Risk communication

Notification to appropriate public health authority and animal health authority

The laboratory is planning to begin diagnostic testing for *Bacillus anthracis* (anthrax) in order to support the detection and diagnosis of a naturally occurring or intentional introduction of the agent affecting local livestock species. The laboratory has completed a laboratory biorisk assessment and has in place appropriate control measures to prevent accidental exposures and release of the agent from the laboratory. The designated laboratory contact person is _____.

Actions to be completed:

1. In consultation with the national animal health authority, identify responsible person(s) and develop a message to be activated in the event of a positive anthrax diagnosis, including a press release, contact lists and a questions and answers document.

2. In consultation with the national animal health authority and/or public health authority as appropriate, identify responsible person(s) and develop a communication message to be activated in the event of a laboratory spill or accident, or accidental human infection with *B. anthracis*.

3. In consultation with the national animal health authority, public health authority and/or security agency as appropriate, identify responsible person(s) and develop a communication message to be activated in the event of a real or apparent deliberate release of the anthrax organism.

Verification, corrective actions, and continuous improvement

Action to be completed: Schedule and conduct internal audits of policy and procedures relating to the handling of organisms that constitute risks such as posed by *B. anthracis*. Exercise (audit) the pathway of activities and actions that would be followed in the laboratory should a potential anthrax disease submission be received. Perform an additional review of the biorisk assessment and control measures should changes be made to the facilities, management practices, or technical procedures associated with the laboratory handling and storage of the anthrax organism.

Example Risk Analysis 2: Foot and mouth disease

Hazard: Foot and mouth disease

Location: Country is FMD-endemic, disease control and eradication efforts are in initial phases. Laboratory located at a fenced site; no susceptible animals within 3 km of the facility.

Pathogen and disease: FMD is a highly contagious viral disease of cattle and swine. It also affects sheep, goats, deer, and other cloven-hooved ruminants. The disease is caused by a non-enveloped virus of the genus *Aphthovirus*, family *Picornaviridae*. There are seven serotypes of FMD virus (FMDV), namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1 with various subtypes. Infection with any one serotype does not confer immunity against another. Within serotypes, many strains can be identified by biochemical and immunological tests.

The transmission of FMDV occurs either by direct contact between infected and susceptible animals, by direct contact of susceptible animals with contaminated material or objects (hands, clothing etc.) or by consumption of untreated contaminated meat products (primarily by pigs). Furthermore, inhalation of infectious aerosols is known. Humans can be carrier of FMDV in their respiratory tract for up to 48 hours.

FMD is characterised by fever and blister-like lesions followed by erosions on the tongue and lips, in the mouth, on the teats, and between the hooves. Most affected animals recover, but the disease leaves them debilitated. The severity of clinical signs varies with the strain of virus, exposure dose, age and breed of animal, host species, and degree of host immunity. Signs can range from mild or inapparent to severe. Morbidity may approach 100%. Mortality in general is low in adult animals (1–5%) but higher in young calves, lambs and piglets (20% or higher). Recovery in uncomplicated cases is usually about 2 weeks.

NOTE: The Table that follows is an example. The cells have been filled in in response to an imaginary scenario. The data are fictitious included to help demonstrate the process. The requirements in the hypothetical examples are based on the country's standards and guidelines, as would be used for an actual risk assessment.

RISK ASSESSMENT (Likelihood and Severity)	RISK MANAGEMENT Administrative Controls	RISK MANAGEMENT Operational Controls	RISK MANAGEMENT Engineering Controls	RISK MANAGEMENT Personal Protective Equipment
Health risk: **Humans** – NO **Animals** – YES (domestic and wild hoofstock) **Transmissible by** direct contact with infected animals, their excretions, or contaminated environment; consumption of contaminated meat; aerosol transmission over distances is documented (references on file).	**Requirements:** **1. Communication plan**: reportable disease list and reporting requirements for animal health authority. **2. Qualified/suitable staff**: training and competency requirements for work with infectious diseases in place and current for all laboratory staff. **3. Health and safety programme** **4. Accident/incident reporting programme** **5. Emergency response plan**: actions including notification list spill/contamination in lab, release from lab, and theft of agent. **6. Agent inventory management programme** **7. Waste management policy** **8. Security programme**: laboratory biosecurity for preventing unauthorised access to FMDV.	**Requirements:** **1.** SOPs and training materials cover **laboratory safety and laboratory biosecurity**. Routine use of Good Microbiology Practices is required of all staff. **2. Disinfection, decontamination, destruction** SOPs are available for all laboratory sections for both solid and liquid wastes. **3. Transport**: the laboratory uses an externally trained and certified staff member for packaging and shipping specimens and infectious substances to the regional reference laboratory. **4.** The **laboratory response plan** has been prepared and includes steps required for containment of an FMDV spill, and steps for notification and control following inadvertent movement of untreated waste materials from the laboratory. **5. Accident/incident reporting**: the laboratory safety officer is responsible for reviewing accident reports submitted by staff. Each accident and immediate response is reviewed, changes made as necessary to prevent a repeat occurrence, staff are notified of the changes, and an audit is scheduled to document effectiveness of the correction. **6. Audit**	**Requirements:** **1. Biocontainment** **2. Separation of incompatible activities** **3.Equipment maintenance, calibration, certification** current **4. Facility security**.	**Requirements:** **PPE**: dedicated laboratory clothing is used at all times in the laboratory, is never removed from the laboratory and is laundered on site.
In the Laboratory **Necropsy:** environment contaminated with fluids, low risk of exposure to outside environment.	**Assessment findings**: 1. Reporting plan FMDV positive animals/herds and contact list (names and numbers of animal health authority) is current.	**Assessment findings**: 1. Staff have been trained by the regional reference laboratory in the technical methods and safe handling requirements	**Assessment findings**: 1. All FMDV handling and diagnostic testing is conducted in a biosafety cabinet. When not in a cabinet, all	**Assessment findings**: Laboratory coats and gloves are required to be worn when working in the receiving or technical areas of the laboratory.

RISK ASSESSMENT (Likelihood and Severity)	RISK MANAGEMENT Administrative Controls	RISK MANAGEMENT Operational Controls	RISK MANAGEMENT Engineering Controls	RISK MANAGEMENT Personal Protective Equipment
Handling of specimens: **u**nknowns via diagnostic submissions including vesicular fluids, saliva (probing), vesicular epithelium containing potentially high virus load. (High risk for laboratory contamination.) Blood and serum (low risk) **Volumes and concentrations:** Anticipate 1200 diagnostic and disease control cases per year; archive 120 × 0.25 ml aliquots at ≥ 10^5 $TCID_{50}$/vial. (Low risk while disease remains endemic. At later stages of eradication effort, risk will progress to high risk). Amplification in cell culture; short and long-term storage of viral isolates (Moderate Risk while disease remains endemic. At later stages of eradication effort, risk will progress to high risk justifying elimination of routine on-site amplification in cell culture and long-term archive of isolates). **Diagnostic test methods:** Identification and characterisation of the agent: Virus isolation, antigen detection (ELISA), nucleic acid detection (real-time PCR). Serology: ELISA, CF, virus neutralisation (VN). (Note: as eradication progresses the risk of amplifying virus and	2. Training and competency in Good Microbiology Practices is current for all staff. Laboratory policy requires that staff agree to not come in direct contact with susceptible species for 96 hours after working with FMDV diagnostic cases and assays. 3. Employee health and safety programme. FMDV is not a human pathogen. 4. Accident/incident reporting programme in place; covers unintentional laboratory contamination associated with diagnostic specimens and derived materials (e.g. FMDV cultures). 5. Response plan addresses accidental and intentional release of FMDV. 6. Agent inventory management plan addresses access, secure storage, transfer, and destruction. Inventory management plan requires annual audit and reconciliation of inventory records. 7. Waste management policy addresses carcasses, tissues, fluids, and all laboratory wastes containing potentially infectious material. All potentially infectious laboratory wastes are autoclaved or incinerated prior to removal from the site. *Gap: cell culture flasks are accumulated in the un-restricted glass-washing/autoclave area prior to disinfection/destruction. Policy and procedure change is being*	for FMDV. Proficiency tests have been successfully passed. SOPs for the handling, testing, archive, and destruction of FMDV and related case materials have been prepared, reviewed by the regional reference laboratory, and used in the training of staff. 2. Decontamination, inactivation, and destruction protocols are in place for all laboratory areas. Specific pH and temperature requirements for inactivation of FMD virus are included. 3. External training and certification was obtained for one staff member responsible for packaging and shipping to reference laboratory. A second individual is currently scheduled for international packaging and shipping training. 4. A laboratory response plan has been prepared, including steps for containment of an FMDV spill, and steps for notification and recovery or destruction following inadvertent movement of untreated waste materials from the laboratory. *Gap: the laboratory has not had an exercise to test the response plan. Additionally the plan does not address the potential for intentional misuse of FMDV: these issues will gain importance as the disease control effort nears completion, and so will be corrected.* 5. The Accident/Incident Reporting process is applicable to work with FMDV. 6. Audit of FMDV biorisk control measures will be included with the laboratory quality system audits (2 per year).	FMDV specimens or assay materials are in a closed container. Note: the regional reference laboratory uses additional measures including FMDV-dedicated rooms with air-lock entry. As the laboratory does not have these facilities, the risk assessment will be reviewed with consideration given to transferring case materials and non-serology FMDV testing to the regional laboratory as the eradication effort progresses. 2. Separation of incompatible activities: The virology testing area where FMDV specimens are handled is separated from other areas of the laboratory by closed doors posted with restricted access signage. A dedicated incubator is used for amplifying FMDV reference viruses and for virus neutralisation assays (e.g. sources of high concentration of FMDV). The incubator is cleaned and decontaminated at the end of each work week. Real-time PCR tubes are not opened following amplification steps. Note: as the eradication effort progresses toward completion, the amplification of reference virus for PCR controls and for VN assays will be discontinued. The risks associated with all testing requiring live virus will be assessed, with the option of transferring samples and testing to the regional reference	Laboratory PPE is not worn outside of the technical areas of the lab. Laboratory wear inadvertently contaminated with FMDV is autoclaved prior to being laundered. Note: additional measures including dedicated clothing and showering after work with the agent are used in the regional reference laboratory. This facility does not have shower facilities, so as the eradication effort progresses toward completion, the risk assessment will be reviewed with consideration given to transferring case materials and non-serology FMDV testing to the regional laboratory.

RISK ASSESSMENT (Likelihood and Severity)	RISK MANAGEMENT Administrative Controls	RISK MANAGEMENT Operational Controls	RISK MANAGEMENT Engineering Controls	RISK MANAGEMENT Personal Protective Equipment
maintaining live FMDV for use in the VN assay will no longer be justified and will be discontinued). **Research:** No experimental work to be conducted on site. Isolates to be transferred to international reference laboratories only. **Viral isolate archives:** Short- and long-term storage of isolates. (Low risk while disease is endemic, progressing to high risk for intentional misuse as eradication of disease nears completion - see Security risks.)	*implemented to insure inactivation of virus prior to transferring the flasks from the virology laboratory* (*e.g. bleach*). 8. Laboratory biosecurity includes signage noting authorised access only to testing and storage areas. Visitors are allowed only with a laboratory escort and prior permission of the laboratory director or his designee. (Note: as eradication progresses, more stringent access control is justified, e.g. padlock or equivalent to be added to archive freezer).	Review of the FMDV risk assessment and biorisk management plan will occur at 14 months following initiation of the FMDV testing or earlier should there be changes made to the facility or to any FMDV-related SOPs or laboratory procedures.	laboratory being given a high priority. 3. The laboratory has maintenance contracts for Biosafety cabinets and thermocyclers. Calibration is performed by a commercial vendor for pipettors and CO_2 incubators. 4. Facility Security: Staff and visitors enter the laboratory grounds via a locked gate in a perimeter fence. The laboratory external doors are locked after business hours. The gate and doors are posted with signage stating "authorised individuals only".	
ECONOMIC RISKS				
Endemic in region: Cattle, sheep, and wildlife (hoofstock).				
Trade barrier: Due to endemic status, there is no current trade. (Note: goal of eradication is to open markets for wool and hides. As eradication effort nears completion the risk of handling the agent in the laboratory will progressively increase, at that time the laboratory must re-evaluate on-site testing and options for transfer of samples to a regional reference laboratory.	Update for current risk assessment is scheduled for 14 months from initiation of control programme, assuming that the disease prevalence will significantly decrease and the severity of economic impact from a laboratory release of FMDV will amplify to high risk.			

RISK ASSESSMENT (Likelihood and Severity)	RISK MANAGEMENT Administrative Controls	RISK MANAGEMENT Operational Controls	RISK MANAGEMENT Engineering Controls	RISK MANAGEMENT Personal Protective Equipment
ENVIRONMENTAL RISKS				
Stabile in environment: YES. Survives drying, may persist for days to weeks in organic matter under moist cool conditions; may persist in contaminated fodder and environment for 1 month (temperature, pH dependent).	Policy regarding recycling/re-use of expendable supplies (e.g. plastic culture flasks) has been modified to state re-use is never allowed for laboratory supplies used in FMDV detection and diagnosis; FMDV related laboratory wastes are placed in a biohazard bag and decontaminated at the end of each day to ensure they are never sent to landfill without prior treatment.	SOP on incineration (destruction) of solid laboratory wastes is in place; SOP on autoclave and chemical inactivation of liquid wastes is in place. Temperature and pH requirements for inactivation of FMDV are included.	Autoclave and incinerator available on-site. Indicator strips used with each run of the autoclave to ensure proper inactivation was achieved.	
SECURITY RISKS				
Short- and long-term storage of isolates. (Low risk while disease is endemic, progressing to high risk for intentional misuse as eradication of disease nears completion - see Security risks.)	Security policy requires visitors to sign a log, be escorted and not allowed in direct contact with diagnostic samples or tests. Note: FMDV has been identified by the international community as a potential biothreat agent.	An inventory log has been created to identify each vial of virus archived, and matches the identification on each vial. The log is electronic and is password protected. Gap: a pre-determined schedule for comparing the inventory to the inventory log has not been formally documented.	The virus inventory is stored on a designated shelf in a freezer with access restricted by signage to authorised staff. *Gap: a padlock is needed to further secure the virus archive.*	

Risk communication

Notification to appropriate animal health authority

The laboratory is planning to begin diagnostic testing for Foot and Mouth Disease in order to support the national FMDV eradication effort. The laboratory has completed a laboratory biorisk assessment and has in place appropriate control measures to prevent release of the agent from the laboratory. The designated laboratory contact for further information is _____.

Actions to be completed:

1. In consultation with the national animal health authority identify responsible person(s) and develop a communication message to be activated in the event of a laboratory spill or accident resulting in the release of FMDV from the laboratory.

2. In consultation with the national animal health authority and security agency as appropriate, identify responsible person(s) and develop a communication message to be activated in the event of theft or apparent deliberate release of FMDV from the laboratory.

Verification, corrective actions, and continuous improvement

Actions to be completed:

Training and exercises are to be scheduled in order to practice procedures for spill decontamination and for proper use of biosafety cabinets, autoclaves and incinerators; and for the proper response and notification procedures should a security breach be detected. Periodic self-assessment and review of standard operating procedures by the biorisk manager and laboratory staff are to be scheduled and completed at an approximate 6-month interval in coordination with the laboratory's quality system audit process. Reporting and documentation of biosafety or biosecurity incidents and breaches involving the FMD virus will automatically trigger a re-assessment of the FMD laboratory biorisks. All incidents will be reviewed by management, the biorisk manager, all impacted staff, and the applicable biosafety committee in order to correct the problem and to identify opportunities for improved laboratory practices and implementation of biorisk control measures.

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* *

NB: First adopted in 2014.

SECTION 2.2.

VALIDATION OF DIAGNOSTIC TESTS

INTRODUCTORY NOTE ON WOAH VALIDATION RECOMMENDATIONS

WOAH has adopted a formal standard entitled Validation of diagnostic assays for infectious diseases of terrestrial animals*, referred to as the WOAH Validation Standard. Its most recent version, adopted in May 2023, may be found as chapter 1.1.6 of this* Terrestrial Manual.

Section 2.2 Validation of Diagnostic Tests *deals with the use of the WOAH Validation Standard and its implementation for specific requirements such as different types of assay* (*antibody, antigen or nucleic acid detection*), *and an elaboration of some of the tools used in validation studies* (*measurement of uncertainty, statistical approaches, reference panels*). *There is also a chapter addressing some of the particular challenges in validating tests for wildlife species. All of the chapters have been written by experts in the respective fields, and have been subjected to an extensive process of consultation in arriving at the final texts.*

None of these chapters should be used in isolation*. Each is designed to complement and inform the application of the WOAH Validation Standard to specific situations.*

*
* *

NB: FIRST ADOPTED IN 2014.

CHAPTER 2.2.1.

DEVELOPMENT AND OPTIMISATION OF ANTIBODY DETECTION ASSAYS

INTRODUCTION

The WOAH Validation Recommendations in Section 2.2 Validation of diagnostic tests *of this* Terrestrial Manual *provide detailed information and examples in support of the WOAH Validation Standard that is published as Chapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals*. The Term "WOAH Validation Standard" in this chapter should be taken as referring to that chapter.*

Detection of antibodies that are elicited in response to infectious agents or their components constitutes an indirect means of laboratory-based disease diagnosis. The most common antibody detection methods are classical virus neutralisation test (VNT), enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition (HAI) and the complement fixation test (CFT). Other, less common, antibody detection tests are the agar gel immunodiffusion (AGID), the indirect fluorescent antibody test (IFAT), the serum agglutination test (SAT), the latex agglutination test (LAT), and the microscopic agglutination test (MAT). More recent novel methods include biosensors, bioluminometry, fluorescence polarisation, chemoluminescence and lateral flow devices also known as point of care or pen-side tests. Other immunological assays that use antibodies in antigen detection tests are described in Chapter 2.2.2.

When considering a candidate assay type for disease diagnosis, one should include antibody detection assays because of their practicality, ease of sample collection and preparation, generally good diagnostic performance characteristics, suitability for automation (high-throughput), low cost and fast turn-around time. They are particularly useful for processing large numbers of samples in epidemiological and population studies, or for mass diagnosis and surveillance programmes. Antibody assays are also widely used for export, import and trade of animals, and still represent the majority of WOAH recommended tests for international trade.

A characteristic of antibody assays is their capacity to indicate prior exposure to an infectious agent in the absence of detectable organisms or their analytes. They are also adaptable to a variety of matrices, such as serum, plasma, whole blood, milk, lacrimal secretions and saliva. Immunoglobulin isotype or subclass-specific test systems may selectively target early or late immune responses, e.g. IgM and IgG, respectively. Specifically designed detection systems allow differentiation between responses to vaccine and field strains and are available as commercial kits, e.g. the detection of antibodies to classical swine fever virus in pigs. Competitive or blocking formats allow use of the same basic assay for a variety of animal species while other formats are species specific. Many types of chemical or physical indicators are used to indicate the presence of specific antibody in a specimen (chromogens, fluorochromes, agglutinins, among many others). Because of the large number of antibody detection methods available, it is not possible to describe the best practices for validation of each of these assay types in this chapter. The most widely used antibody detection system, the ELISA, will therefore be used as an example for application of best practices in antibody assays. Most of the basic processes used to validate other types of assay systems will become evident by extension of those used to validate ELISAs.

A. ANTIBODY DETECTION ASSAY DEVELOPMENT PATHWAY

1. Intended purpose(s) of the antibody assay

The first consideration in assay development is to define clearly the specific purpose and application of the test to be developed. Many decisions in developing assays will be based on these first considerations. For antibody detection assays (hereafter in this chapter designated as "antibody assays") such as ELISA, such knowledge will guide the selection of the most appropriate type of antibody detection system to achieve the intended purpose. Many factors related to the assay's intended purpose, use, and suitability need to be taken into account (see the WOAH Validation Standard for other possible purposes).

The six basic intended purposes for diagnostic assays are stated in the WOAH Validation Standard, and listed in the footnote to Table 1 below. Because antibody assays have such a broad range of applications, and can be configured for very specific purposes, it is useful to consider and evaluate several parameters when establishing the specific purpose(s) for the candidate assay. Table 1 summarises characteristics of antibody assays when applied for different purposes. Consideration of these characteristics will provide guidance in establishing the specific purposes for which the candidate assay will be fit.

Note – The reader is advised to read Section B.4. *Programme implementation*, as a primer for the following discussions. That section describes the inter-relationships between diagnostic sensitivity and specificity, false positive and negative test errors, and positive and negative predictive values. For a more in-depth discussion of predictive values as a function of prevalence, see Jacobson, 1998.

Table 1. Determinants of an antibody assay's fitness for its intended purpose

Assay characteristics	*Determinants of fitness for purpose*						
	*1**		*2**	*3**	*4**	*5**	*6**
	a	b					
Diagnostic sensitivity (DSe)	+++	+++	+++	+++	+++	+	+
Diagnostic specificity (DSp)	+	+	+	+	+++	+	+++
Positive predictive value (PPV)	+	+	+	+	+++	+	+++
Negative predictive value (NPV)	+++	+++	+++	+++	+++	+	+
Throughput capacity	+	+++	++	+	–	++	++
Turn-around time of test	+	+	+	+	+++	–	+
QA capability	+++	+++	+++	+++	+++	+++	+++
Reproducibility	+++	+++	+++	+++	+++	+++	+++
Repeatability	+++	+++	+++	+++	+++	+++	+++

Other characteristics such as the technical sophistication of the assay, and the skill required for interpretation will be related to the disease or infection under investigation.

Symbols: +++ = essential; + = of less importance; – = not important.

*Basic purposes for which an assay may be deemed fit: 1. Contribute to the demonstration of freedom from infection in a defined population. 2. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes; 3. Contribute to the eradication of disease or elimination of infection from defined populations; 4. Confirmatory diagnosis of clinical cases (includes confirmation of positive screening test); 5. Estimate prevalence of infection or exposure to facilitate risk analysis; 6. Determine immune status in individual animals or populations (post-vaccination)

1.1. Purpose 1

For disease freedom categories as given in purposes 1a and 1b (Table 1), antibody screening tests of high diagnostic sensitivity (DSe) are the tests of choice. As indicated in the purposes above, these tests would be applied to populations that have an apparent prevalence of zero. Tests of high DSe demonstrate low false negative (FN) rates and when applied to low prevalence populations, the negative predictive value (NPV) is at its highest level. However, DSe and diagnostic specificity (DSp) are usually inversely related and as such, a decrease in DSp will result in an elevated false positive (FP) rate. Other considerations, if

this is to involve a continuous volume of surveillance samples, would include high throughput, low cost and technical simplicity. All screening test positive results should be subjected to some form of confirmatory testing to evaluate their true status. Confirmatory tests characteristically have high DSp and therefore a low FP rate. These tests are often more sophisticated, more costly and may require enhanced interpretive skills.

If demonstration of freedom from infection is to be achieved after an outbreak, in which vaccination has been used for disease control, then screening of massive numbers of sera is often required. In addition to the considerations above, this also necessitates an antibody detection test which is able to distinguish between infected and vaccinated animals (i.e. a DIVA [differentiation of infected from vaccinated animals] test). At the same time an antigen or nucleic acid detection test may be warranted in some situations to prove that shedding and/or circulation of the infectious agent has ceased.

1.2. Purpose 2

If the purpose is to qualify individual animals for international movement, antibody screening tests of high DSe are again the tests of choice. The same rationale as stated above applies with respect to the NPV. Again, all positive reactors will need to be subjected to some form of confirmatory testing to evaluate their true status or may be excluded from shipment without further testing. In cases where borderline positives are observed, it may be wise to request a repeat sampling of the animal(s) at a suitable time interval to ensure that herd/flock has not been very recently infected.

1.3. Purpose 3

If the purpose of the test is the eradication of disease or elimination of infection from defined populations, antibody screening tests of moderate to high DSe are the tests of choice. However, the rationale is slightly different in that the testing will likely be done at herd or compartment level. At the beginning of the campaign, when the disease prevalence is high, moderate DSe and DSp are suitable as both FP and FN rates are less relevant at this juncture and a moderate level of test error is tolerable. Depending on the nature of the disease and rapidity of spread, high throughput and fast turn-around-times may become critical. Usually decisions are made without confirmatory testing at this point.

In the latter stages of the campaign, a higher DSe is warranted as the FN rate becomes the more critical factor. Much like Purposes 1 and 2, positive reactors will need to be subjected to some form of confirmatory testing to evaluate their true status. In these latter stages, antibody detection tests are often applied in conjunction with antigen and/or nucleic acid detection systems to detect subclinical cases and possibly, latent carriers.

1.4. Purpose 4

For the confirmatory diagnosis of clinical cases, antibody tests of high DSp are the tests of choice. In these cases, the idea is to minimise the FP rate and enhance the PPV of the test. As a general rule, infection is well established and the immune response is usually well underway. In some situations it may be preferable to carry out a screening test of high DSn but a lower DSp, then following up positives with a high DSp confirmatory test. For some clinical cases, e.g. vesicular diseases in terrestrial animals, several tests may be required to rule out select pathogens that present similar clinical signs. In some cases, antigen and/or nucleic acid detection tests may be a better choice for confirmation of clinical cases provided that they offer a fast turn-around-time. A prime example would be highly pathogenic avian influenza infections where mortality may occur before an immune response is even detectable.

1.5. Purpose 5

For estimates of prevalence of infection or exposure to facilitate risk analysis, e.g. for health surveys, herd health status and to monitor disease control measures, antibody tests of moderate DSe & DSp are the tests of choice. In general, this would balance both FN & FP rates and result in a more accurate estimate of the true prevalence of infection in the target population. However, if accurate estimates of both DSe and DSp have been established, statistical approaches can be used to minimise bias attributable to FN & FP rates (see Chapter 2.2.5 *Statistical approaches to validation*).

1.6. Purpose 6

For the determination of the immune status in individual animals or populations, e.g. post-vaccination, antibody tests of high DSp are required. Such tests have very low FP rates and as such provide a high degree of confidence in the PPV of the result. For use in individual animals, the use of virus neutralisation (VN) tests in cell culture for the detection of vaccine-induced neutralising antibodies against rabies virus in dogs and cats would be a prime example of a test with high DSp used for expression of titres in international units. However, these tests are technically sophisticated, expensive to maintain and run, and require strict biosafety procedures. For larger volume applications, such as monitoring regional vaccination programmes, ELISA-based tests would be more applicable, given their simplicity, cost effectiveness and high throughput. The same DSp considerations should be applied to these types of tests.

The experience of laboratory diagnosticians is not only essential in the choice of an appropriate test that will achieve the desired purpose, but is also required to determine reliably the scientific limitations of an assay and practical considerations such as cost, equipment and reagent availability, throughput capacity of the laboratory and test turn-around-times.

2. Assay development – experimentation

2.1. Reference materials, reagents and controls

Designing the method

- Has the design been shaped by the intended purpose of the assay?
- What is the specific application?
- What are the types and statistically relevant numbers of samples to be tested? (See Chapter 2.2.5)
- Will the test be field or lab based?

2.1.1. Test samples

Samples to be tested in antibody assays should be handled as described in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens*. The sample matrix in which antibodies are usually detected is serum, but may also include plasma, whole blood, milk, meat juice, egg yoke, lacrimal secretions and saliva.

2.1.2. Reference Standards

Antisera directed against the reference strain of a pathogen are known as reference sera or reference standards (Wright *et al.,* 1993; WOAH Validation Standard; Section 1.4 of Chapter 2.2.6 *Selection and use of reference samples and panels*). Such sera containing antibody of known concentration/activity are useful in the initial development of an assay. For a number of WOAH listed diseases, (e.g. avian influenza, foot and mouth disease, classical swine fever, etc.) international reference standards are available through WOAH Reference Laboratories and Collaborating Centres. When not available from other sources, it may be necessary to produce in-house reference standards against which working standards (process or quality controls) are calibrated.

2.1.3. Positive and negative reference panel

These sera, containing concentrations of antibody over the intended operating range, (also known as dynamic range) of the assay, should be used throughout the development and standardisation of an antibody assay. It is recommended that they be prepared in sufficient quantities so that they may be used in various aspects of validation. These samples should represent known infected and uninfected animals from the population that eventually will become the target of the validated assay. They should preferably be derived from individual animals, but they may represent pools of samples from several animals (Chapter 2.2.6).

2.1.4. Monoclonal antibody reagents

The advent of monoclonal antibodies has greatly enhanced enzyme immunoassays. Whereas polyclonal anti-immunoglobulin conjugates are used in most indirect ELISAs, monoclonal antibody conjugates can be directed to specific immunoglobulin isotypes. Depending on the immunoglobulin epitope targeted, many of these monoclonals can be effectively used to detect

antibodies in related species, e.g. ruminants. Using monoclonal conjugates to either light or heavy chain epitopes can effectively modulate the DSp and DSe of the indirect ELISA.

Monoclonal antibodies are best known for their application in competitive or blocking ELISAs. In this case, the monoclonal specificity is directed to epitopes on the pathogen in question. Depending on the epitope targeted, the analytical specificity of the assay can be modulated.

Critical points to be addressed:

- Have you considered that concentrations of analyte in matrix significantly impact the lower limit of antibody detection and the operating range of the assay?
- Are the required antibody reagents (mono/polyclonal) available?
- Is available antigen sufficiently purified?
- Are reagents commercially available? If not, is it practical to produce them in-house?
- Are reference standard reagents available? If not, how are you going to resolve this deficiency? (See WOAH Validation Standard, Section A.2.6.)

Monoclonal antibodies can also be used in sandwich ELISAs, either for trapping antigen to the plate or for subsequently detecting antigens that have been trapped. Depending on the size and complexity of the antigen in question, it is sometimes preferable to use a polyclonal antibody preparation for trapping as they generally contain antibodies of high binding affinity.

2.1.5. Antigens

Aspects affecting choice of test

- Is the assay to be used for screening or confirmatory purposes, or both?
- Will it be used for one or more species? Which ones?
- Is the test intended for detection of early or late infection?
- Will the test be used to measure serotype- or subtype-specific antibodies?
- Will the assay be used to confirm sero-conversion after vaccination?
- Will it be a DIVA assay (differentiation of infected from vaccinated animals)?
- Will the test be applied to trade?

Antigens used in ELISAs are of critical importance to diagnostic performance given a particular application. Antigens expressing highly conserved epitopes, such as those found in some viral matrix or nucleoproteins, are generally useful in group-specific assays, such as ELISAs for the detection of responses to all Influenza A viruses. Other antigen epitopes can be used to restrict detection to certain serotypes. The choice of antigen must be carefully researched and considered.

Crude antigen preparations like cell lysates have had widespread use in the past, and are still deployed for some assays. However, antigens improved greatly as purification techniques advanced, e.g. affinity chromatography. Further improvements were achieved through the application of molecular cloning. Recombinant antigen technologies have greatly enhanced all aspects of ELISA performance, from analytical through diagnostic characteristics.

2.2. Design of test method

In designing a test, its intended application will influence the choice of assay format that is best suited for the task. For example, if its use is primarily for surveillance, then the type of ELISA needs to be conducive to achieving high DSe, as described in the 'Purposes' above. If, however, the screening assay's DSe is set so high that it generates many false positives, then a companion confirmatory test should also be considered at the same time. Many ELISA formats are available, each with their advantages and disadvantages that allow customisation of assays for very specific purposes (Table 2).

Practical matters in selecting an assay format

- Is high-throughput essential? Will it be automated?
- What is the anticipated turnaround time? Is that suitable?
- What level of sophistication is needed to run the assay?
- What skills are required to interpret the test?
- Will that assay be feasible for use in my laboratory?
- Will it be easily transferrable to other laboratories?

Important factors that influence the choice of an antibody assay format are availability of reagents and likely continuity of supply, not only for the design and optimisation stage but for operational scale application of the test. A limitation may be the unavailability of relevant antibody reagents for a particular format, e.g. competitive or blocking formats generally require antigen-specific monoclonal antibodies. Another example would be the need for an effective capturing antigen: a rather crude antigen may be acceptable for use in a sandwich-based ELISA screening assay, whereas a purified antigen would be necessary for a confirmatory assay. Other important considerations for choosing a particular ELISA format are which antibody isotypes, concentrations, avidities and antigenic specificities are diagnostically relevant; which antigen, and in particular which epitopes are relevant; and what is the desired operating range of the assay. All will play a large role in selecting a particular type of ELISA (Table 2). If it is anticipated that the test will be used in different species, including wildlife, a competitive/blocking format may be useful. Deciding on an assay format also requires that application of the assay be considered. Questions that should be addressed are detailed in the box above on "*Practical matters in selecting an assay format*" and practical questions in the boxes below Table 2. It is essential to deal with such questions at this point in assay development as they are essential to a positive outcome and application.

Table 2. ELISA formats: advantages and disadvantages*

Type of ELISA	*Advantage*	*Disadvantage*
Indirect – bound Ab detected by anti-species conjugate or by Protein A/G conjugates	Use and availability of high variety of antispecies-specific conjugates often targeting particular antibody subsets, such as anti IgM, IgG1, IgG2, etc. Protein A and Protein G conjugates have a wide species specificity and may give lower background signals than anti-Ig reagents. Wide use for screening large numbers of samples	Variation in degree of nonspecific binding in individual sera To compensate for this problem high starting dilutions are required This can lead to a decrease in DSe in comparison to competitive/blocking formats Can only be used for one or a few species at a time
Sandwich – Ag presented on a solid-bound-phase capture antibody	The capture antibody on the solid phase can help to orient the antigenic molecule, which improves the chance that the sample antibody will bind. Unpurified antigen preparations can be used because capture antibody selectively binds crude antigen. Pre-coating with capture antibody can reduce the potential for subsequent binding of nonspecific proteins during the test.	Antigens must have at least two antigenic sites or epitopes which limits this type to relatively large antigenic complexes or more complex proteins Size and spatial relationship of epitopes can affect the assay
Competition (indirect and sandwich types) – Test antibody in sample mixed with pre-titrated detection antibody, then added to wells coated with capture antigen, either in direct or inhibition/blocking format.	Easy adaptation for use as antibody detection tests When highly specific MAbs are used the antigen does not have to be highly purified Can be used in different species for which no conjugated antibodies exist Advantage of competitive/blocking sandwich type relies on antigen capture Sera can be tested in low dilutions without risk of interference due to non-specific antibodies binding. This may contribute to a higher sensitivity of this format Different antibody concentrations can be used to favour either analytical sensitivity or specificity. This is particularly relevant for assays using polyclonal antibodies which are much more affected through the use of different dilutions of sera	Generally more steps and more optimisation may be needed, e.g. pre-titration and optimisation for liquid and solid phase reagents. Higher level of technical sophistication required

*Primary source is Crowther (2001).

2.3. Proof of concept experiments (feasibility studies)

After choice of an ELISA format, initial experiments are designed to determine if the proposed assay is viable. A reference panel such as described in Section A.2.1.3 should be tested in the prototype assay. If a reference standard is to be used for normalisation of test data, it should be selected and incorporated at this point in assay development. To provide continuity in data assessment throughout, both the reference panel and any reference standards should be included in all remaining aspects of the validation studies. The reference panel used in the feasibility study should span the entire anticipated operating range of the candidate assay and be run in replicates as a quick check for repeatability.

Proof of concept

- Was the feasibility study conducted with at least 4 to 5 samples spanning the operating range of the assay?
- Did you include one or more reference standards if required for data normalisation?
- Was separation of results between negative, low positive and high positive samples adequate?

The assay should achieve good separation in OD values, spanning the operating range of antibody activity. Adequate separation is particularly important between the negative and low positive samples. The lower OD range should be 0.1 or less for the negative control in indirect ELISAs, or for the strong positive control in competitive/blocking ELISAs. OD values at the upper end of the operating range should not exceed 2.0, as above this value plate readers become rather inaccurate. If the assay appears promising, optimisation is the next step.

2.4. Samples and data expression

2.4.1. Preparation and storage serum panels for optimisation studies

A best practice for antibody assays to select several (a minimum of four to five) serum samples that range from negative to high levels of antibodies against the infectious agent in question. These samples are initially used in experiments designed to demonstrate proof of concept. A large volume (e.g. a minimum of 10 ml) of each serum sample is acquired and divided it into 0.1 ml aliquots for storage at or below –20°C. One aliquot of each sample is thawed, used for experiments, and ideally then discarded. If it is impractical to discard the aliquot, it may be held at 4°C between experiments for up to about 2 weeks; however, there is a possibility of sample deterioration under these circumstances. Then, another aliquot is thawed for further experimentation. This method provides the same source of serum with the same number of freeze–thaw cycles for all experiments (repeated freezing and thawing of serum can denature antibodies so should be avoided). Also, variation is reduced when the experimenter uses the same source of serum for all experiments rather than switching among various sera between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples.

After the initial stages of assay validation are completed, one or more of the samples may be suitable as a reference standard for data expression and the entire panel may be used for repeatability assessments both within and between runs of the assay (Jacobson, 1998). They may also serve as in-house working standards, i.e. quality or process controls given that their reactivity has been well characterised; such controls provide assurance that runs of the assay are producing accurate data (Wright *et al.,* 1993).

2.4.2. Normalisation of results and their expression

An optical density (OD) reading in ELISA is a measurement of colour development that is a function the amount of antibody present in a sample. Because colour development is a function of a reaction of enzyme and substrate in the presence of a chromogen, results from day to day are subject to variation attributable to external factors such as temperature, reaction time, etc. Comparison of OD results for the same samples between runs of an assay in the same laboratory, or between laboratories, lacks precision because of variation in results of reference standards included in each run of the assay. Therefore, OD results of test samples need to be adjusted as a function of the OD(s) of one or more reference standards in a specific assay run. This process is known as “normalisation” of ELISA results (see the WOAH Validation Standard, Section A.2.7 for

details). The method of normalisation and expression of data should be determined, preferably no later than at the end of the feasibility studies.

OD values may be expressed in several ways (Wright *et al.,* 1993). A simple method is to express all OD values as a percentage of a single high-positive serum control that is included on each plate. For such calculations, this control must yield results that are in the linear segment of the operating range of the assay. A more rigorous normalisation procedure is to calculate results from a standard curve generated by plotting observed OD values against concentration (or dilution) of antibody for several serum controls that span the range of antibody activity of the assay. It requires a more sophisticated algorithm, such as linear regression, log-logit, or 4 or 5 parameter logistic regression analysis, among others. This approach is more precise because it does not rely on only one high-positive control sample for data normalisation, but rather uses several serum controls, adjusted to expected values, to plot a standard curve from which the sample value is extrapolated. This method also allows for exclusion of a control value that may fall outside expected confidence limits.

2.5. Optimisation

For ELISAs, the most important variables that need to be optimised are concentration/dilution of antigen adsorbed to the solid phase, test serum working dilution, enzyme molarity, antibody-conjugate dilution, and substrate solution concentration. These are evaluated through checkerboard assessments (each variable compared against all other variables within one run of an assay that is repeated several times). Other variables that need consideration are pH and ionicity of reagents, molecular factors such as valency and epitope density of antigens, isotype of targeted antibody and antibody affinity. Precision of test results can be graphically depicted or expressed numerically by various statistical methods (Crowther, 2001). ELISA studies require that instrumentation (plate washers and readers, etc.) must be properly calibrated prior to use – part of the laboratory's quality control programme.

Optimisation and standardisation

- Have all critical reagents been tested against each other in checker board titrations?
- Did you find optimal concentration/dilutions for each reagent?
- Did you incorporate quality or process control procedures and reagents?
- Did you incorporate methods for normalisation of test data?

2.6. Inhibitory factors in sample matrix

Although ELISA antibody detection systems are rather resistant to inhibitory factors, the WOAH Validation Standard, Section A.2.4, and Greiner *et al.* (1997) provide descriptions of the type of inhibitors that could affect the assay. These references should be reviewed carefully to assure that all inhibitory factors are accounted for and controlled.

2.7. Calibration to reference standard sera

If international, national, or other-source reference sera are available, the assay should be calibrated to match the analytical sensitivity in terms of the metrological units ascribed to the calibration sera (Wright, 1998).

B. ASSAY VALIDATION PATHWAY

1. Stage 1 – Analytical performance characteristics

1.1. Repeatability

Analytical performance characteristics

- Has repeatability been established for a range of positive and negative samples within and between runs of the assay
- Have upper and lower control limits of the assay been established
- Have you defined ASe and ASp for this assay?
- Does the candidate assay compare favourably with a standard test method, based on objective quantitative and qualitative criteria?

Repeatability is the level of agreement between results of replicates of a sample, both within and between runs of the same method in one laboratory. The same or similar panel of samples used in the feasibility study is adequate. No less than three (preferably 5) samples covering the operating range of the assay, and of sufficient quantity for at least 20 runs of the assay over several days. Specifics of how the samples should be prepared and handled are provided in Chapter 2.2.6 and in the WOAH Validation Standard, Section B.1.1. It is valuable to include at least one reference sample in an indirect ELISA (a positive serum control) to which the test samples can be normalised by per cent of the positive control. The within run variation can be determined by the mean OD and coefficient of variation (CV) of the replicates of each sample. The CV should not exceed about 15% (with the possible exception of negative and very low positive samples which may have higher (and meaningless) CVs). If all of the samples have previously been calibrated to reference standards, and their expected ODs are thus known, the observed ODs for each sample in each run can normalised as a function of their expected ODs in linear regression analysis. This provides a correlation coefficient as evidence of closeness of fit to the expected value, and allows for normalised values to be plotted in control charts (Crowther, 2001).

1.2. Analytical specificity

Analytical specificity (ASp) is determined by testing sera from animals that are known to have been infected/exposed to all species/strains that the test should detect (Chapter 2.2.6, Section B.1). Cross reactivity with sera from animals infected with related species is used to evaluate the ASp. ELISAs are also subject to false positive results attributable to exogenous factors, such as nonspecific binding of serum or conjugate to the plastic surface that may require use of blocking agents. Care must be taken to eliminate this source of error. Blocking and competitive ELISAs may also suffer specificity problems due to stearic hindrance preventing proteins binding to their target sites.

1.3. Analytical sensitivity

Analytical sensitivity (ASe) is synonymous with the lower limit of detection (LOD) of antibody concentration in a sample. The different types of antibody assays vary considerably in their inherent limit in antibody detection. For instance, LODs for eight different types of antibody assays range from 1000 ng/ml (radial immunodiffusion) to 0.01 ng/ml (chemiluminescence) (Nielsen *et al.,* 1996). LODs are usually determined by endpoint dilution in which replicates (preferably 10) of each dilution in a $\log_2$ dilution series are run in the assay.

1.4. Standard test method comparison with the candidate test method

The candidate test method should be run in parallel with an WOAH or other accepted reference test method, using the same panel of samples on both, to determine whether the candidate method exhibits the same quantitative and qualitative characteristics as the standard method. Favourable comparability lends strength to the belief that candidate method will be a successful substitute for the reference method (see also methods comparison studies, Chapter 2.2.5).

2. Stage 2 – Diagnostic performance characteristics

DSe and DSp are the primary performance indicators of the validation process. Antibody assays are subject to the same general procedures to achieve estimates of DSe and DSp as required of all other assay types (see the WOAH Validation Standard, Section B.2 for essential details). The number of samples needed to establish these estimates for a particular antibody assay require a sampling design that considers many variables. This includes creation of a sample panel that is tailored particularly for the intended purpose of the assay (e.g. a screening versus confirmatory test). It also requires predetermined desired levels of DSe and DSp (indicating acceptable levels of false negative and false positive results), allowable error in the estimates of such DSe and DSp, and the confidence level required for these estimates.

The number of animals required to establish acceptable DSe and DSp estimates is a function of the level of confidence desired in DSe and DSp estimates and the accepted allowable error. For instance, for a pathogenic disease like FMD, it is necessary to reduce the likelihood that infected animals will be misclassified as uninfected, which reduces allowable error in the test result which, in turn, increases the number of samples needed to establish a high level of confidence in the DSe estimates. Alternatively, for a confirmatory assay it is desirable to reduce the likelihood that uninfected animals will be classified as infected. A high DSp is then desired with minimal allowable error, requiring a larger sample size of uninfected animals. All of these general issues related to sample size, confidence intervals and allowable error in the DSe and DSp estimates are described in the WOAH Validation Standard, Section B.2, with additional detail and tables of sample numbers required available elsewhere (Jacobson, 1998).

It is often challenging to obtain a sufficient number of well characterised sera to achieve estimates of DSe and DSp that are sufficient for the intended purpose of the assay. Initially, it may be a compromise between what is statistically meaningful and practically feasible, resulting in an assay that is provisionally recognised (WOAH Validation Standard, Section B.2.6). However, over time, with accumulation of more well characterised samples, the estimates of DSe and DSp may be strengthened (see Section 5.4 below).

2.1. The challenge in establishing accurate estimates of DSe and DSp for antibody assays

Antibody assays undergoing validation pose unique problems when attempting to assemble known positive and known negative samples in sufficient quantity to establish assay performance characteristics. Antibody is an indirect indicator of the presence of, or prior exposure to, an infectious agent or its components. Inferences from detection of antibody (or the lack thereof) depend on the host's qualitative and quantitative responses to the organism. Factors that affect the concentration and composition of specific antibody in serum samples are inherent to the host (e.g. age, sex, breed, nutritional status, pregnancy, immunological responsiveness) or acquired (e.g. passively acquired antibody, or active immunity elicited by vaccination or infection). Theoretically, samples from animals that represent all of these variables should be included in the panels used for establishing DSe and DSp estimates. Clearly, this becomes a daunting, if not impossible task. To surmount this problem, the initial sample panels should be representative of the majority of animals in the target population to achieve initial estimates of DSe and DSp. In reality, it is necessary to enhance DSe and DSp estimates after the assay has been implemented as more well characterised samples become available (see Section 5.4, below).

Diagnostic performance characteristics

- Are the criteria used to determine the positive and negative reference populations legitimate?
- Do the reference samples fully represent the population targeted by the assay?
- Were there difficulties in obtaining a sufficient number of samples? If so, how was the problem addressed?

Because it is often desirable to stretch the application of antibody detection assays to a huge number of animals spanning large geographical areas (e.g. as in screening assays for an entire continent), assembly of fully representative sample panels for such a large diagnostic window of variables may be nearly impossible. A useful alternative is to first establish DSe and DSp estimates for a rather homogeneous population of animals. If the assay is destined for use in disparate populations of animals, which may harbour a different infectious agent profile (with possibility of cross reactions not seen in the original targeted population), a reassessment of DSe and DSp may be necessary, drawing from data acquired using new sample panels that are representative of the population(s) targeted.

2.2. Reference animal populations

2.2.1. Animals of "known infection status"

Reference animals of known infected and known uninfected status are the ideal source of samples for determining DSe and DSp. However, such samples are rare and difficult to establish. The most familiar term for reference animals or samples used in establishing DSe and DSp is the so called "gold standard", a misnomer commonly used to classify almost any reference animal as infected/exposed or uninfected, with samples from such animals classified as positive or negative (see the WOAH Validation Standard, Section B.2.1–2.3).

Assay developers should be aware of the advantages, and particularly the pitfalls, associated with various methods that are used to classify reference animals as infected or uninfected. The samples from such animals are deemed either positive or negative, and collectively become the *reference standard* upon which the candidate assay's DSe and DSp are based. It is, therefore, crucial to carefully consider the validity of various reference standards as exemplified in the following four examples:

i) An unequivocal reference standard: presence of the agent in the host or evidence of definitive (pathognomonic) histopathology

If an infectious agent or definitive histopathological criterion is detected in an animal, this generally constitutes an unequivocal reference standard for that animal. Serum samples derived from such animals usually are considered to be unequivocal serum reference standards for determining DSe and DSp of the candidate assay. However, such samples may have their limitations. At the population level, a pathogen may be unequivocally present in some animals, but if the serum sample was taken from the animal early in the infection process, the immune response may not yet have produced detectable antibody. In this case, such serum samples used as reference standards would have been FN for the subset of animals in an early stage of infection. In contrast, for more chronic types of infection, using only reference animals that have confirmatory culture or histopathology may produce higher estimates of DSe than are realistic for the population targeted by the assay because the immune response will always be well established.

ii) A composite reference standard: verification of uninfected or unexposed animals

This standard is achieved by selecting reference animals from geographical areas where herd histories, clinical profiles, prior testing results and other parameters provide evidence suggesting the absence of the pathogen, and thus no specific host antibody response to the pathogen targeted by the candidate assay. These types of reference materials, their strengths and limitations are described elsewhere (Jacobson, 1998), and must be considered carefully when using samples from such sources for establishing DSe and DSp for a candidate assay.

iii) A relative reference standard: comparative serology

This standard is characterised by reference animals that have been classified for their infection status by comparison with the test results of another serological assay on the same samples. It often is the only practical source of reference material available for evaluation of a new serological test. If results of such a reference test are chosen as the standard for determining diagnostic performance characteristics of the candidate assay, the resultant estimates of DSe and DSp are useful only insofar as the reference test has documentable, established and acceptable performance characteristics. A deficiency of relative reference standards is that they have their own established levels of FP and FN test results, which are sources of error that will be compounded in estimates of DSe and DSp for the new assay. Generally, however, the use of other well described test methods is regarded as good practice to determine the status of reference animals, but only if the inherent bias introduced by the relative reference standard is accounted for.

iv) *An adjunct reference standard*: experimental infection or vaccination

(See the WOAH Validation Standard, Section B.2.3 for significant limitations of this type of standard.) In some cases, the only way to obtain positive samples is by experimental

infection. This approach is highly suitable to model the dynamics of the infection and to determine the 'diagnostic window' with the new assay. For example, it is possible to get estimates of the time interval between exposure to a pathogen and when antibody is first detectable, or when 25, 50, 75 and 100% of the infected animals return a positive test result. Nevertheless there are pitfalls in use of time-series data that must be avoided. Data representing repeated observations from the same animals cannot be used in calculation of DSe and DSe because the statistical models used to establish DSe and DSp require independent observations (only one sample from each anima). For statistically legitimate time-course studies, or when single samples are used from each of many experimental animals, the strain of cultured organism, route and dose of exposure, infection with other related, cross-reactive and non-related, non-cross-reactive organisms are variables which may produce quantitatively and qualitatively atypical responses which are not found in natural infections in the target population. Experimental conditions typically lead to an overestimation of sensitivity and specificity for example by artificially high challenge doses and by using specific pathogen free animals as negative controls.

The time point of sample collection (days post-infection) must be indicated. Sources and history of experimental animals should be described. The validation should not be based solely on experimental animals as they do not represent natural populations of animals subject to pathogens by natural exposure.

2.2.2. Latent-class models for estimation of DSe and DSp

For a discussion of this approach for estimation of diagnostic performance, see the WOAH Validation Standard, Section B.2.5 and Chapter 2.2.5.

2.3. Threshold (cut-off) determination

The procedures for establishing the cut-off between negative and positive results of antibody assays are as described in the WOAH Validation Standard, Section B.2.4.

3. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility is the measure of precision of an assay when used in several laboratories located in distinct regions or countries using the identical assay (protocol, reagents and controls) to test the same panel of samples. Reproducibility assessments for antibody assays are not uniquely different from similar assessments for any other type of assay. Therefore the reader is directed to the WOAH Validation Standard, Section 3, for details on reproducibility analysis and for reference samples and panels to Chapter 2.2.6.

4. Stage 4 – Programme implementation

4.1. Interpretation of results and determination of predictive values

Best practices for programme implementation are general to all assay types (WOAH Validation Standard, Section B.4). However, as ELISA is often the assay of choice for surveillance programs to affirm absence of disease, or for eradication of disease or elimination of infection from defined populations, the issue of false positive results can be a significant problem even if the diagnostic specificity is very high.

A common misperception is that a test with 99% DSp and DSe will only mis-classify animals as FP or FN 1% of the time. The FN and FP rates vary depending on the prevalence of infection in the targeted population. False positive reactions in a disease eradication campaign can vary significantly from the beginning of the campaign when prevalence is relatively high (for example, 10%) to near the end of the campaign when it has decreased to 0.1%. The predictive values of test results then become very important. Predictive values are probabilities that a test result is truly positive or truly negative. In our example using an assay with 99% DSe and DSp for testing a population of animals with a 10% prevalence of disease, the predictive value of a positive test result (PPV) is 91.7%, meaning that there is a 91.7% probability that the animal is truly infected. The predictive value of a negative test result (NPV) is 99.9. When the prevalence drops to 5%, the PPV and NPV are 83.9% and 99.9%, respectively. However, if the prevalence drops further to 0.1%, by successfully removing infected animals from the population, the same test will produce a PPV of 9% and a NPV of 99.9%, meaning that there is only a 9% chance that a

positive test result is detecting a truly infected animal (of 1000 animals tested, only about 1 in 10 positive test results is indicative of an infected animal – the other 9 are false positive). So, if the test is intended for the purpose of eradication of a disease or elimination of infection from a population, the test developer is advised to consider moving the assay to a second cut-off that yields a higher DSp late in the campaign to reduce the probability of false positive reactions. It is instructive to examine a predictive value chart for assays of varying DSe and DSp, to visualise the effects of reduced prevalence on predictive values of an assay (WOAH Validation Standard, Table 2, and Jacobson, 1998).

5. Monitoring assay performance

5.1. Monitoring the assay

Once the assay is in routine use, internal quality control is accomplished by consistently monitoring the assay using quality control charts for assessment of repeatability and accuracy. Charts representing at least 30 runs will reveal trends or shifts in values of controls and standards. Lines representing the mean value of a control sample in at least 30 runs, plus/minus 3 standard deviations, are useful decision criteria for inclusion or exclusion of a run of the assay. The run is rejected if one control/standard exceeds ± 3 standard deviations (STD) or if 2 controls (or more) exceed ± 2 STD (Crowther, 2001). Decision criteria may need to be customised for a given assay because of inherent differences between assays attributable to the host pathogen system. Chapter 2.2.4 provides an example of how to apply measurement uncertainty for an antibody ELISA using a positive internal control sample.

Reproducibility of test results between laboratories should be assessed by External Quality Assurance at least once per year and is an essential requirement of ISO 17025 accredited laboratories. Membership in a consortium of laboratories that are interested in evaluating their output is valuable.

5.2. Minor modifications of the assay – replacement of depleted reagents

When quality or process control samples are nearing depletion, it is essential to prepare and repeatedly test the replacement samples. The replacement samples should be included in at least 10 routine runs of the assay, with their results normalised against the existing reference standard. The activity of the replacement control should be comparable to the replaced control. If the reference standard requires replacement, care must be taken to select a replacement that matches all of the original serum characteristics as closely as possible, thus allowing use of the replacement to normalise test results with comparable outcomes (see also Chapter 2.2.8 *Comparability of assays after changes in a validated test method*).

When other reagents such as antigen for capture of antibody, must be replaced they should be produced or procured using the same protocols or criteria as used for the original reagents. They need to be assessed using sera from routine submissions in 5–10 parallel runs that include the current and the new reagent(s). A panel of representative samples, such as a proficiency panel, is also a useful tool for assessing the comparability of the reagents (Chapter 2.2.6).

Monitoring assay performance

- Has the purpose of the assay changed?
- Has the epidemiology of the disease in question changed, e.g. prevalence, new serotypes or strains, etc.?
- Have critical reagents been changed, and if so, was comparability of the new reagents assessed?
- Are performance indicators included in day to day use of the assay (control charts, basic statistics)?
- Are upper and lower limits in control charts updated periodically as more experience with the control samples is achieved?
- Are test panels shared with other laboratories to assess reproducibility?
- Is proficiency testing included as part of continuing evaluation of the assay?

5.3. Major modifications of the assay – changing to a new ELISA type

If the assay is to be changed from, say, a sandwich ELISA to a competitive/blocking format, the assay will require revalidation because of the many variables that may affect the performance characteristics of the assay. For an assay considered for implementation in another geographic region, e.g. from the northern to the southern hemisphere, it is essential to revalidate the assay by subjecting it to sera from populations of animals that reside under local conditions. Evaluation of reference sera that represent those populations is done by using stages 3–5 in Figure 1 in the WOAH Validation Standard. It is the only way to assure that the assay is valid for populations that are of different composition compared with the original population targeted by the assay.

5.4. Enhancing confidence in validation criteria

Due to the extensive set of variables that have an impact on the performance of serodiagnostic assays, it is useful to expand the number of reference sera wherever possible, recognising the principle that error is reduced with increasing sample size. An expanded reference serum bank should be accumulated with well characterised sera, and used periodically to update estimates for DSe and DSp for the population targeted by the assay.

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CHAPTER 2.2.2.

DEVELOPMENT AND OPTIMISATION OF ANTIGEN DETECTION ASSAYS

INTRODUCTION

The WOAH Validation Recommendations in Section 2.2 Validation of diagnostic tests *of this* Terrestrial Manual *provide detailed information and examples in support of the WOAH Validation Standard that is published as Chapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals. *The Term "WOAH Validation Standard" in this chapter should be taken as referring to that chapter.*

The detection and identification of an agent is confirmatory evidence of either infection with or disease caused by a particular pathogen. There are many different direct and indirect test methodologies available. Classical direct[1] agent detection assays include electron microscopy, light microscopy (*e.g. observation of unique histopathological or pathognomonic features, identification of parasites* in situ*, etc.*)*, virus isolation, bacterial culture and parasitic digestion techniques. Many direct techniques require secondary procedures to assist in the characterisation and identification of these agents* (*e.g. haemagglutination inhibition or virus neutralisation tests, special bacteria or fungal stains, etc.*)*. Representatives of indirect agent and in particular, antigen* (*Ag*) *detection tests include enzyme linked immunosorbent assays* (*ELISA*)*, immunofluorescence or immunoperoxidase assays, Western blotting techniques, micro-arrays, fluorescence activated cell sorting* (*FACS*) *and biosensors. Often direct and indirect methods are used in tandem, for example a direct technique may be used to isolate, enrich and/or extract the organism followed by indirect techniques to characterise and identify the agent.*

Practical matters in selecting an assay format

- Is high-throughput essential? Will the test be automated?
- Will the test be used as a herd test or for testing individuals?
- What is the anticipated turnaround time? Is that suitable?
- What level of sophistication is needed to run the assay?
- What skills are required to interpret test results?
- Will the assay be feasible for use in my laboratory?
- Will it be easily transferrable to other laboratories?

The examples given above vary widely in terms of laboratory requirements. Pen-side tests (*including lateral flow devices*) *may also be antigen detection tests. They are easy to use and were developed for testing of animals in the field but may have applications in laboratories. Cost, laboratory infrastructure, biocontainment/safety, technical sophistication, interpretation skills, turn-around time, throughput capacity, diagnostic performance characteristics, repeatability and reproducibility are important parameters which need consideration when selecting the most appropriate assay. They also vary with respect to suitability for different diagnostic applications.*

1 Definitions: In the context of direct and indirect detection of an analyte, the term "direct" is relevant to the organism or its antigens, e.g. direct detection by microscopy. The term "indirect" is relevant to the host response to the organism, (e.g., indirect detection inferred by detection of antibodies to the organism). In the context of diagnostic methods that are used to detect infection, microscopy is a means of direct observation of the organism, whereas using a reagent that infers the presence of the agent (such as an enzyme conjugated to a purified antibody that is specific for the agent) is an indirect method.

In contrast to serological or antibody detection tests, Ag detection tests depend heavily on the time of clinical onset and pathogenesis of the disease and the concentration of the pathogen in certain tissues and/or fluids. Successful diagnosis depends on appropriate timing and selection of the sampling site (affected tissue/lesions, scrapings, swabs, blood, and other body fluids), storage conditions *and specimen integrity during transport. For certain applications, the testing of individual animals and/or samples may be appropriate (e.g. confirmatory testing); while for other purposes (e.g. screening), pooling of animals may be efficient and effective. Selection of the appropriate specimen requires good understanding of the disease and the effect of the sample matrix on the pathogen (e.g. cloacal or tracheal swabs for avian influenza).*

Nucleic acid detection (NAD) tests are increasingly replacing classical antigen detection systems. For development and optimisation of NADs, please refer to Chapter 2.2.3. Although these NAD tests may seem to be the tests of choice for many applications, they are not always the most practical or efficient. In most cases it is still necessary and prudent, at least for an index case, to culture the agent on selective media or in susceptible cell lines or eggs to facilitate further characterisation and identification. While genotyping is an important consideration, especially in molecular epidemiology, other means of agent characterisation such as serotyping, pathotyping or biotyping are also important. Cultured and preserved agents have tremendous historical value and are also an important source of reference materials.

Due to its worldwide application, the Ag ELISA is used as an example for application of best practices in antigen detection assays in this chapter. Most of the basic processes used to validate other types of antigen detection assays will become evident by extension of those used to validate ELISAs. Because of the many conceptual similarities between antigen and antibody detection assays this chapter frequently cross-refers to Chapter 2.2.1 on antibody detection.

A. ANTIGEN DETECTION ASSAY DEVELOPMENT PATHWAY

1. Intended purpose(s) of the antigen assay

The intended purpose of the test is a key factor which will guide decisions in the selection and early development of the candidate assay. Given the WOAH-defined 'fitness for purpose' categories in Table 1, Ag detection systems may be appropriate for certain applications. Support for disease eradication or surveillance programmes generally require testing of high numbers of samples, with an emphasis on diagnostic sensitivity and throughput capacity. In contrast, confirmation of clinical cases does not entail high numbers to be tested, but diagnostic specificity and turn-around-times become very important. At the outset, the questions posed in the text box, above, should be carefully considered.

Table 1. Determinants of an antigen assay's fitness for its intended purpose

Assay characteristics	*Determinants of fitness for purpose*						
	*1**		*2**	*3**	*4**	*5**	*6**
	a	b					
Diagnostic sensitivity (DSe)		+++	+++	+++	+++		
Diagnostic specificity (DSp)		+	+	+	+++		
Positive predictive value (PPV)		+	+	+	+++		
Negative predictive value (NPV)		+++	+++	+++	+++		
Throughput capacity		+++	++	+	–		
Turn-around time of test		+	+	+	+++		

Assay characteristics	*Determinants of fitness for purpose*						
	*1**		*2**	*3**	*4**	*5**	*6**
	a	b					
QA capability		+++	+++	+++	+++		
Reproducibility		+++	+++	+++	+++		
Repeatability		+++	+++	+++	+++		

Other characteristics such as the technical sophistication of the assay, and the skill required for interpretation will be related to the disease or infection under investigation.
NB NAD tests may also be used for agent detection, and are considered in Chapter 2.2.3.
Symbols: +++ = essential; + = of less importance; – = not important.
*Basic purposes for which an assay may be deemed fit: 1. Contribute to the demonstration of freedom from infection in a defined population. 2. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes; 3. Contribute to the eradication of disease or elimination of infection from defined populations; 4. Confirmatory diagnosis of clinical cases (includes confirmation of positive screening test); 5. Estimate prevalence of infection or exposure to facilitate risk analysis; 6. Determine immune status in individual animals or populations (post-vaccination)

1.1. Purpose 1

For disease freedom categories as given in purpose (Table 1), antibody screening tests of high diagnostic sensitivity (DSe) are usually the tests of choice provided that a detectable immune response is a significant indicator of infection. However, there may be certain disease situations where the humoral immune response can be misleading and pathogen detection may be more appropriate (e.g. mycobacterial or trypanosomal infections). In these cases, Ag detection tests may be more effective. The antigen screening test should demonstrate a high DSe. Tests of high DSe demonstrate low false negative (FN) rates and when applied to low prevalence populations, the NPV is at its highest level. As DSe and diagnostic specificity (DSp) are usually inversely related, a decrease in DSp will result in an elevated false positive (FP) rate. Therefore, all screening test positive results should be subjected to some form of confirmatory testing to evaluate their true status. Confirmatory tests characteristically have high DSp and therefore a low FP rate. These tests are often more sophisticated and require enhanced interpretive skills.

1.2. Purpose 2

For a number of diseases included in the *Terrestrial Manual*, agent identification is listed as the preferred test method for the purpose of determining "Individual animal freedom from infection prior to movement". Although in many cases this involves culture of the organism or detection of nucleic acid, there may be situations where an antigen detection test is appropriate for this purpose. To avoid risk of disease spread through trade, a test offering high DSe is to be preferred.

1.3. Purpose 3

If the purpose of the test is the eradication of disease or elimination of infection from defined populations, antigen screening tests, like antibody screening tests, of moderate to high DSe, are the tests of choice. However, the rationale is slightly different in that the testing will likely be done at the herd or compartment level. At the beginning of the eradication campaign, when the disease prevalence is high, moderate DSe and DSp are suitable as both FP and FN rates are less relevant at this juncture and a moderate level of error is tolerable. Depending on the nature of the disease and rapidity of spread, high throughput and fast turn-around-times may become critical. Usually decisions are made without confirmatory testing at this point.

In the latter stages of the campaign, a higher DSe is warranted as the FN rate becomes the more critical factor. Much like Purpose 1, positive reactors will need to be subjected to some form of confirmatory testing to evaluate their true status. In these latter stages, antigen and/or nucleic acid detection systems are critical in the detection of sub-clinical cases, shedders and possibly, latent carriers.

1.4. Purpose 4

Although antibody tests of high DSp are often the tests of choice for confirmatory diagnosis of clinical cases, antibody tests may not always be the tests of choice, especially if clinical signs appear before an immune response is mounted. A prime example would be highly pathogenic avian influenza infections where mortality may occur before an immune response is even detectable. Antigen and/or nucleic acid detection tests are usually a better choice for confirmation of clinical cases provided that they offer a fast turn-around-time. In these cases, the idea is to maximise DSp and thereby minimise any potential FP reactions. For some clinical cases, e.g. vesicular diseases in terrestrial animals, several tests may be required to quickly rule out select pathogens that present similar clinical signs. In this category of test, fast turn-around-times are extremely critical in identifying potential outbreaks.

1.5. Purpose 5

For estimates of prevalence of infection and/or shedding to facilitate risk analysis, e.g. for health surveys, herd health status and to monitor disease control measures, antigen detection tests of moderate DSe and DSp are the tests of choice. In general, this would balance both FN and FP rates and result in a more accurate estimate of the true prevalence in the target population. However, if accurate estimates of both DSe and DSp have been established, statistical approaches can be used to minimise bias attributable to FN and FP rates (see Chapter 2.2.5 *Statistical approaches to validation*).

1.6. Purpose 6

Not applicable to antigen detection assays.

2. Assay development – experimentation

2.1. Reference materials, reagents and controls

2.1.1. Test samples

Samples required for antigen detection assays should be handled as described in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens*. Sample matrixes for antigen detection assays can be very heterogeneous (e.g. blood, faeces, milk, skin, semen, saliva, blisters, vesicles or swabs from affected tissues such as oropharynx (Probang), trachea, genitals, cloaca, oesophagus, etc.). The ideal specimen is the one that is easy to obtain and with a high concentration of the analyte. In many cases blood or swabs are the specimens of choice but depending on the pathogen, other tissues or fluids are needed, e.g. skin, organs such as brain (rabies, transmissible spongiform encephalopathies [TSE]), lymphatic organs, such as spleen and lymph nodes (classical swine fever), kidney, liver, heart, parts of respiratory tract (avian influenza), digestive tract (parvovirus), milk, faeces, semen, saliva, tumour material (enzootic bovine leukosis), etc.

As stated in Chapter 1.1.2, the usual considerations apply to limit bacterial and fungal contamination of specimens. The use of preservatives and fixatives is not usually recommended and samples should be sent with minimal delay and under refrigeration to a diagnostic laboratory. During transport and storage it is important to be aware of the physical and chemical requirements of the pathogen (e.g. foot and mouth disease [FMD] virus is highly labile at low pH and requires equal amounts of glycerol and phosphate buffer to maintain a pH over 7).

If samples are to be tested as pools, experiments need to be undertaken to demonstrate that the assay is fit for that purpose (e.g. that the analytical sensitivity is sufficiently high to detect one infected animal in a pool of 5, 10, 50 or more samples from uninfected animals).

2.1.2. Reference Standards

See Chapters 2.2.1 *Development and optimisation of antibody detection assays* and 2.2.6 *Selection and use of reference samples and panels*.

2.1.3. Positive and negative reference panels

Samples, containing concentrations of antigen over the intended operating range of the assay, should be used as controls during the development and standardisation of an antigen detection assay. They may be obtained from field specimens or produced in the laboratory as spiked samples (see Chapter 2.2.6). Negative samples should be obtained from known uninfected animals and this same matrix should be used when spiked samples are produced.

2.1.4. Purified and crude antigens for antibody production

In general, antigens to be used for the production of immunological reagents should be as natural as possible in terms of conformation to ensure that the presentation of epitopes mimics the orientation on the live organism. Therefore, isolation and/or purification methods used should preserve the antigenic integrity of the agent as much as possible.

For very large pathogens such as poxviruses, bacteria and protozoal parasites, protein microarrays may be useful to identify and select antigens which elicit strong immune responses.

2.1.5. Monoclonal and polyclonal antibodies for indirect antigen detection assays

Monoclonal antibodies demonstrate unique analytical specificities and are very useful in the detection of agent-specific epitopes at the group, strain or sub-strain levels. As such, they need to be considered carefully with respect to the application at hand and the desired specificity of the assay. Polyclonal antibodies, by nature, tend to demonstrate a broader range of specificities. Purified or semi-purified polyclonal antibodies are often the reagent of choice for trapping complex antigens because they usually demonstrate higher affinities than their monoclonal counterparts.

2.2. Design of test method

2.2.1. Choice of test

The prologue to a proper test design requires careful consideration of many variables in the context of performance requirements. The choice of assay must be coupled with its intended application, which usually necessitates consensus between the assay developer, statisticians, and other stakeholders such as epidemiologists and regulatory bodies. If the purpose is to develop a screening test (e.g. during a post-outbreak surveillance period) the emphasis will be on high DSe, high throughput, low cost, technical simplicity, low interpretative skill, etc. If the purpose is to develop a confirmatory test (e.g. for the confirmation of clinical cases or confirmation of positive screening test reactors), a different set of priorities will come into play including high DSp, fast turn-around-times, technical sophistication and interpretative skills. There are a growing number of point-of-care or pen-side tests that have their own set of additional robustness and ruggedness requirements given the variable conditions of the environment in which they will be used and the skill level of the operator who will performing and interpreting the test.

Aspects affecting choice of test

- Is the assay to be used for screening or confirmatory purposes, or both?
- Will the assay be used as a laboratory or field based test?
- Will it be used for one or more species? Which ones?
- Will the test be used for typing organisms to group, serotype or strain-specific levels?
- Will the test be applied nationally or internationally?

The antigen ELISA is conceptually the same method employed for the antibody ELISA (Chapter 2.2.1), with the exception that antigen is the targeted analyte, and antibodies are the primary reagents used for capture and detection of antigens. Depending on whether the antigen is adsorbed directly on the microplate or captured by antibodies on a solid phase, along with subsequent detection steps, different formats are available.

It is not always apparent which assay format should be used to best fit the intended purpose. Availability of reagents and the limit of detection of the assay for a particular application may be significant factors in limiting the choice. Since many of the systems now target highly specific antigens, the choice of antibody for both trapping and/or detection becomes critical.

Preparation of the test sample is also a critical consideration depending on the test format being used. The use of trapping or capture antibody in sandwich-type assays enhances selectivity and reduces potential matrix effects. For assays requiring direct application of the analyte to the solid phase, preparatory extraction, centrifugation or filtration methods may be necessary to remove extraneous material. For a more in depth discussion of these different assay formats, please see Crowther (2001).

Of critical importance is the size and complexity of antigen and the availability of relevant reagents, such as capture antibodies (e.g. antigens to be detected in sandwich assays must have at least two unfettered epitopes) which limits this assay type to relatively large antigens or whole pathogens. The affinities of the immunological reagents come into play as the stability of the resulting antibody-antigen complexes in the microplate or on beads will affect the performance characteristics of the assay.

Practical concerns are availability and use of antigen standards for quality control and assurance purposes, repeatability, reproducibility, throughput capacity, turn-around-time of a test result, cost and technical sophistication and interpretation skills.

Working with exotic and/or zoonotic agents requires particular attention to biosafety and biosecurity regulations (see also Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

2.3. Proof of concept experiments (feasibility studies)

The same types of experiments used in antibody detection tests are required for antigen detection tests (see Chapter 2.2.1).

2.4. Samples and data expression

2.4.1. Selection, storage and use of control samples for test development and validation studies

It is important to assess and monitor sensitivity and specificity of the test during development and validation. This is achieved by selecting several samples (4–5 is adequate) that range from negative to high levels of antigen of the analyte in question. These samples are used in experiments designed to optimise the assay. To achieve continuity of evidence requires care and forethought in preparation and storage of samples. A large volume (e.g. 10 ml) of each control sample is acquired and divided into 0.1 ml aliquots for storage at or below –80°C. One aliquot of each sample is thawed, used for experiments, and ideally then discarded. If it is impractical to discard the aliquot, it may be held at 4°C between experiments for up to about 2 weeks; however, there is a possibility of sample deterioration under these circumstances. Then, another aliquot is thawed for further experimentation. This method provides the same source of sample with the same number of freeze–thaw cycles for all experiments (repeated freezing and thawing of samples can denature antigen and/or facilitates the growth of other unwanted microorganisms and should be avoided). Also, variation is reduced when the experimenter uses the same source of sample for all experiments rather than switching among various samples between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples. After the initial stages of assay validation are completed, one or more of the samples can become reference reagent(s) that are the basis for data expression and repeatability assessments both within and between runs of the assay (Jacobson, 1998). They may also serve as in-house working standards if their activity has been predetermined; such standards provide assurance that runs of the assay are producing accurate data (Wright, 1998).

2.4.2. Normalisation of results and their expression

The same normalisation procedures as used for antibody detection assays are applicable to antigen detection assays (see Chapter 2.2.1 for details).

2.5. Optimisation

The aim of this stage is to finalise test parameters related to reagents, consumables and equipment that will lead to a fixed protocol and will be used during the assay validation pathway Part B. For details, see Chapter 2.2.1.

Optimisation and standardisation

- Is the test able to distinguish between samples with and without the analyte (what is the limit of detection = analytical sensitivity?)
- Does the test cross-react with non-target antigen in the sample or sample matrix (analytical specificity)?
- What is the noise or background activity in a negative sample?
- Has the repeatability been assessed for a range of control samples over a number of days
- Are sufficient positive and negative samples on hand to carry out the experiments for optimisation and validation
- If yes, are reference and control samples dispensed and stored properly to avoid introduction of sample related bias (sample deterioration)?
- Have all critical reagents been run against each other in checker board titrations?
- Did you find optimal concentration/dilutions for each reagent?
- Did you include reference reagents and working standards/controls and did you normalise the OD data to achieve the best possible comparative results?

2.6. Inhibitory factors in sample matrix

Due to a higher variety and complexity of specimens, antigen detection assays are more likely to be influenced by matrix factors than antibody detection assays, which normally detect antibodies in serum. Inhibitory substances are frequently found in complex matrixes such as pus, semen, tracheal/nasal/cloacal swabs and may have an impact on the test result. ELISA antigen detection systems are rather resistant to inhibitory factors, please see the WOAH Validation Standard, Section A.2.4, and Greiner *et al.* (1997) for descriptions of the type of inhibitors that could affect the assay. These references should be reviewed carefully to assure that all inhibitory factors are accounted for and controlled.

2.7. Calibration to reference sample and comparison to standard test method

Chapter 2.2.1 contains information relevant to this procedure.

B. ASSAY VALIDATION PATHWAY

1. Stage 1 – Analytical performance characteristics

1.1. Repeatability

Chapter 2.2.1.

1.2. Analytical specificity

Analytical specificity (ASp) is defined as the degree to which the assay distinguishes between the target analyte and other components that may be detected in the sample matrix (see Chapter 2.2.6, Section B.1). The higher the ASp, the lower the number of false positive results. ASp should be determined by testing well characterised samples from similar or related pathogens, which produce similar lesions as the target pathogen or are frequently found in samples containing the target pathogen. For example, to assess the ASp of a

Analytical performance characteristics

- Has repeatability been established for a range of positive and negative samples within and between runs of the assay?
- Have upper and lower control limits of the assay been established?
- Have you defined ASe and ASp for this assay?
- Does the candidate assay compare favourably with a standard test method, based on objective quantitative and qualitative criteria?

FMD antigen detection ELISA for one particular serotype (e.g. serotype O), it is necessary to assess its reactivity of all sub-strains within this serotype (e.g. O Campos, O Manisa, etc.) to assess inclusivity. At the same time it is important to show that the test does not cross-react with other serotypes such as A, Asia 1, C, SAT 1, 2 and 3. Finally, there is also a need to assess whether the test cross-reacts with agents from diseases which may produce similar signs, e.g. vesicular stomatitis, swine vesicular disease and swine vesicular exanthema. Another example is an ELISA designed to detect avian influenza virus: as a screening test the assay should detect the nucleoprotein or matrix antigen of all subtypes, e.g. H1-H16 and N1-N9. However it should not cross-react with viruses which cause similar clinical signs such as Newcastle disease or infectious bursal disease (diagnostic specificity) or with other non-specific components present in the matrix or on the solid phase. Some ELISAs may be subject to false positive results attributable to non-specific factors, such as non-specific binding of antibody conjugates to the plastic surface and may require use of blocking agents. Care must be taken to eliminate these types of errors.

1.3. Analytical sensitivity

Analytical sensitivity (ASe) is synonymous with the lower limit of detection (LOD) of antigen concentration in a sample. LODs are usually determined by endpoint dilution in which replicates (preferably 10) of each dilution in a $\log_2$ dilution series are run in the assay. The larger the number of replicates, the more precise the determination of the dilution at which the antigen is no longer detectable. Further information on LODs and ASe is given in the WOAH Validation Standard and in Chapter 2.2.5.

Screening assays or assays which are designed to detect sub-clinical infections or carriers should have a very high ASe. In these cases it may be difficult to obtain suitable samples and to determine the comparative ASe by running a panel of samples on the candidate assay and on another independent assay. If available, serial samples from experimentally infected animals could provide temporal information about the assay's capacity to detect antigen over the course of infection.

1.4. Standard test method comparison with the candidate test method

Chapter 2.2.1.

2. Stage 2 – Diagnostic performance characteristics

See also Chapter 2.2.1.

2.1. The challenge in establishing accurate estimates of DSe and DSp for antigen assays

For all antigen detection assays including ELISAs, particular consideration must be given to the timing when the sample is taken as the probability of detecting antigen or the pathogen itself is very closely linked to the stage of infection. The diagnostic window will likely be much smaller in antigen/pathogen detection assays than in antibody detection tests as immune responses can normally be measured over an extended period of time. Dynamics of infection, e.g. acute, persistent, sub-acute, chronic or carrier-state are important determinants for sampling recommendations. For example, during an acute viral infection, the sample should be taken as early as possible after the onset of clinical signs. In persistent, sub-acute, chronic or carrier animals there is a balanced relation between the pathogen and the host and the agent may be present in minute concentrations which may be very difficult to detect. During the course of pathogenesis, other organ systems may become involved and different tissues or fluids may be more appropriate target tissues for sampling as the disease progresses.

Diagnostic performance characteristics

- Are the criteria used to determine the positive and negative reference populations legitimate?
- Do the reference samples fully represent the population targeted by the assay?
- Were there difficulties in obtaining a sufficient number of samples? If so, how was the problem addressed?

2.2. Reference animal populations

2.2.1. Animals of "known infection status"

Depending on the composition of positive and negative reference samples, the same test may have different estimates for DSe and DSp. Ideally the composition of reference samples and animals should match as closely as possible the samples which are expected from the target population for which the test was developed. It is important to have a clear case definition. This is a set of criteria used to decide whether an individual or group of animals is infected or not. The reference status must relate to the purpose of the testing. For example, if the purpose of the assay is to be used as a screening test to detect early infection of FMD (e.g. in free-ranging cattle with vesicles), then the majority of samples to determine DSe and DSp should be taken from this target population. Relevant information should be collected and summarised for all animals involved at this stage of validation (e.g. species, age, sex, breed), and information on other factors that are known to influence DSe and DSp (e.g. date and place of sampling, immunological status, vaccination and disease history, pathognomonic and surrogate tests used to define status of animals, prevalence within population and description how the reference status was derived).

A sample from a negative reference animal refers to lack of exposure to or infection with the agent in question. For example a classical swine fever negative population could be defined as a region with pig herds without confirmed clinical cases of the disease in recent years, supported by negative serological tests and negative virological test results of suspected cases. Samples from these animals fulfil the status of negative reference samples. The negative reference population should be chosen with care so as to ensure that it is representative and matches the positive reference population (e.g. as to the breed and exposure to environmental challenge).

If vaccination is carried out, it may interfere with antigen detection (e.g. vaccination with modified live viral vaccines). Samples from these animals should not qualify as negative reference samples.

The types and limitations of reference standards commonly used for evaluation of the performance characteristics of a new assay are listed here. An expanded description of each reference standard is provided in Chapter 2.2.1, Section B.2.2.1 and in Jacobson, 1998. The strengths and limitations of these reference standards must be considered carefully when using samples, derived from animals that fall into any of the following four categories, as sources for establishing DSe and DSp for a candidate assay (Jacobson, 1998).

i) An unequivocal reference standard

   An unequivocal reference standard: presence of the agent in the host or evidence of definitive (pathognomonic) histopathology.

ii) A composite reference standard

   A composite reference standard: verification of uninfected or unexposed animals.

iii) A relative reference standard

   A relative reference standard: reference animals that have been classified for their infection status by comparison with the test results of another assay for antigen or nucleic acid detection on the same samples. As with antibody assays, the estimates of DSe and DSp are useful only insofar as the reference test has documented, established and acceptable performance characteristics. A deficiency of relative reference standards is that they have their own established levels of FP and FN test results, which are sources of error that will be compounded in estimates of DSe and DSp for the new assay. Generally, however, the use of other well described test methods is regarded as good practice to determine the status of reference animals, but only if the inherent bias introduced by the relative reference standard is accounted for.

iv) An adjunct reference standard: experimental infection or vaccination

See the WOAH Validation Standard, Section B.2.3 for significant limitations of this type of standard. Note: In the context of antigen detection, "comparative" tests should include pathogen detection by isolation, culture, NAD tests or histopathology or other *in-situ* techniques.

2.2.2. Latent-class models for sample selection

For a discussion of this approach to sample selection, see the WOAH Validation Standard, Section B.2.5 and Chapter 2.2.6.

2.3. Threshold (cut-off) determination

It is important to clearly describe the method and the samples used for selecting a cut-off. It is strongly recommended to conduct a receiver operating characteristic (ROC) analysis to show the potential performance of the test in other epidemiological settings.

3. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility assessments for antigen detection assays are not uniquely different from similar assessments for any other type of assay. Therefore the reader is directed to the WOAH Validation Standard, Section 3, for details on reproducibility analysis and for reference samples and panels to Chapter 2.2.6. WOAH provides guidelines for laboratory proficiency testing (https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-5).

4. Stage 4 – Programme implementation

4.1. Interpretation of results

See the Chapter 2.2.1, Section B.4.1.

5. Monitoring assay performance

5.1. Monitoring the assay

After performance characteristics of the new test have been established, on-going monitoring, maintenance and enhancement are required. It is important to continue monitoring the repeatability and reproducibility of the assay over time, on a per run basis, quality control samples have to fall within pre-established limits. If not, the test result is not valid and has to be repeated. Monitoring the assay controls' performance over time is an important way to detect changes or trends in the assay. Simple analysis of results, e.g. statistical assessment of mean values, standard deviation and coefficient of variation are useful in this process and results can be plotted in a control chart. Participation in external quality control or proficiency testing programs is useful to identify random and systematic errors and provide credibility in test results. Further information about this topic can be obtained from Crowther *et al.* (2006) and in the Chapter 2.2.1, Section B.5.1.

5.2. Minor modifications of the assay – replacement of depleted reagents

Over time changes in the test protocol may be necessary due to better or less costly reagents or because the target analyte has changed. Batch-to-batch variation of biological reagents is considered a major contributor to test variation. When reagents such as antibodies or antigen must be replaced they should be produced or procured using the same protocols or criteria as used for the original reagents. New biological reagents (e.g. control samples, antigen, capture or detection antibodies, conjugate, chemicals or consumables) need to be assessed for comparability. Chapter 2.2.8 *Comparability of assays after changes in a validated test method* gives an overview of acceptable comparability studies. A ground rule is never to change more than one reagent at a time in order to avoid the compound problem of evaluating more than one variable concurrently (see also Chapter 2.2.1, Section B.5.2).

5.3. Major modifications of the assay – changing to a new ELISA type

It is a major challenge of laboratory diagnosis to keep up with the evolving nature of infectious pathogens. Over time pathogens may change their antigenic characteristics and new strains may emerge, e.g. the emergence of Bluetongue virus serotype 8 in northern Europe in 2009. This change necessitates a full test development and validation study. Another major change is the use of a test in a different species than that for which it was originally validated, e.g. there may be a requirement to use an FMD Ag detection ELISA, validated in cattle, for testing camelids or buffalo in different geographical and climatic regions. Evaluation of reference samples that represent those populations of Stage 2 in Figure 1 of the WOAH Validation Standard will accomplish this requirement (see also Chapter 2.2.1, Section B.5.3).

5.4. Enhancing confidence in validation criteria

Due to the extensive set of variables that have an impact on the performance of antigen detection assays, it is useful to expand the number of reference samples when possible, due to the principle that error is reduced with increasing sample size (see also Chapter 2.2.1, Section B.5.4).

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CHAPTER 2.2.3.

DEVELOPMENT AND OPTIMISATION OF NUCLEIC ACID DETECTION ASSAYS

INTRODUCTION

The WOAH Validation Recommendations in Section 2.2 Validation of diagnostic tests *of this* Terrestrial Manual *provide detailed information and examples in support of the WOAH Validation Standard that is published as Chapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals*. The Term "WOAH Validation Standard" in this chapter should be taken as referring to that chapter.*

An ever increasing number of nucleic acid detection (*NAD*) *tests are now being used for diagnosis of infectious diseases in various species of animals and man. The most common methods are the polymerase chain reaction* (*PCR*), *of which there are a number of variations and isothermal amplification methods* (*such as loop-mediated isothermal amplification*)*. In addition, solid-phase or liquid phase microarrays are appearing as new tools of biotechnology-based diagnosis. The amplification techniques employed in these assays provide them with high analytical sensitivity. The products of the amplification reaction can be detected in a number of ways, for example, by visualisation in agarose gels, using labelled probes* (*e.g. TaqMan probes*), *by the detection of the accumulation of the products in real time or by using arrays where specific probes are captured on a solid-surface matrix or on beads* (*Lauerman, 2004; Viljoen* et al., *2005*)*. Different PCR assays can be multiplexed together to detect several targets in one tube or to combine targeted analytes and controls that generate different amplification products, all in one reaction vessel. Whilst this has obvious advantages, great care must be taken during optimisation to ensure that assay performance is not compromised. Similarly a multiple outcome PCR can be created using one set of primers, but employing tagged probes, which bind to different target sequences in the different species or strains detected by the PCR* (*Wakeley* et al., *2005; 2006*).

NAD amplification techniques are usually based upon the principle that there is an exponential amplification of the specific sequence targeted in the reaction. This provides a NAD with high analytical sensitivity and specificity. Due to this characteristic high level of sensitivity reached with NAD, special care, and indeed special precautionary steps have to be taken to prevent NAD amplicon contamination to subsequent sample analyses. This is more likely to be a problem where reaction tubes are opened in the laboratory for further processing, for example, to run gels or to perform nested assays. To avoid such contamination, strict laboratory protocols should be employed involving separate rooms or cabinets for particular stages of the assays, changes of laboratory gowns and gloves, and stringent cleaning programmes (*Viljoen* et al., *2005; Subcommittee of Animal Health Laboratory Standards*)*. For these reasons, tests based on closed tube systems are generally more suitable for diagnostic assays* (*Sawyer* et al., *2006*). *As with all assays, it is important to use appropriate controls to prove that the assay is performing as expected. All samples used in assay development should have well established provenance and the development should be carried out within the framework of a quality system to ensure appropriate levels of training, equipment maintenance and monitoring, etc.* (*Burkhardt, 2000*).

A. ASSAY DEVELOPMENT PATHWAY

1. Definition of the intended purpose(s) for an assay

Purpose of the assay:

- Is it for a screening or confirmatory test, or both?
- Is it for detection of a group of pathogens?
- Is it for detecting a single disease agent?
- Is it for discriminating between vaccinated and infected animals?

The first consideration in assay development is to define clearly the specific purpose and application of the test to be developed and to understand how it will be applied because this informs many of the decisions of the development pathway. For example one might choose to develop a screening test to detect all avian influenza (AI) subtypes or variants in birds for which an inclusive and broadly reactive test is necessary, or to determine the haemagglutinin type, in which case a more specific test is needed. For some tests the requirement is to detect a group of viruses, e.g. the pan-pestivirus PCR that detects bovine viral diarrhoea (BVD), border disease (BD) and classical swine fever (CSF) viruses. For other tests, the requirement may be to detect a single agent, or sometimes even to allow a DIVA (differentiating infected from vaccinated animals) approach. An example of such a method is the recently published real-time RT PCR assay for CSF virus which was developed for the genetic differentiation of naturally infected from vaccinated wild boar (Liu *et al.,* 2009).

2. Assay development – experimentation

2.1. Quality assurance

It is important that assays are developed in laboratories where high standards of quality assurance and control are employed (see Chapter 1.1.5 *Quality management in veterinary testing laboratories*). The validation data for test performance and accuracy determined during the development and validation phase must be robust as it will form the basis for interpretation of disease status and consequent actions when the assay is routinely used.

2.2. Reference materials

Sample selection (see Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens*).

- What kinds of samples will be used from the target population?
- What are the predilection sites for the agent in the host?
- What sample collection, storage and transport methods are anticipated and what are the possible effects on results?
- Will samples be single or pooled?
- Will samples in the validation panel be representative of the target population?

For most diseases, the samples required for NAD assays are likely to be similar to those used for current detection methods such as bacterial cultivation or virus isolation. For example, detection of AI by real-time RT-PCR and virus isolation use the same samples. Recently, swabbing of fresh cuts in organ samples proved to be a practical approach, replacing the laborious homogenisation of organ samples. Similarly, there is a trend to use samples, which can be obtained by non-invasive methods, such as saliva for detection of porcine reproductive and respiratory syndrome (PRRS) in pigs (Prickett *et al.,* 2008) or bulk milk samples for determination of herd BVD status. Prior to the selection of pooled samples, such as bulk milk, test developers need to consider and assess the implications of dilution of the target analyte on diagnostic sensitivity and incorporate this into the validation plan (see below).

It is important to understand the biology of the pathogen concerned and the nature of the sample collection devices. As in the case of AI, different subtypes or variants of the viruses have different predilection sites in host birds. For example cloacal samples are appropriate for some subtypes, whereas buccal samples are acceptable for others. Therefore, a test for use in a surveillance programme would need to incorporate both sampling sites and be validated using both types of sample. Another significant factor is the matrix in which the analyte resides in the host. Cloacal samples are more likely to contain PCR inhibitors than buccal samples. Another potential confounding factor is

the type of swab used to collect the samples; some contain materials that inhibit PCR assays. Therefore, it is very important to specify precisely the preferred sample material and to describe fully the swabs and swabbing protocol, including the preferred buffers or transport media and storage conditions.

Consideration also should be given to whether samples should be tested singly or in pools and whether pools should be of different samples from a single or multiple animals. Any pooling strategy should be precisely defined and validated prior to use. Finally, if the target population is "birds", the validation study should cover a large representative population of different species to demonstrate that the assay is widely applicable, whilst concentrating on the most prevalent species or those used as sentinels of infection.

2.3. Design of test method (fitness for purposes)

2.3.1. Choice of test

For most surveillance activities large numbers of samples may be tested first by a screening assay. In the above example for AI, the screening assay described must detect all known subtypes or variants of Influenza A, either in a particular region or throughout the world. The test must be highly sensitive so it does not miss true positive samples, and analytically specific (inclusive) for detection of all viruses in the Influenza A group.

Avian Influenza is a high profile disease. If a new test should generate a high proportion of false positive results that cannot otherwise be confirmed, the infection status of the birds would be difficult to resolve. Exclusion of closely related agents that are not of interest within the context of the intended purpose of the assay is therefore essential.

- Is the test for use only in a particular region or world-wide?
- Is it sufficiently sensitive and is the analytical target inclusive enough to not miss positive samples?
- Consider the impact of a high proportion of false positive results that cannot be confirmed
- Are rapid results possible and/or necessary?
- Will confirmatory tests (an analytical tool) be used to determine/confirm pathogen pathotype (strain)?
- Will determination of the strain have important bearing on the action taken?

To continue with the AI screening example, it is also important for the test to produce rapid results so that disease control measures can be swiftly applied. So, for the detection of avian influenza, the logical choice of tests would be a real time PCR using TaqMan probes or a field based assay. For an AI screening assay, the primers and probes are likely to be based upon the Matrix (M) gene which is known to be present in all influenza type A isolates. Dependent on the assigned fitness for purpose, the scientists may choose to develop an additional assay for use in conjunction with the screening test to determine which particular strain of AI is present and whether the strain is highly pathogenic, because this has an important bearing on the notification requirements and control measures that may follow. Some test methods may qualify as secondary analytic tools that are applied to the analyte detected in the primary assays, and may be used to further characterise the product detected in the screening assay (see the WOAH Validation Standard, Section B.1.4). An example of such an analytic method would include sequence analysis of the matrix PCR amplicon detected during AI screening. Development of paired confirmatory assays such as real-time PCR assays that target other genes, such as the AI haemagglutinin or neuramindase to identify subtype or pathotype, would require that those assays also undergo all steps of development, optimisation and validation. Recently a novel, plexus-primers-cased PCR assay was developed at the WOAH Collaborating Centre for the Biotechnology-based Diagnosis of Infectious Diseases in Uppsala, Sweden. This assay allows the simple and rapid determination of various RNA-virus pathotypes. For example, avian influenza viruses and Newcastle disease viruses can be detected and evaluated with low-cost equipment (PCR machine) and in a very short period of time (less than three hours), in simply equipped laboratories or even on site, in the field, during outbreak situations (Leijon *et al*., 2011; Yacoub *et al.,* 2012). This indicates a very rapid development and simplification in the field of NAD tests in diagnostics towards field/on-site applications.

2.3.2. Method design

The method should be carefully designed to fulfil the purpose that was defined at the outset. Important factors to consider will include application, types, and numbers of samples to be tested. This, in turn, leads to logistical and practical considerations for the candidate assay, such as whether it will be conducted in a laboratory, with or without automation to achieve high throughput, or in the field using a pen-side assay. All available information such as publications, sequences deposited in databases and in house sequence data should be used. Sophisticated software is available to optimise primer and probe design; computer modelling of probable sequences for use in the assay is a first step.

- Was the assay first evaluated on a small sample panel (six to eight samples) to assess viability of the approach?
- Was there good separation between test results of negative and positive samples?
- Was a preliminary test made on a dilution series in matrix to assess preliminary relative analytical sensitivity?

2.4. Feasibility study

Before embarking on the validation of a newly developed prototype assay, a feasibility study should be carried out using a small panel of approximately six or eight well-characterised samples to assess whether the system is viable. The panel should consist of samples distributed across the operating range of the assay, i.e. at least two negatives, at least two unambiguous positives, and ideally samples falling mid-range. Ideally these samples should come from different animals. The assay should achieve as wide-as-possible separation of test results for the high positive and negative samples in this panel. It may be useful to test a dilution series (analyte diluted in matrix) at this point to assess relative analytical sensitivity if the test is a potential replacement for another method where sensitivity is an important criterion (see Chapter 2.2.8 *Comparability of assays after changes in a validated test method*).

2.5. Development and optimisation

- Optimal location? Use a clean room or cabinet system to minimise contamination
- Optimal method of sample extraction to prepare samples? Done manually or by automation?

The aim of this stage is to define and optimise the method that will be used to carry out the test in future routine testing. This includes description of appropriate facilities to carry out the assay. It is important to consider both the method of sample extraction required to prepare samples for the test and the assay procedure. For any PCR assay, definition of clean room protocols is required to minimise contamination. If large numbers of samples are anticipated, selection of an automated extraction procedure may be essential (Jungkind, 2001).

Usually, it is necessary to assess a number of different test methodologies and vary concentrations of reagents, template additions and reaction times to optimise the extraction and the assay. It is important to either change only one variable at a time or use a multi-factorial design and analysis (see the WOAH Validation Standard, Section A.2.5 on Robustness). It is important to identify which factors have only a narrow range of optimal activity, as these are critical points in the assay procedure and may affect an assay's robustness. Generally, the assay stages are relatively simple to optimise, but care needs to be taken to ensure a robust nucleic acid extraction, and that all assay reagents, and procedural steps are optimised and suitable for routine laboratory application (Burkhardt, 2000).

- Have concentrations of reagents been tested for optimal reactivity?
- Has extraction been optimised?
- What are the template additions and reaction times to optimise the assay?
- Which factors have a narrow range in which they perform optimally?
  - Has that range been defined?

Controls for PCR are many and varied. It is important to include appropriate controls to show that the assay is performing as expected. The various controls that should be considered include:

2.5.1. A host-species control

This control demonstrates that the target species has been appropriately sampled. In the AI screening assay example, the control verifies that the sample swab had been in contact with a target species and that the sample contained "bird" nucleic acid, which is available for extraction using the defined protocol. A possible target for this control is a housekeeping gene such as beta actin, which is present in the target host species. This is relatively simple if the host species is a single species e.g. chickens, but it will be more challenging to find a suitable target for all "bird" samples. This type of control may not be required if a sample is added directly to the extraction process, such as a piece of tissue or blood, but is recommended for "indirect" samples such as those collected on swabs; in this case, the host-species control should be used for every sample tested.

Have you included all necessary controls to prove the assay is performing as expected?

- Template control?
- Inhibition control?
- Positive sample control?
- For RNA assay – reverse transcription control?

2.5.2. No-template control

This control reveals whether contamination of the sample has occurred, resulting in amplified product when no amplified product should be present, as in a sample containing no target. Consideration should be given to the number and placement of no template controls in the assay set up template. Generally, a number (approximately 5% of wells) of no-template controls are distributed randomly within the assay or over the plate when 96 and 386 well-plate formats are used.

2.5.3. Analyte-positive control

This positive control, having a real-time PCR cycle threshold (Ct) activity within the defined operating range of the assay, is used on each plate. A suitable choice for this control may be a plasmid containing the target sequence, which can be used to check for the expected level of amplification in the assay. However, this does not assess the efficacy of the extraction process, which requires a known field sample or its equivalent (such as a sample from an experimental infection).

2.5.4. Inhibition control

- Have both the advantages and disadvantages of including an internal control been considered?
- Is the purpose and application of the inhibition control clearly specified in the assay protocol?

This control is needed to detect possible inhibitors of the PCR reaction. If an inhibition control yields a negative result, this infers that the sample contains inhibitory substances and that a negative test result for the test sample cannot be interpreted as "negative" because the assay did not perform correctly. Certain samples such as faeces and semen often contain inhibitors whereas this may be less of a problem when testing blood samples or cultured organisms (Ballagi-Pordány & Belák, 1996). Data collected during the validation process concerning assay performance using the sample matrices being targeted will allow for a risk-based decision as to whether an inhibition control should be included for each sample or whether the test system is unlikely to be affected by inhibition. If inhibitory substances are a significant problem an inhibition control must be included for each test sample.

There is considerable debate about the most suitable and effective inhibition control. Examples include the following:

i) An artificial target, such as a length of DNA contained in a plasmid, which is added to the extracted sample and amplified with the same primers as the test target, but is of a different size or contains a different internal sequence so that it is identified as the internal control when the detection method is applied (Sawyer *et al.,* 2006).The advantage of this approach is that it utilises the same primers employed in the test, and the control can be added at precise concentrations. Because the target for the assay and for the control is the

same, competition for the primer and dNTPs may reduce the analytic performance of the assay. Care must be taken during assay optimisation, so that the analytical sensitivity of the assay is not detrimentally affected. Another disadvantage is that as an added component, this control only verifies the assay stage of the test and does not act as a control for the extraction stage.

ii) An alternative strategy for an inhibition control is to amplify a housekeeping or structural gene such as β-actin which always is present in the target tissue, and thus the sample. If the housekeeping gene has been inhibited by substances in the sample, the inference is that amplification of the gene targeted by the test may also be inhibited. This conclusion is not always warranted. Housekeeping genes are often present in abundance, so sometimes can be detected even in the presence of inhibitory substances while the more limited amounts of the assay's targeted sequences may be inhibited from amplification. In this case, the amplified and detected housekeeping gene was not a sufficient control for inhibitors, resulting in significant risk of a false negative inference for the test result. However, with knowledge of the risk associated with housekeeping genes, which are naturally present in the sample housekeeping genes can be a useful control for inhibitors for the whole assay including sampling, storage and extraction.

2.6. Inhibitory factors in the sample matrix

Generally for NAD methods, pure cultures, blood and most tissues are the preferred samples because extraction and recovery of amplifiable NA is generally successful. Faecal samples, semen and autolysed tissue can be more challenging because they generally contain more inhibitors for NAD assays. It is vital to have a robust and repeatable sample extraction procedure, which is appropriate for the numbers of samples to be handled (automated if necessary) and to utilise inhibition controls as necessary (see section above).

2.7. Operating range of the assay

- For determining the operating range of the assay, were samples diluted in the matrix for which the test is intended?
- Does the operating range of the assay conform with the expected norms for such an assay?

The operating range of the assay should be determined by diluting a strong positive sample and plotting the range of results obtained versus known amounts of nucleic acid (concentration, dilution, number of genomic copies, etc.). This reference sample must be in the same matrix as the test sample, i.e. it is not appropriate to determine the operating range of a sample diluted out in buffer, if the usual matrix is blood.

2.8. Robustness

An assay should tolerate small changes in concentrations of reagents and/or slight variations in processing times and temperatures used for different stages of the assay in order to be effectively deployed and provide reproducible results when used in multiple laboratories. This can be determined during assay optimisation when critical procedural steps, reagents, and equipment are identified. Such factors that when not adhered to cause unacceptable variability should be well described in the assay protocol so that particularly exacting processes are assured for carrying them out. This is a laborious process that ultimately is monitored for precision and accuracy by internal and external quality control samples run in the assay.

2.9. Calibration of the assay to reference samples

- Have you calibrated the assay to external reference standards?
- If external reference standards not available, have you created an in-house reference standard?
- Have you made working reference standards in sufficient amounts for use in all development and validation experiments?

Ideally, international or national reference standards should be used to calibrate the assay. However, these are not always available so it may be necessary to create an in-house reference standard (see Chapter 2.2.6 *Selection and use of reference samples and panels*). A working standard(s), for inclusion in all runs of the assay, needs to be created, aliquotted, and stored in sufficient quantities for use in every run of

the validation process and for routine use after the validation has been completed. The working standard(s) could be multiple aliquots of a particular sample, which can be used within each assay run. They could also consist of a plasmid containing the sequence of interest, spiked into sample matrix. Use of the latter allows the test developer to determine the number of genome copies that can be detected by the assay. In some instances test sample results are "normalised" by comparison to the working standard sample(s) included in each run of the assay. This allows direct comparison of data between runs (Huggett *et al.,* 2005; and WOAH Validation Standard).

B. ASSAY VALIDATION PATHWAY

Once the protocol for the assay has been developed and optimised, it must be fixed and held constant while being evaluated through the stages of the validation pathway and during routine use. Minor changes to a validated assay can be addressed using comparison studies to document that the assay continues to perform as originally defined (see Chapter 2.2.8).

1. Stage 1 – Analytical performance criteria

1.1. Repeatability (Ct or qualitative conventional PCR)

- Has intra- and inter-assay repeatability been determined?
- Is repeatability within the accepted range of coefficient of variation (CV) limits?

Repeatability of the assay is a measure of agreement between results (within and between runs) using the same test method in one laboratory. Usually a small panel of three (preferably five) samples covering the operating range of the assay is selected and tested using the entire assay procedure (including nucleic acid extraction). Within assay (intra-assay) variation is determined using multiple (at least five) replicates of each sample in this panel in one assay run. Between run (inter-assay) variations are determined by testing these samples over several days, using several operators and at least 20 runs. The repeatability panel should be tested treating all samples and each of their replicates exactly like individual diagnostic samples, subjected to every step from sample preparation to data analysis. Accordingly, every replicate of every sample is subjected to an independent extraction. This allows for determination of repeatability, both within and between runs of the assay that mimic future runs of the assay when implemented for diagnostic use. Minimal variation in repeatability is important, particularly near the cut-off(s) that establish positive, inconclusive and negative ranges, because higher variability can result in incorrect interpretations (see Chapter 2.2.4 *Measurement uncertainty*).

Repeatability can be expressed as a coefficient of variation (see Chapter 2.2.5 *Statistical approaches to validation*). The assay should be designed so that the decision point (cut-off) lies on the steepest part of the real-time PCR Ct curve. If this is achieved, repeatability will be optimal at the critical point of the assay. (Larger CVs at the clearly negative and highly positive ends of the operating range of the assay do occur and have little impact on test result interpretation).

1.2. Analytical specificity (ASp)

Depending upon the intended purpose of an assay, its analytical specificity is determined by the selected genetic sequence(s) of organism(s) targeted by the assay. The assay can be designed to be highly selective, with analytically specificity for a single genetic sequence that is not known to be present in other organisms or strains of the targeted organism. Such an assay is said to exhibit exclusivity and connotes a confirmatory assay of high ASp.

Alternatively, the assay may be designed to target a conserved genetic sequence that is common to several strains of a given species, or several species of a genus. Such an assay has an ASp that exhibits inclusivity, making it useful as a screening test. For an inclusive screening assay, the analytical specificity should be determined by testing all lineages, strains, species, etc., that the assay is expected to detect. The assay should then be evaluated for its capacity to exclude related organisms such as non-pathogenic strains that are not of interest to the intended purpose of the candidate assay.

In the example of an AI screening test, the assay should be evaluated against as many well-characterised isolates of AI virus as are available to assure that all strains from a variety of geographical areas and hosts are detected (i.e. to demonstrate inclusivity). This is generally done using laboratory strains/cultures or extracted nucleic acid. For AI, different lineages of the virus exist such as Asian, European and North American. It is important to consider how the assay will be used and in which geographical regions. That will aid in determining whether it is necessary to evaluate some or all of these lineages. This important requirement is clearly demonstrated with the recently developed rapid and simple RNA-virus pathotyping assays, which were tested on a large collection of AI and Newcastle disease viruses, originating both from the Eastern and from the Western hemispheres (Leijon *et al.,* 2011; Yacoub *et al.,* 2012), in a collaboration between various WOAH Collaborating Centres. Another important factor is that viruses can change rapidly and the resultant mutations can render a diagnostic test sub-optimal. An example of this is the appearance of the 2009 pandemic strain of Influenza A H1N1 and the 2013 low pathogenic AI H7N9 outbreak in Southern China. The analytical sensitivity of the PCR for the traditional M gene was impaired for this strain because the sequence that rendered the assay specific had mutated. In most countries new primers have been introduced and used either as a new test or in combination with the traditional M gene primers as a combination test.

- Has a panel of as many well-characterised isolates of the target pathogen as possible, including isolates from a variety of geographical areas and hosts, been tested?
- Have related organisms and pathogens, which cause similar clinical syndromes, been tested?

The discriminatory power of the assay should be checked by testing organisms related to the AI virus. These would include pathogens which cause similar clinical syndromes such as Newcastle disease, infectious bursal disease, etc., and other organisms, which are likely to be found in the target sample (i.e. to demonstrate exclusivity).

1.3. Analytical sensitivity (ASe)

There are two common approaches to assessing analytical sensitivity, also known as the limit of detection. The first is to use a dilution series of the target pathogen (in this case AI virus) diluted in sample matrix and not buffer. The dilution series is usually tested using the assay under validation and a standard method. For AI, the standard method could be virus isolation or another in-house standard method of detection. This approach yields a comparative measure of the two methods (see Chapter 2.2.5). The second approach is to use a plasmid construct, containing the target sequence and test this as a dilution series in sample matrix. In this manner, the number of genome copies detectable by the test method can be estimated.

1.4. Standard test method for comparison with the candidate test method

On some occasions it is not possible to complete a full validation exercise either because samples of known analyte status are scarce (e.g. exotic diseases) or, when an emergency situation arises, the assay is required for use before it can be fully validated. Provisional recognition can be achieved provided that results through Stage 1 of the validation process compare favourably with results of a standard test method or a known established and preferably published method. Another choice for a standard method is the one used routinely in the laboratory. It is important to recognise that different methods identify different morphological or functional entities of the organism. It is therefore possible that comparison between a standard culture-based method and a new NAD technique will give rise to discrepant results (see Section B.2, Stage 2, below, for discussion on resolution of discrepancies). The choice of assessment panel is also very important and it should be as extensive as possible (see Chapter 2.2.6). If only a small panel of samples is available for evaluation, it is useful to determine if the assay passes reproducibility expectation, i.e. withstands the rigors of use in other laboratories. This requires that both laboratories use the same protocol, and same reagents, same panel of samples and similar (if not identical) equipment.

- Has the new test performed in a satisfactory manner compared to a standard method of comparison?
- Is preliminary reproducibility data acceptable
- Does the assay merit proceeding to studies on full validation (Stages 2–4)?

If lack of samples prevents continuing through the next stage of the validation pathway (Diagnostic Performance of the assay), it is acceptable to use a NAD assay that has been provisionally accepted by having been thoroughly validated through Stage 1 of validation (see the WOAH Validation Standard, Section B.2.6). Acceptance of provisional validation is fully dependent upon approval by local authorities, or through bilateral agreements between countries.

1.5. Analytical accuracy

Analytical tools, designed to provide information to characterise samples that are detected using a screening assay, only require validation of their analytical performance characteristics. So, there is no requirement to determine diagnostic sensitivity or diagnostic specificity in such cases. Examples of such approaches include PCR typing assays to determine whether a matrix-positive influenza A strain is H5 or H7, or methods to determine antibiotic resistance which are only applied to cultured bacteria. A nucleic acid based technology employed for such tests includes nano-array based methods, which introduce their own challenges due to the large amount of data generated for each sample tested. Before implementing such assays, consideration should be given to how this analysis can be accomplished. A simpler approach is to compare the results of a new analytical tool to a standard tool and permit its use as long as the results of the new and existing techniques compare favourably (Anjum *et al.,* 2007; Batchelor *et al.,* 2008).

2. Stage 2 – Diagnostic performance criteria

2.1. Diagnostic sensitivity and diagnostic specificity

Diagnostic sensitivity (DSe) and specificity (DSp) provide the principle performance indicators for use of a diagnostic assay. When determining these estimates it is vital to select sufficient numbers of samples which represent the target population for the test under assessment).It can be difficult to obtain large numbers of samples (particularly positive samples) for some exotic diseases, and negative samples for endemic diseases. In such cases, with few samples available, the amount of error allowed in estimates of DSe and DSp, of necessity, may be rather large (see the WOAH Validation Standard, Section B.2, Table 2).). If an appropriate sampling design can be employed and different independent test methods can be used to test the samples, it is possible to obtain estimates of DSe and DSp by using Bayesian methods (latent class models) (see Chapter 2.2.5). Negative reference samples are often selected from animals living in regions where the disease is not present, while positive samples are usually obtained from animals with clinical signs which have been confirmed in the laboratory. This can lead to overly optimistic estimates of DSe and DSp because the samples do not represent the whole spectrum of the disease process, ranging from non-clinical animals which may have pathogen loads that are much different from animals experiencing fulminant or chronic disease.

- **Cut-off** is the test (Ct) value selected for distinguishing between negative and positive results on a continuous scale of test values.
- **Indeterminate, intermediate, suspicious, borderline, grey zone or equivocal** are terms used synonymously for a zone of test values falling between the positive and negative cut-offs.

Samples are often categorised using current test methods such as virus isolation (VI) or bacterial culture. However, this can be problematic when validating new molecular tests, because the basis of the two test systems is different. For example, a positive bacterial culture is dependent upon the presence of a viable organism whereas nucleic acid based methods detect genomic sequences of both live and dead organisms as long as the nucleic acid is still present in the sample.VI methods can be particularly susceptible to inhibitors and contaminants present in the sample matrix, leading to an underestimate of “true positives”. This can result in apparent discrepancies where samples are positive using the new molecular test and negative by traditional methods. Various strategies for resolving such anomalies include, but are not limited to, sequencing (which can demonstrate that the pathogen of interest was present in a particular sample), or testing using another molecular approach.

To calculate DSe and DSp estimates of the candidate assay, the test results first must be reduced to categorical (positive, negative, or indeterminate) status. This is accomplished by insertion of one or two cut-off points (decision limits) on the continuous scale of test results. For example, in some circumstances it is appropriate to use a cut-off for a real time PCR assay in the region of 35 Ct, which

means that some samples that produce a higher Ct value are categorised as negative or inconclusive. For a different PCR assay, however, any sample which merely registers a Ct may be categorised as positive. The performance of a particular real-time PCR, comparative validation data, the ultimate application of the results generated, and any relevant veterinary information should be taken into account when considering the use of a cut-off.

3. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility is a measure of the agreement between results obtained in different laboratories using the same protocol, similar (preferably the same) equipment and the same panel of samples. Ideally the panel would consist of 20–30 samples including a few which are present as quadruplicates. The panel should consist of samples that cover the dynamic range of the test with several that have activity close to, and on either side of, the test-cut-off(s). The same panel used for determination of repeatability could be used for this evaluation, but with enhanced numbers of replicates. Measurements of precision can be estimated for both the repeatability and reproducibility data (see Chapter 2.2.4 for further explanation of this topic and its application). Chapter 2.2.6 provides further information about the selection and use of reference panels.

4. Stage 4 – Programme implementation

4.1. Interpretation of test results

Best practices for programme implementation are general to all assay types (see WOAH Validation Standard). For NADs, an inherent advantage is the possibility of follow-up genomic sequencing to resolve apparent false positive results. Assays including PCRs are often validated using similar numbers of positive and negative samples. However, in surveillance programs, assay results are often applied to affirm the absence of the disease in question in locales where disease prevalence is very low and often approaching zero. In such circumstances, false positive results can be a significant problem even if the diagnostic specificity of a particular assay is high. If the DSp of an assay is 99.5% this means that one in 200 test-positive results will be false if the prevalence is close to zero. If a large number of samples are tested from a population of zero or very low prevalence, such false positive results can significantly out-number true positive results (see the WOAH Validation Standard, Section B.4.2 for further explanation of predictive values of test results as a function of disease prevalence). For NAD assays used in such circumstances, it would be good practise to confirm PCR-positive results by sequencing.

5. Monitoring of assay performance after initial validation

5.1. Monitoring the assay

Monitoring of repeatability by charting the Ct values obtained for working standard control samples provides re-assurance that the assay is performing as expected. Similarly, participation in proficiency testing schemes issued by external providers of quality assurance samples provides evidence of on-going reproducibility and also allows comparison of test accuracy if a reference standard(s) are included in each run for “normalisation” of data. Re-testing of a proportion (usually in the range of 1–5% depending on throughput) of retained samples is also employed by some laboratories to demonstrate that the assay is performing consistently between runs.

In time it may be necessary to modify the assay because the target analyte has changed, e.g. if the assay for avian influenza is to be applied in another part of the world or if new strains or lineages of a virus have emerged (see Section B.1.2, above, on the evolution of the new pandemic lineage of H1N1). RNA viruses evolve rapidly and point mutations can occur, so it is advisable to regularly confirm the nucleotide sequences of the primer and probe sites to ensure that they remain appropriate.

5.2. Minor modification of an existing validated assay

5.2.1. Technical modifications

Modifications of an assay are likely to be required, over time. For example, use of different equipment, use of a different extraction protocol or automation of particular stages will

minimally require comparison of the original validated assay with the modified version (see Chapter 2.2.8). If results of the modified version fall outside of the operating range or performance expectation of the original assay, a revalidation may be necessary.

5.2.2. Replacement of depleted reagents

It is important to assign unique identification numbers to all batches of reagents and to record the components used for particular assays. The most critical components in PCR based assays are the probes, the primers and the enzymes. Current and new batches of critical reagents should be tested in parallel prior to their introduction. However, for other reagents such as buffers and nucleotides it is usually sufficient to monitor batches to inform troubleshooting, should that become a necessity.

5.3. Major change in assay requiring revalidation

Upon occasion, application of the assay may need to be extended beyond the scope of the original intended purpose of the assay. Examples are inclusion of another host species or a population of animals from a different geographical area. In such cases it is important to revalidate the assay because of new biological considerations with their many associated variables. The precise details will depend on the extent of the change. Moving the assay into a new geographical area might mean that the analytical characteristics of the assay are still valid but that the diagnostic criteria need to be re-defined. Similarly modifications may be made to PCR primer or probe sequences to allow detection of new strains. It will then be necessary to demonstrate how the new reagents behave in terms of analytical and diagnostic accuracy compared with the previous version of the assay.

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CHAPTER 2.2.4.

MEASUREMENT UNCERTAINTY

INTRODUCTION

The WOAH Validation Recommendations in Section 2.2 Validation of diagnostic tests *of this* Terrestrial Manual *provide detailed information and examples in support of the WOAH Validation Standard that is published as Chapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals. *The Term "WOAH Validation Standard" in this chapter should be taken as referring to that chapter.*

Estimation of measurement uncertainty (*MU*)*, sometimes termed measurement imprecision, is a requirement for testing laboratories based on international quality standards such as ISO/IEC 17025-2005, General requirements for the competence of testing and calibration laboratories* (*ISO/IEC 17025*)*. The measurement process for detection of an analyte in a diagnostic sample is not entirely reproducible and hence, there is no exact value that can be associated with the measured analyte. Therefore the result is most accurately expressed as an estimate together with an associated level of imprecision. This imprecision is the measurement uncertainty* (*MU*)*. MU is limited to the measurement process. It is not a question of whether the measurement is appropriate and fit for whatever use to which it may be applied. It is not an alternative to test validation, but is rightly considered a component of that process* (*see the WOAH Validation Standard, Section B.1.1*).

A. THE NECESSITY OF DETERMINING MU

To assure compliance with ISO/IEC 17025-2005 requirements, national accreditation bodies for diagnostic laboratories require MU estimates for test methods that produce quantitative results, e.g. optical densities (OD), percentage of positivity or inhibition (PP, PI), titres, cycle threshold (CT) values, etc. This includes tests, where numeric results are calculated and then are expressed as a positive or negative result at a cut-off value. For the purpose of estimating MU in serology and RT PCR, suitable statistical measures are mean target values ± 2 standard deviations (SD), which is approximately equal to a 95% confidence interval (CI), relative standard deviation (RSD = SD / mean of replicates) and coefficient of variation (CV = RSD × 100%). The concept of MU does not apply to strictly binary results (positive or negative).

1. Samples for use in determining MU

Repeatability is the level of agreement between results of replicates of a sample both within and between runs of the same test method in a given laboratory. During assay development, repeatability is estimated by evaluating variation in results of independent replicates from a minimum of three (preferably five) samples representing analyte activity within the operating range of the assay (see the WOAH Validation Standard, Sections A.2.5 and B.1.1, and Chapter 2.2.6 *Selection and use of reference samples and panels*, Section 3.1). Typically, the variation in replicate results is expressed as RSD or CV. The significant feature is that repeatability studies can be used to define the expected precision of the assay in the detection of a range of analyte concentrations.

The use of internal quality or process controls over a range of expected results has become part of daily quality control and quality assurance operations of accredited facilities (see the WOAH Validation Standard, Sections A.2.6 and B.5.1, and Chapter 2.2.6, Section 1.4). These results provide a continuous monitor relative to different aspects of repeatability, e.g. intra- and inter-assay variation, intra- and inter-operator variation and intra- and inter-batch variation, which, when subjected to statistical analysis, provide an expression of the level of robustness (precision) of a test procedure. The monitoring of assay quality control parameters for repeatability provides evidence that the assay is, or is not performing as expected. In order for control samples to provide valid inferences about assay precision, they should be treated in exactly the same way as test samples in each run of

the assay, e.g. including sample preparation such as extraction steps or dilution of serum samples for an antibody enzyme-linked immunosorbent assay (ELISA).

The variation of the results for control samples can also be used as an estimate of those combined sources of uncertainty and is called the "top-down" approach. This approach recognises that the components of precision will be manifest in the ultimate measurement. So monitoring the precision of the measurement over time will effectively show the combined effects of the imprecision associated with component steps.

The imprecision or uncertainty of the measurement process associated with a test result becomes increasingly more important the closer the test value is to the diagnostic cut-off value. This is because an interpretation is made relative to the assay threshold regarding the status of the test result as positive, negative, or inconclusive (as will be described in the following example). In this context, low positive samples, like those used in repeatability studies or as the low positive control, are most appropriate for estimation of MU. The rationale being that MU, which is a function of assay precision, is most critical at decision-making points (i.e. thresholds) which are usually near the lower limit of detection for the assay. In this chapter, the application of MU with respect to cut-off (threshold) values, whether recommended by test-kit manufacturers or determined in the diagnostic laboratory, is described.

2. Example of MU calculations in ELISA serology

For most antibody detection tests, it is important to remember that the majority of tests are measurements of antibody activity relative to a threshold against which a dichotomous interpretation of positive or negative is applied. This is important because it helps to decide where application of MU is appropriate. In serology, uncertainty is frequently most relevant at the threshold between positive and negative determinations. Results falling into this zone are also described as intermediate, inconclusive, suspicious or equivocal (see the WOAH Validation Standard, Section B.2.4).

A limited data set from a competitive ELISA for antibody to avian influenza virus is used as an example of a "top-down" approach for serology. A low positive control sample was used to calculate MU at the cut-off level.

2.1. Method of expression of MU

As the uncertainty is to be estimated at the threshold, which is not necessarily the reaction level of the low positive control serum, the relative standard deviation (RSD), or coefficient of variation (CV), if expressed as a percentage, provides a convenient transformation:

$$RSD\ (X) = SD\ (X) / (\bar{X})$$

To simplify assessment, the transformed result is regarded as the assay output result, which is the averaged across the number of replicates ($\bar{X}$). In the case of this example, a competitive ELISA, results are "normalised" (as defined in the WOAH Validation Standard, Section A.2.7) to a working standard by forming a ratio of all optical density (OD) values to the OD result of a non-reactive (negative) control (OD_N). This ratio is subtracted from 1 to set the level of antibody activity on a positive correlation scale; the greater the level, the greater the calculated value. This adjusted value is expressed as a per cent and referred to as the percentage inhibition or PI value. So for the low positive control serum (OD_L), the transformation to obtain the per cent inhibition values for the low positive control (PI_L) is:

$$PI_L = 100 \times [1 - \{OD_L / OD_N\}]$$

The relative standard deviation becomes:

$$RSD\ (PI_L) = SD\ (PI_L) / (PI_L)$$

2.2. Example

A limited data set for the AI competitive ELISA example is shown below. In the experiment, the operator tested the low positive control serum ten times in the same run. Ideally in the application of this "top down" method, a larger data set would be used, which would enable accounting for effects on precision resulting from changes in operator and assay components (other than only the control serum).

Test	*PI(%)*
1	56
2	56
3	61
4	64
5	51
6	49
7	59
8	70
9	55
10	42

Mean PI = 56.3; Std Dev (SD) = 7.9; Assays (*n*) = 10

2.3. Calculating uncertainty

From the limited data set,

RSD (PI_L) = SD/Mean 7.9/56.3 = 0.14 (or as coefficient of variation = 14%)

Expanded uncertainty (U) is the statistic defining the interval within which the value of the measure and is believed to lie within a specified level of confidence, usually 95%. Expanding the uncertainty is done by multiplying the RSD (PI_L) by a factor of 2; this allows the calculation of an approximate 95% confidence interval around the threshold value (in this case at PI = 50%), assuming normally distributed data.

U (95%CI) = 2 × RSD = 0.28

This estimate can then be applied at the threshold level

95% CI = 50 ± (50 × 0.28) = 50 ± 14%

2.4. Interpretation

Any positive result (PI > 50%) that is less than 64% is not positive with 95% confidence. Similarly, a negative result (PI < 50%) that is higher or equal to a PI of 36 is not negative at the 95% confidence level. This zone of lower confidence may correlate with the "grey zone" or "inconclusive/suspect zone" for interpretation that should be established for all tests (Greiner *et al., 1995*).

B. OTHER APPLICATIONS

The top-down approach should be broadly applicable for a range of diagnostic tests including molecular tests. For the calculation of tests using a typical two-fold dilution series for the positive control such as virus neutralisation, complement fixation and haemagglutination inhibition tests geometric mean titre (i.e. mean and SD of log base 2 titre values) of the positive control serum should be calculated. Relative standard deviations based on these log scale values may then be applied at the threshold (log) titre, and finally transformed to represent the uncertainty at the threshold. However, in all cases, the approach assumes that the variance about the positive control used to estimate the RSD is proportionally similar at the point of application of the MU, for example at the threshold. If the RSD varies significantly over the measurement scale, the positive control serum used to estimate the MU at the threshold should be selected for an activity level close to that threshold. The Australian Government, Department of Agriculture and Water Resources, has compiled worked examples for a number of diagnostic tests, which are available online at:

http://www.agriculture.gov.au/animal/health/laboratories/tests/worked-example-measurement

For real-time PCRs, replicates of positive controls with their respective cycle threshold (CT) values can be used to estimate MU using the top-down approach.

Other approaches and variations have been described, i.e. for serological tests (Dimech *et al.,* 2006; Goris *et al.,* 2009; Toussaint *et al.,* 2007). Additional work and policy documents are available from the National Pathology Accreditation Advisory Group and Life Science. The central document to MU is the Guide to the expression of uncertainty in measurement (GUM), ISO/IEC Guide (1995).

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CHAPTER 2.2.5.

STATISTICAL APPROACHES TO VALIDATION

INTRODUCTION

The WOAH Validation Recommendations in Section 2.2 Validation of diagnostic tests *of this* Terrestrial Manual *provide detailed information and examples in support of the WOAH Validation Standard that is published as Chapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals. *The Term "WOAH Validation Standard" in this chapter should be taken as referring to that chapter.*

The choice of statistical methods for analysis of test validation data from laboratory experiments and from evaluation of field-based samples depends on considerations such as the experimental design and sample selection (source, number of samples, number of replicates of tests, etc.). Specific guidance about the "best approach" should be made in consultation with a statistician and should be done during the design phase before validation studies commence.

Definitions of scales of measurement:

Binary (dichotomous): Either positive or negative because that is how the test result is presented, or positive/negative at a selected threshold (cut-off) value when results are measured on an ordinal or continuous scale.

Ordinal: Measured on a scale with discrete values where higher values typically indicate more analyte, e.g. serum virus neutralisation titres.

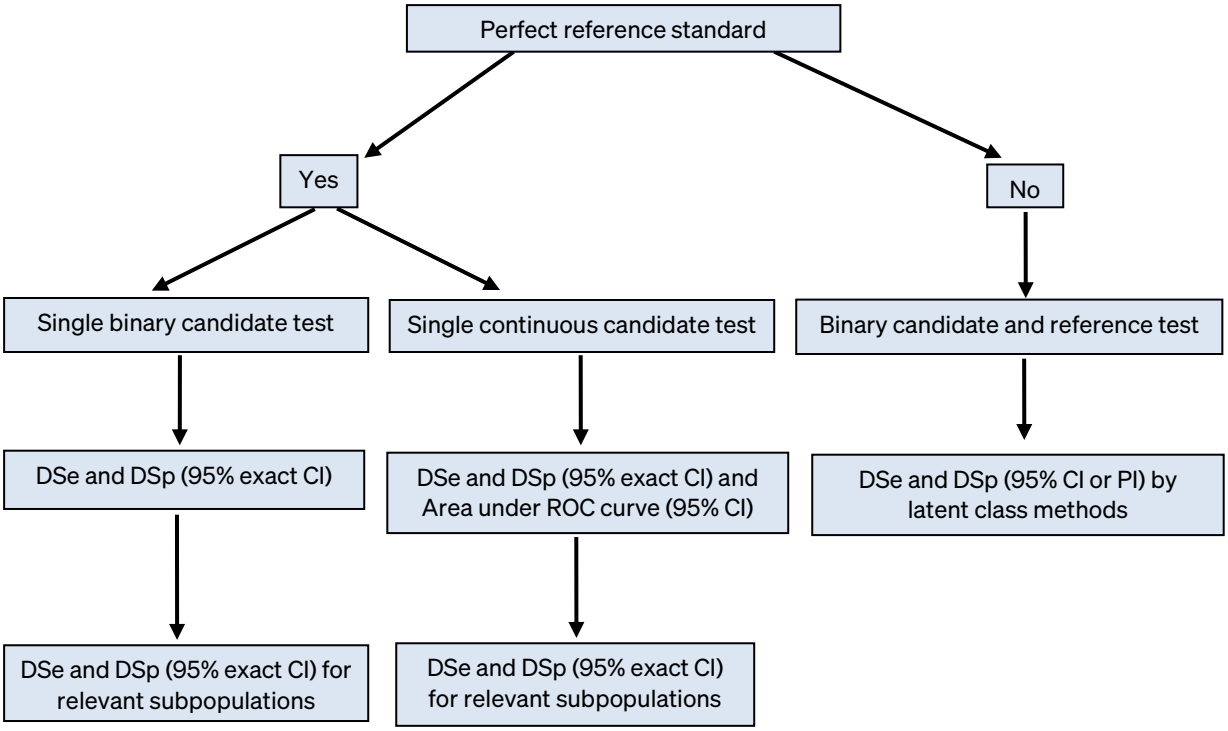
Continuous: An infinite number of measured values are theoretically possible, depending on the measurement system, e.g. optical density or per cent positive values in an enzyme-linked immunosorbent assay, and cycle threshold values from real-time polymerase chain reaction assays that are lower than the maximum number of cycles that are run in the assay.

For brevity, this annex considers commonly-used approaches for validation of a candidate test and hence, does not consider all statistical methods that might be used in practice. Methods are described to estimate the precision of an assay when repeated multiple times (repeatability and reproducibility), analytical characteristics (analytical sensitivity and specificity) and diagnostic characteristics (e.g. diagnostic sensitivity [DSe] and specificity [DSp], and area under the receiver-operating characteristic curve of an assay) used to detect an analyte in individual animals. Similar principles apply when tests are used to detect the same analyte in naturally or artificially-created sample pools from animals in aggregates (e.g. herds or flocks). In this case, the epidemiological unit is the aggregate rather than individual animals.

Statistical methods differ depending on whether a single or multiple tests are evaluated, their scales of measurement (binary, ordinal, or continuous), whether independent or dependent (paired) samples are used, and whether there is a perfectly accurate reference standard (often termed a gold standard) for comparison (Wilks, 2001). Flow charts to guide selection of statistical methods for evaluation of diagnostic accuracy measures such as sensitivity and specificity are in Figures 1 and 2.

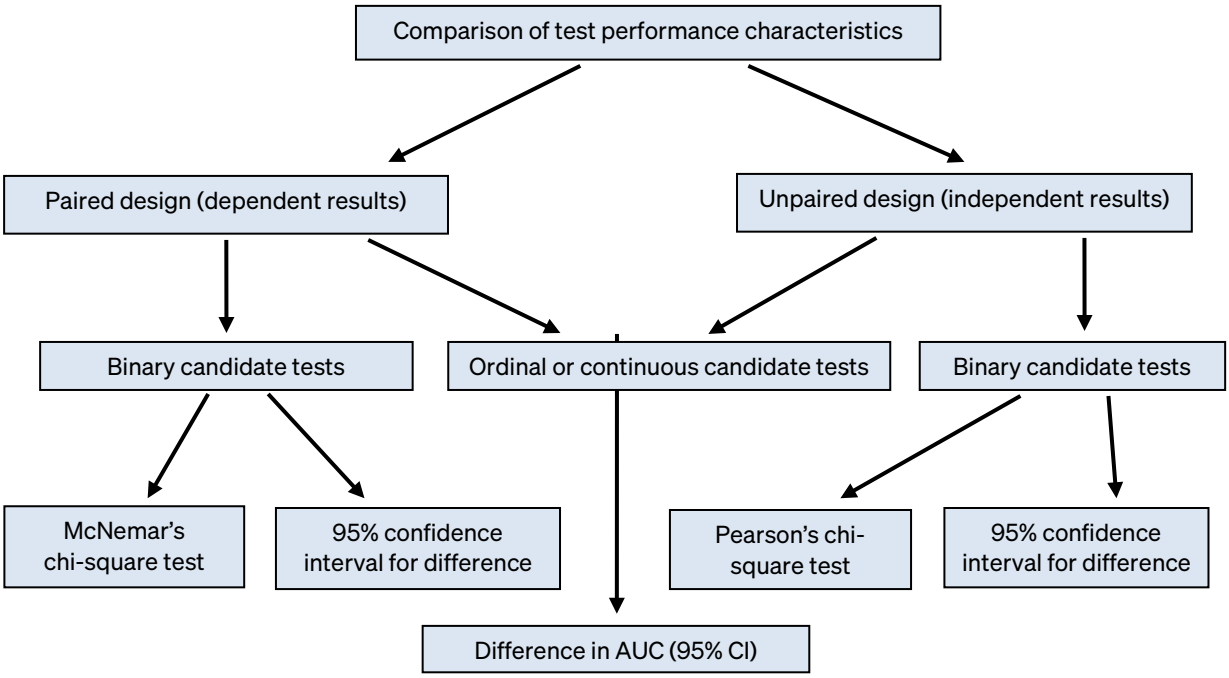
*The adequacy of the design of the study and statistical analysis may not always be reflected in the quality of reporting in scientific publications and hence, test developers and evaluators are encouraged to follow the STARD (**Sta**ndards for **R**eporting of **D**iagnostic Accuracy) checklist (Bossuyt* et al., *2003) to ensure complete reporting of all relevant information in validation studies of infectious diseases in animals.*

For guidance on analysis of measurement uncertainty data and for data for methods comparison studies refer to Chapters 2.2.4 and 2.2.8, respectively.



Abbreviations: DSe = diagnostic sensitivity; DSp = diagnostic specificity; ROC = receiver operating characteristic; CI = confidence interval; PI = probability interval.

Fig. 1. Flow chart for suggested methods of statistical analysis when a single candidate test is evaluated with and without a perfect reference standard.



Abbreviation: AUC = area under the receiver operating characteristic curve

Fig. 2. Flow chart for suggested methods of statistical analysis when sensitivities (DSe) and specificities (DSp), and the AUC of multiple tests, are evaluated with a perfect reference standard. Ordinal and continuous data should be analysed in their original form and as binary results at the recommended thresholds. Analyses should be done for both DSe and DSp where these data are available.

A. ASSAY REPEATABILITY WITHIN A SINGLE LABORATORY

An assessment of within-laboratory repeatability of an assay (often termed *precision* when the measurement is on a continuous scale) requires that a minimum of three samples having analyte concentrations within the operating range of the assay are tested in replicate by a single operator using a single test-kit lot or batch. Typically these runs will be on the same day but separate days are also possible. Use of three or four replicates of a sample rather than two is encouraged because it better captures the inherent variability in within-run assay results. Because of cost considerations, use of more than two replicates may not be feasible for all assay types (e.g. nucleic-acid detection). As described in the WOAH Validation Standard, between-run variation can be evaluated in multiple runs involving two or more operators on multiple days. The following two sections describe approaches to analysis of continuous and binary data for assay repeatability.

1. Continuous outcomes

For continuous outcomes, the simplest approach is to estimate the standard deviation (SD) of replicates of a set of samples representing the operating range of the assay. These results initially should be evaluated in a scatter plot or diagram of the mean of the replicates plotted against the SD. For assays, where the SD is proportional to the mean, the within-sample coefficient of variation (CV) is often calculated. CV is often used even when proportionality does not exist. In this case, the CV should be reported for levels of the target analyte (e.g. low, moderate and high). This is necessary because it is a common finding that CV is often larger when the concentration of target analyte is low. In general, an estimate of uncertainty in the CV values (e.g. 95% confidence interval [CI]) should also be calculated. Where the CV values are fairly constant over the range of test values, this can be done using the results of all samples. Where CV differs according to analyte concentration, separate 95% CI should be calculated for each analyte category based on the number of samples tested at each level. Methods for CI calculation for CV and the difference in two CV for normal data are described in Donner & Zou (2012).

$$CV = \frac{\text{SD of replicates}}{\text{Mean of replicates}}$$

where: CV = Coefficient of variation
SD = Standard deviation

If the experimental design includes evaluation of multiple factors such as different operators and run days, other approaches such as variance component models (mixed models) may be needed should the goal be to decompose the variation into the sum of several components that can be readily interpreted. Variance components models can also be used for reproducibility data (see Section B).

2. Binary outcomes

In general, quantitative results should be used for evaluation of assay precision when data are available in that form, even though results might be dichtomotised for reporting purposes. For inherently binary tests which yield positive or negative results, the kappa statistic can be used to quantify the agreement of test results beyond chance. Kappa ranges from 0 (no agreement beyond chance) to 1 (perfect agreement beyond chance) but there is much conjecture about how kappa values should be interpreted (Fleiss *et al*., 2003; Landis & Koch 1977). Better agreement is typically expected when test results are well away from the cut-off points and hence, some samples with intermediate/suspicious values should be tested to avoid overly optimistic assessments of agreement. A weighted version of kappa for ordinal results (e.g. negative, suspicious, and positive) can be used to recognise that a large discrepancy (e.g. two category difference) is more serious than a smaller discrepancy (e.g. one category difference). Ninety-five per cent CI should be reported for unweighted or weighted estimates of kappa (Fleiss *et al.,* 2003).

Table 1. Examples of Kappa calculations for binary outcomes

Example 1: Kappa calculation based on repeated test results classified as positive or negative

Test result	***Positive***	***Negative***
Positive	90	5
Negative	10	95
	100	100

Kappa = 0.85 (95% CI = 0.78 to 0.92)

Example 2: Kappa calculation based on repeated test results classified into three categories (positive, suspicious, or negative)

Test result	*Positive*	*Suspicious*	*Negative*
Positive	80	10	10
Suspicious	15	75	10
Negative	5	15	80
	100	100	100

Kappa = 0.68 (95% CI = 0.61 to 0.75). Weighted kappa = 0.70. (95% CI = 0.61 to 0.79)

B. ASSAY REPRODUCIBILITY AMONG LABORATORIES

Assay precision will vary according to routine implementation, e.g. different operators, different test sites, using different kit lots, or on different days. Most commonly, the term *reproducibility* is applied to assessment of precision of the selected assay in multiple laboratories. Factors held constant should be described to allow interpretation of results in context to the actual testing situation. Reproducibility studies can be done independently of or in association with repeatability studies but should be done in a blinded fashion. As suggested in the WOAH Validation Standard, at least three laboratories should test a minimum of 20 samples with identical aliquots going to each laboratory.

Intraclass correlation coefficient represents the similarity or correlation of any two measurements made on the same sample. The ICC takes values between 0 and 1 with values closes to 1 indicating minimal measurement error. Conversely, values close to 0 indicate a large amount of measurement error.

Statistical methods for analysis of studies of assay reproducibility among laboratories are similar to those used for assessment of within-laboratory repeatability. However, as part of an among laboratory study, it might be considered important to assess and rank variability in test results from multiple sources (often termed a class). For example, if a study was designed to test an assay in three laboratories each using two highly-trained technicians and running the samples in duplicate on two kit lots, each test sample would be tested 24 times. The selected factors (laboratory, technician, kit lot, replicate result) can be considered to be fixed or random depending on how they are selected and whether they are representative of the target population. For this study design, variance components can be estimated for each class (example is Dargatz *et al.,* 2004) and the intraclass (intracluster) correlation coefficient (ICC) can be estimated as a measure of the similarity of sample results (Bartlett & Frost, 2008).

1. When a technical modification of the test method is made

After an assay has been validated for use in a controlled laboratory environment, it may be considered for use in a very different environment (such as a pen-side application). Because of the more extreme changes, for example severe temperature fluctuations which often occur at pen-side, it would be expected that the two tests might behave very differently in their different environments. In fact, rather than random measurement error which applies to the assessment of within or among laboratory measurement error, it is anticipated that the values in such a study likely would be interpreted as a systematic measurement error, which would be the case if the values provide an over- or under-estimate of the true value. For the example of a test run on split samples pen-side and in a laboratory, the mean of the differences between the pen-side value and the within laboratory value (true value) for the same sample should be reported with a 95% CI. If the 95% CI excludes zero, there is evidence of systematic deviation of test results when used pen-side compared within the laboratory. When such a systematic deviation in test results occurs, the pen-side test results are not comparable with those from the laboratory-based validated assay. To validate the pen-side assay, either it is subjected to a “technical modification” that is then evaluated in a methods comparison study (see Chapter 2.2.8) or a full re-validation of the pen-side application is required.

Similar approaches can be used to assess method changes within a laboratory to determine whether there is systematic or random variation in the results.

Example: The following unpublished data were obtained comparing two extraction methods (old and new on split samples) on cycle threshold (CT) values for a real-time polymerase chain reaction (PCR) for bluetongue. The data (n=10) represent means of sample duplicates.

Old method: 25.6, 24.5, 21.3, 26.8, 25.2, 30.2, 31.2, 32.8, 31.8, 34.9

New method: 23.1, 21.0, 18.2, 25.2, 24.7, 28.6, 30.4, 32.2, 31.3, 34.7

The mean difference between the two methods (old minus new) was –1.49 (95% CI = –2.33 to –0.64) with a two-tailed probability of p=0.003. Because the 95% CI excludes zero, this indicates a systematically lower CT value when the new extraction method is used. A Bland–Altman plot (Bland & Altman, 1999; Fig. 3) can be used to graphically depict how the difference changes as a function of the mean value of the old and new method. For these data, the difference appears to decrease for higher CT values but the sample size is small.

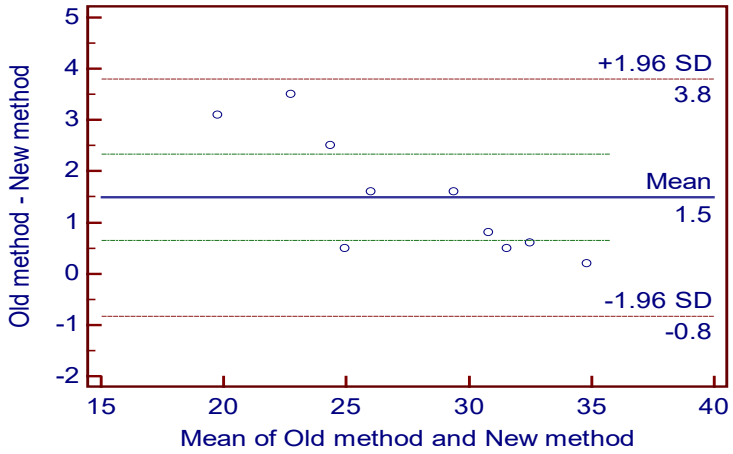


Fig. 3. Bland-Altman plot of mean difference (y axis) in CT values as a function of the mean value of the old method and new method (n=10).

C. ANALYTICAL SENSITIVITY (ASE, SYNONYM = LIMIT OF DETECTION: LOD)

Analytical sensitivity can be estimated using a dilution-to-extinction (DTE) experiment in which serial dilutions of a known quantified amount of target analyte are made into the appropriate sample matrix. This known quantified amount might be from an in-house or national/international reference standard or a field sample whose analyte concentration has been determined. Parallel runs of a comparison standard can be done but are not essential, unless the study is one in which a minor change in a validated assay is being compared with the original validated assay. The DTE approach can be used if the analyte is measured qualitatively or quantitatively. In the latter case, the test result is reclassified as positive or negative.

The approach to analysis of LOD data depends on the experimental design. For example, suppose that a study was done in which 10^8 colony-forming units (CFU) of a bacterium were spiked into 10 g faeces to achieve a concentration of 10^7 CFU/g. This sample was then diluted in tenfold serial dilutions to 10^1 CFU/g. The experiment was repeated three times. If all replicates at 10^3 CFU/g were detected but none at 10^2 CFU/g, the LOD could be conservatively estimated as 10^3 CFU/g. If a precise estimate of the LOD were needed, a second stage experiment could be designed to determine the LOD with a greater certainty using a series of finer dilutions, e.g. twofold, encompassing the interval between 100% detection and 0% detection identified in the first experiment. The LOD endpoint often is chosen to be 95%; in an experiment with 20 replicates, this corresponds to the dilution where 19 replicates for analyte were positive. The important point is that the chosen probability point for the LOD (whether 95%. 50% of another value) should be specified and used consistently if results of multiple tests are being compared. The LOD can be estimated using the Spearman-Kärber non-parametric approach, or by logistic regression or probit analysis. The greater the number of replicates for each dilution, the more precise the estimate of LOD.

Example: Guthrie *et al.* (2013) made a twofold dilution series of an AHSV-positive horse blood (10^{-3} dilution), which covered the non-linear range of the assay. The extraction was repeated 25 times and samples were tested by

AHSV real-time PCR. The real-time PCR results for the 15 dilution points were used in a probit analysis to calculate the 95% LOD (i.e. input concentration giving a positive real-time real-time PCR result in 95% of the replicates (Burns & Valdivia, 2008). The 95% LOD was estimated to be at a dilution of 3.02×10^{-6}, as shown in Figure 4, and corresponded to a quantification cycle of 35.71 in the real-time PCR. CI for the estimate were not reported.

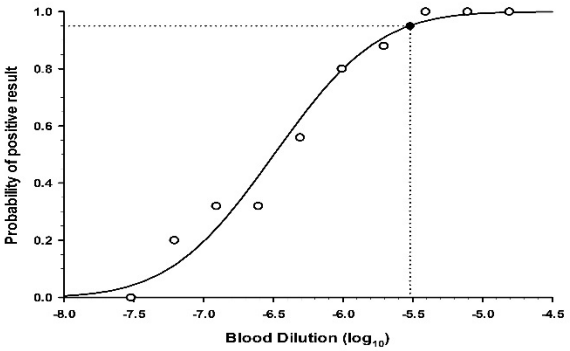


Fig. 4. Estimated 95% limit of detection of AHSV in horse blood (*log_{10}*), which is shown by the dashed line.

D. ANALYTICAL SPECIFICITY (ASP)

Analytical specificity can be described in at least three distinctive ways: selectivity, exclusivity (synonym: cross-reaction profile), and inclusivity (as described in the WOAH Validation Standard). The latter two measures should be reported on a per lineage, isolate, species or genus basis, as appropriate for the target analyte and the intended purpose of the test. For screening tests, a broader and more inclusive specificity is required than for a confirmatory test that may distinguish between isolates that, for instance, vary in pathogenicity. Because the choice of related organisms is subjective and often dependent on the types and numbers of samples, the exclusivity result should be reported qualitatively, e.g. the percentage of related agents that cross-reacted in the assay with a listing of potential cross-reacting agents that were evaluated. Similarly, inclusivity is reported as a percentage of the serovars, strains, genera and species detected by the assay, as appropriate for the target analyte.

E. DIAGNOSTIC PERFORMANCE OF THE ASSAY

Diagnostic performance of an assay is mostly commonly measured as sensitivity (DSe) or specificity (DSp) or a combined measure of DSe and DSp such as the likelihood ratio of a positive of negative result. Likelihood ratios for intervals of test results can also be calculated when it is important to retain information on the magnitude of the test result rather than use it in a dichotomised form. For more information on use and calculation of likelihood ratios, see Gardner & Greiner (2006) and Gardner *et al.* (2010). The latter paper includes an example for porcine toxoplasmosis with CI calculations by two methods.

Statistical uncertainty about diagnostic performance parameters, e.g. DSe and DSp, should be presented as confidence intervals (CI). Typically, a 95% CI is used and its width (precision of the estimated value) depends strongly on the sample size used for parameter estimation. Exact CI are preferred to normal approximations because they avoid upper limits that exceed 100%.

DSe and DSp can be estimated when the reference or comparison method is perfectly sensitive and specific or when the reference standard is imperfect. In general, most ante-mortem reference standards in common use in diagnostic laboratories are imperfect and hence, necropsy with testing of multiple tissues by ancillary tests such as culture and/or histopathology often is necessary if the results of the reference standard are to be considered to be the truth. For most test validation studies for animal diseases, this latter option is not feasible or cost-effective except for a limited number of samples.

1. DSe and DSp with a perfect reference standard

The candidate test may yield results on binary (dichotomous), ordinal (e.g. titre) or continuous scales. For the latter two scales, results need to be dichotomised before DSe and DSp can be calculated, i.e. a cut-off (threshold) needs to be established. Exact binomial 95% CI are recommended for DSe and DSp (Greiner & Gardner, 2000) because the normal approximation may not yield appropriate CI when parameter estimates are close to 1.

Example: indirect enzyme-linked immunosorbent assay (I-ELISA)

		Number of animals	
		Known antibody positive (369)	Known antibody negative (198)
Test results	Positive	287 TP	FP 1
	Negative	FN 82	TN 197
		Diagnostic sensitivity* TP/(TP + FN) **77.8%** (73.2 – 81.9%)*	Diagnostic specificity* TN/(TN + FP) **99.5%** (97.2 – 99.9%)*

TP and FP = true positive and false positive, respectively

TN and FN = true negative and false negative, respectively

*95% exact binomial confidence limits for DSe and DSp

When the reference standard is not applied to all positive and negative test results (partial verification), corrected estimates of DSe and DSp should be made as described in Greiner & Gardner (2000) to account for different sampling probabilities in the test-positive and test-negative groups.

For assays yielding ordinal (e.g. titre values) or continuous results (e.g. ratios of test sample to positive control sample values in an ELISA), estimates of DSe and DSp should be complemented with estimates of the area under the receiver-operating characteristic (ROC) curve. ROC analysis provides a cut-off-independent approach for evaluation of the global accuracy of a test where results are measured as ordinal or continuous values. The area under the ROC curve provides a single numerical estimate of overall accuracy ranging from 0.5 (useless test) to 1 (perfect test). The main justification for ROC analysis is that cut-off values for test interpretation may change depending on the purpose of testing (e.g. screening versus confirmation) and with the prevalence of infection, the costs of test errors, and the availability of other tests. Detailed descriptions of ROC analysis are presented elsewhere (Gardner & Greiner, 2006; Greiner *et al.,* 2000; Zweig & Campbell, 1993). When multiple ordinal or continuous tests are compared, the difference in the area under the curve with a 95% CI should be calculated. Methods for calculating differences vary for independent and dependent samples and are implemented in many statistical programs (Gardner & Greiner, 2006). Examples of a dot diagram for results of a single ELISA and ROC curves for two ELISAs are shown in Figures 5 and 6.

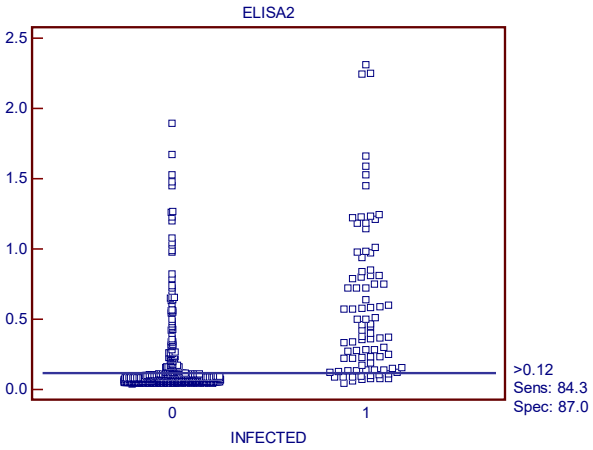


Fig. 5. Dot diagram of ELISA results for non-infected (Code = 0) and infected (Code = 1) animals.

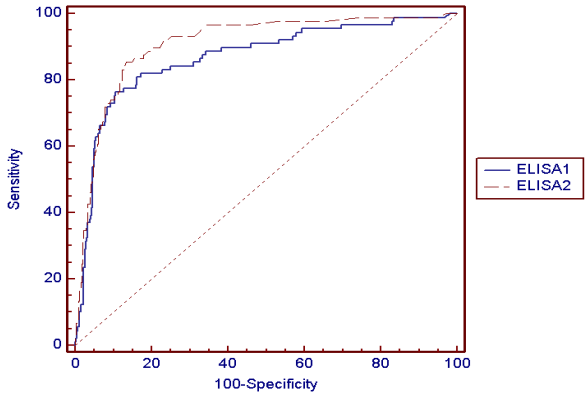


Fig. 6. Receiver-operator characteristic curve for two ELISAs.

In the absence of a perfect reference standard, it is also possible to estimate the AUC using latent class (LC) models. For example, LC models can be applied to normally distributed data from two dependent tests (see for example Choi *et al.,* 2003) and using semiparametric approaches (Branscum *et al.,* 2008). LC models for continuous data including censored or truncated data that occur with real-time PCR assays are not described in this validation guideline because of their complexity. However, LC models for binary test results and an example application are described in Section E.3.

2. Comparison of DSe and DSp estimates for two tests with a perfect reference standard

Often, investigators might wish to compare DSe values in subpopulations of infected animals, e.g. clinically versus subclinically infected, or DSp values in different geographical areas. Since these are independent samples, the comparisons can be made statistically by Pearson's chi-square test for homogeneity. Alternately, separate 95% CI and a 95% CI for a difference in two proportions can be calculated. When the DSe (or DSp) of two tests are compared on the same set of infected (or non-infected) samples in a paired design, the test results are no longer independent. Statistical methods such as McNemar's chi-square can be used to test the hypothesis of equal sensitivities (specificities) when testing is done on the same samples.

Example: Five antibody detection tests were evaluated for diagnosis of bovine paratuberculosis in dairy cows in known infected and non-infected herds, as determined by faecal culture results and herd history. The following data tables were generated based on the original data before subsequent publication in Collins *et al*. (2005). In the publication, one herd was removed from the analysis. The example is used for demonstration purposes to show the tabular layout for calculation of DSe and DSp and statistical evaluation.

Infected

	T_{2+}	T_{2-}	
T_{1+}	124	74	198
T_{1-}	8	243	251
	132	317	449

Non-infected

	T_{2+}	T_{2-}	
T_{1+}	3	27	30
T_{1-}	16	366	382
	19	393	412

Sensitivity of T1 = 198/449 = 44.1%

Sensitivity of T2 =132/449 = 29.4%

Specificity of T1 = 382/412 = 92.7%

Specificity of T2 = 393/412 = 95.4%

Sensitivities differed significantly ($p<0.0001$), but specificities did not ($p= 0.126$) based on a two-tailed McNemar's chi-square test. Sensitivity and specificity covariances (see Gardner *et al*., 2000 for details) can also be calculated to indicate whether the tests are conditionally independent or dependent, given infection status. For these data, the sensitivity covariance (calculated using the infected table on the left) was 0.147 ($p < 0.0001$ by Pearson's chi-square) indicating strong dependence of the two tests when used in infected animals. The specificity covariance (calculated using the non-infected table on the right) was 0.004 ($p = 0.152$ by Pearson's chi-square) indicating no significant dependence.

An additional example based on porcine toxoplasmosis data is presented in Gardner *et al*. (2010).

3. DSe and DSp without a perfect reference standard

Advances in statistical methodology, specifically the development of latent class (sometimes termed "no-gold-standard") models, now allow investigators to liberate themselves from the restrictive assumption of a perfect reference test and estimate the accuracy of the candidate test(s) and the reference standard with the same data (Enoe *et al.,* 2000; Hui & Walter, 1980).

Latent class (LC) models, either using maximum likelihood or Bayesian methods, can be used for estimation of DSe and DSp when joint test results are available from multiple tests applied to animals in multiple populations (e.g. herds or geographical areas). Not all LC models for estimation of DSe and DSp will be statistically identifiable for inference. A model is identifiable if it is theoretically possible to determine the true value of model parameters after obtaining an infinite number of observations from it. In essence, this equates to having a unique set of values for the parameters of interest (DSe, DSp). Bayesian approaches are especially suited to situations where prior information is available about DSe and/or DSp and when the estimation problem is not identifiable (Branscum *et al*., 2005).

The simplest one-population LC model that is identifiable is when three conditionally independent are run on the same samples. The constraint of independence of three tests may be difficult to achieve in practice unless the target analyte differs among tests. Hence, a commonly used approach in animal health is to run two tests on all samples from animals in two populations because it is less costly and assumptions of conditional independence may be more reasonable. The two-test two-population model also requires the assumptions of constant sensitivity and specificity across the two populations, and distinct prevalences. The assumption about constant sensitivity may be difficult to verify and is unlikely to be correct if one population has clinically affected animals and the other population has subclinically affected animals because many published studies have shown that test sensitivity is greater in clinically affected animals. If one of the two populations is known to be pathogen-free (prevalence is zero) while the other population is known to have a non-zero prevalence, the former population can be used for estimation of DSp and this will facilitate estimation of DSe in the infected population.

WOAH-listed diseases where DSe and DSp have been estimated with Bayesian methods include ovine brucellosis (Praud *et al*., 2012), Q fever (Paul *et al*., 2013), trypanosomosis (Bronsvoort *et al*., 2010), bovine tuberculosis (Clegg *et al*., 2011), foot and mouth disease (Bronsvoort *et al*., 2006), African horse sickness (Guthrie *et al*., 2013) and infectious salmon anaemia virus (Caraguel *et al*., 2012).

The WinBUGS software[1] allows easy implementation of Markov-chain Monte Carlo methods for Bayesian estimation (Lunn *et al.,* 2000) and simple maximum likelihood analyses can be done using a web-based interface (Poulliot *et al.,* 2002). Prior information about model parameters used in the Bayesian analyses may affect the final estimates depending on the relative strength of evidence provided by the priors (level of prior uncertainty) and the data (uncertainty attributable to finite sample sizes). Therefore, the sources of prior information must be well documented in Bayesian analyses and it may be desirable to repeat the analysis using non-informative priors on all parameters when the model is identifiable.

Maximum likelihood – a method for estimation of the most likely values for the parameters of interest based on the value of likelihood function for the data.

Bayesian methods – incorporate relevant prior information or knowledge about one or more tests in addition to the likelihood function for the data. With large sample size, maximum likelihood and Bayesian methods will yield similar inferences.

It is important to note that LC analysis cannot correct for biases inherent in poorly designed studies. The methods should be used carefully and include a thorough evaluation of underlying assumptions (e.g. conditional dependence, constant sensitivity and specificity across populations, and distinct prevalences), the effects of use of the selected prior distributions on posterior inferences as described in the previous paragraph, and convergence of Markov chains in a Bayesian analysis (Toft *et al.,* 2005).

Example: Guthrie *et al*. (2013) estimated the DSe and DSp of a quantitative real-time PCR and conventional virus isolation (VI) for detection of African horse sickness (AHS) virus in whole blood samples using a two-test two-population Bayesian latent class model. Two populations of South African thoroughbred horses (503 AHS suspect cases and 503 healthy horses from the AHS virus controlled zone) were tested by PCR and VI. For the 503 suspect cases the joint test results were: PCR+VI+ (*n*=156). PCR+VI– (*n*=184), PCR–VI+ (*n*=0), and PCR–VI– (*n*=163). All 503 healthy horses were PCR–VI–. Various models (conditional independence and conditional dependence) were fitted to the data and a second population of healthy horses was also included in some analyses.

Models were run in WinBUGS 1.4.3 (Lunn *et al.,* 2000) with the first 5000 iterations discarded and the next 50,000 iterations used for posterior inferences (medians and 95% probability intervals for DSe and DSp. Model convergence was assessed by visual inspection of trace plots of iterated values and running multiple chains from dispersed initial values. The conditional independence model fitted with non-informative beta (1,1) priors on DSe and DSp of both tests yielded almost identical results to the model which used a highly informative beta (9999,1) prior for the DSp of VI. Estimated medians values and 95% probability intervals (sometimes termed credible intervals) in parentheses from the conditional independence model with non-informative priors were:

PCR sensitivity = 0.996 (0.977–0.999)

PCR specificity = 0.999 (0.993–1.0)

VI sensitivity = 0.458 (0.404–0.51)

VI specificity = 0.999 (0.998–1.0)

The results indicated a twofold higher DSe of PCR compared with VI and comparable DSp of both tests. For a complete description of the modelling approach see Guthrie *et al.* (2013).

4. Comparison of DSe and DSp estimates for two tests without a perfect reference standard

If a Bayesian approach is used in WinBUGS to analyse the joint test data from multiple populations, the difference in sensitivities (specificities) can be readily estimated and the probability that the sensitivity (specificity) of one test exceeds the other can be estimated with the STEP function.

Example: For the results of the Guthrie *et al.* (2013) data in Section E.3, the 95% probability intervals (PI) for DSe did not overlap but there was marked overlap in the 95% PI for DSp. The corresponding probability values obtained from the STEP function were 1 and 0.24, respectively. These values indicate certainty that the DSe differ but the probability that the DSp differ is small (less than 0.5).

1 Available at http://www.mrc-bsu.cam.ac.uk/bugs/winbugs/contents.shtml

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NB: FIRST ADOPTED IN 2014.

CHAPTER 2.2.6.

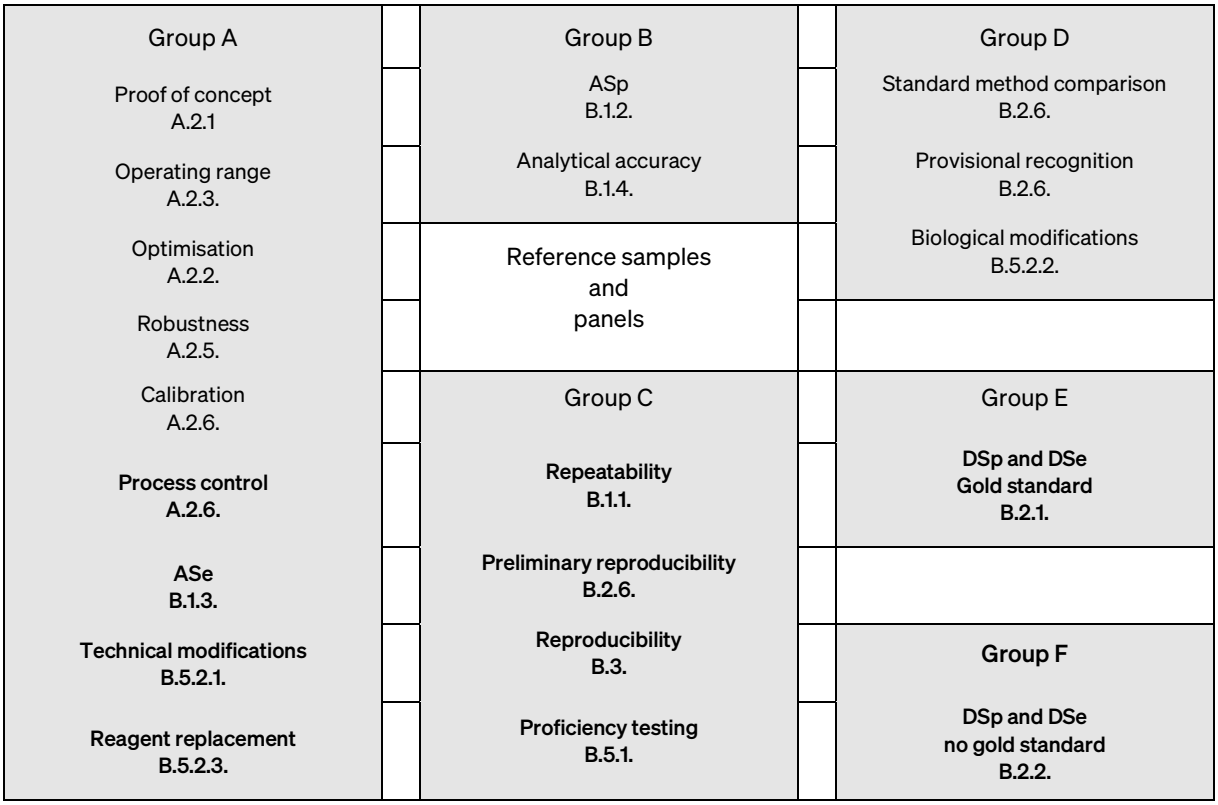
SELECTION AND USE OF REFERENCE SAMPLES AND PANELS

INTRODUCTION

The WOAH Validation Recommendations in Section 2.2 Validation of diagnostic tests *of this* Terrestrial Manual *provide detailed information and examples in support of the WOAH Validation Standard that is published as Chapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals. *The Term "WOAH Validation Standard" in this chapter should be taken as referring to that chapter.*

Reference samples and panels are essential from the initial proof of concept in the development laboratory through to the maintenance and monitoring assay performance in the diagnostic laboratory and all of the stages in between. The critical importance of reference samples and panels cannot be over-emphasised. The wrong choice of reference materials can lead to bias and flawed conclusions right from development through to validation and use. Therefore, care must be exercised in selecting reference samples and designing panels.

Fig. 1. Reference samples & panels grouped based on similar characteristics and composition. The topics and alphanumeric subheadings (e.g. Proof of Concept, A.2.1) refer to the relevant section in the WOAH Validation Standard.



ASp = Analytical specificity; ASe = Analytical sensitivity; DSp = diagnostic specificity; DSe = diagnostic sensitivity

As can be seen in Figure 1, reference samples and/or panels are mentioned throughout the WOAH Validation Standard. Reference materials are substances whose properties are sufficiently homogenous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials[1]. In the context of test method validation, reference materials or samples contain the analyte of interest in varying concentrations or activities and are used in developing and evaluating the candidate assay's analytical and diagnostic performance characteristics. In our case, analyte means the specific component of a test sample that is detected or measured by the test method, e.g. antibody, antigen or nucleic acid. These reference samples may be sera, fluids, tissues, excreta, feed and environmental samples that contain the analyte of interest and are usually harvested from infected animals and their environments. However, in some cases, they may be prepared in the laboratory from an original starting material (e.g. a dilution of a high positive serum in negative serum) or perhaps created by spiking the chosen matrix with a derived analyte (e.g. a bacterial or viral culture, a recombinant/expressed protein, or a genomic construct). Whether natural or prepared, they are used in experiments throughout the development process, carry over into the validation pathway and can be used to monitor performance throughout the lifespan of the assay.

In Figure 1, reference samples and panels are grouped based on similar characteristics and composition and these groupings will be the basis for the following descriptions. As a cross-reference, the appropriate Section of the WOAH Validation Standard is indicated under each particular application of the reference sample or panel.

Reference samples may be used for multiple purposes from the initial stages of development and optimisation, through Stage 1 and into continual monitoring and maintenance of the assay. Wherever possible, large quantities of these reference samples should be collected or prepared and preserved for long-term use. Switching reference samples during the validation process introduces an intractable variable that can severely undermine interpretation of experimental data and therefore, the integrity of the development and validation process. For assays that may target multiple species, the samples should be representative of the primary species of interest. It is critical that these samples reflect both the target analyte and the matrix in which it is found in the population for which the assay is intended. The reference materials should appropriately represent the range of analyte concentration to be detected by the assay.

It is important to emphasise that, no matter whether reference samples are selected from natural sources or prepared in the laboratory, all selection criteria or preparation procedures, as well as testing requirements, need to be fully described and put into document control. Not only is this good quality management practice, but it will provide both an enhanced level of continuity and confidence throughout the lifespan of the assay.

A. GROUP A

The question of pooling of samples to create a reference sample is often asked. If reference material is harvested from a single animal, it is important to ascertain whether or not it is representative of a typical course and stage of infection within the context of the population to be tested. If not, this could lead to bias and flawed conclusions related to validation. Pooling is a good alternative but it is imperative to pool from animals that are in a similar phase of infection. This is particularly important for antibody detection systems. Pooling also addresses the issue of the larger quantities of reference material to be stored for long term use, especially when dealing with smaller host species. Before pooling any samples, it is preferable that they be independently tested to demonstrate that they are similar with respect to analyte concentration and/or reactivity. There should be an assessment following pooling to ensure that unforeseen interference is not introduced by the pooling of multiple samples, for example differing blood types or antibody composition within the independent samples could cross-react within the pool, thus causing the pooled sample to behave differently in the specified assay than the individual samples when tested independently.

It is often difficult to obtain individual samples that truly represent analyte concentrations or reactivities across the spectrum of the expected range. Given the dynamics of many infections or responses to pathogens,

1 https://www.techlab.fr/Commun/UK_Def_MRC.asp

intermediate ranges are often very transient. In the case of antibody responses, early infection phases in individual animals often result in highly variable and heterogeneous populations of antibody isotypes and avidities. In general, these do not make good reference samples for assessing the analytical characteristics of an assay. They are nonetheless important for different types of reference panels as will be discussed later. For most applications in Group A, it is acceptable to use prepared samples that are spiked with known concentrations of analyte or a dilution series of a high positive in negative matrix to create a range of concentrations.

Whether natural or prepared, reference samples should represent the anticipated range of analyte concentrations, from low to high positive, which would be expected during a typical course of infection. A negative reference sample should be included as a background monitor. If a negative (matrix) is used as diluent for preparation of a positive reference sample (e.g. a negative serum used to dilute a high positive serum or tissue spiked with a construct), that negative should definitely be included as the negative reference sample.

As mentioned above, all reference samples should be well characterised. This includes documentation on both the pathogen and donor host. For pathogens, this may include details related to strain, serotype, genotype, lineage, etc. The source of the host material should be well described with respect to species, breed, age, sex, reproductive status, vaccination history, herd history, etc. Wherever possible, the phase of infection should be noted. This could include details related to clinical signs, antibody profiles, pathogen load or shedding, etc. Equally important, tests that are used to determine disease/infection status need to be well documented (see Section E of this chapter for further explanation). In some cases, experimental infection/exposure may be the only viable option for the production of reference material. In this case, all of the above considerations plus the experimental protocol should be detailed.

Above all else, natural or prepared, reference materials must be unequivocal with respect to their status as representing either a true positive or a true negative sample. This may require that the status be confirmed using another test or battery of tests. For example, many antibody reference sera are characterised using multiple serological tests. This provides not only confidence but additional documented characteristics that may be required when attempting to replace or duplicate this reference material in the future.

Recommendations regarding stability and storage of reference materials are available: https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-4

1. Proof of concept (WOAH Validation Standard, Section A.2.1)

The WOAH Validation Standard states that test methods and related procedures must be appropriate for specific diagnostic applications in order for the test results to be of relevance. In other words, the assay must be 'fit for purpose'. Many assays are developed with good intentions but without a specific application in mind. At the very outset, it is critical that the diagnostic purpose(s) should be defined with respect to the population(s) to be tested. The most common purposes are listed in broad terms in Section A of the WOAH Validation Standard. As such, they are inclusive of more narrow and specific applications. However, these specific purpose(s) need to be clearly defined from the outset and are critically important in the context of a fully validated assay. As will be seen in the following descriptions, clearly defining the application will have impact on both the selection of reference samples and panels and the design of analytical and diagnostic evaluations.

2. Operating range (WOAH Validation Standard, Section A.2.3) and analytical sensitivity (WOAH Validation Standard, Section B.1.3)

2.1. Analytical approaches

The operating range of the assay is the interval of analyte concentrations (amounts) over which the method provides suitable accuracy and precision. It also defines the lower and upper detection limits the assay. To establish this range, a high positive reference sample is selected. This high positive sample, natural or prepared, is serially diluted to extinction in a negative matrix representative of sample matrix of samples from animals in the population targeted by the assay. This includes antibody assays where a high positive reference serum should be diluted in a negative reference serum to create the dilution series. Analytical sensitivity (ASe) is measured by the lower limit of detection (LOD) of an analyte in an assay. The same high positive reference sample may be used to determine both the operating range and the analytical LOD.

2.2. Comparative approaches

If the intended purpose is to detect low levels of analyte or subclinical infections, it may be difficult to obtain the appropriate reference materials from early stages of the infection process. In some cases, it may be useful to determine a comparative ASe by running a panel of samples on the candidate assay and on another independent assay. Ideally this panel of samples would be serially collected from either naturally or experimentally infected animals and should represent infected animals early after infection, on through to the development of clinical or fulminating disease if possible. This would provide a relative comparison of ASe between the assays, as well as, a temporal comparison of the earliest point of detection relative to the pathogenesis of the disease.

An experiment like the one described above, provides a unique opportunity to collect reference samples representing a natural range of concentrations that would be useful for other validation purposes. Care must be taken to avoid use of such samples when inappropriate (consult Group D below). Wherever possible serial samples should be collected from at least five animals throughout the course of infection. In cases where sampling is lethal (e.g. requiring the harvest of internal organ tissues), the number of animals required would be a minimum of five per sampling event. For smaller host species, this number may need to be increased in order to collect sufficient reference material. Given that experiments like this require a high commitment of resources, it would be wise to maximise the collection of not only the currently targeted reference samples but additional materials (e.g. multiple tissues, fluids, etc.) that may be useful as reference materials in the future.

3. Optimisation (WOAH Validation Standard, Section A.2.2) and preliminary repeatability (WOAH Validation Standard, Section A.2.8)

Optimisation is the process by which the most important physical, chemical and biological parameters of an assay are evaluated and adjusted to ensure that the performance characteristics of the assay are best suited to the intended application. At least three reference samples representing negative, low and high positive may be chosen from either natural or prepared reference samples. Optimisation experiments are rather exhaustive especially when assays with multiple preparatory and testing steps are involved. It is very important that a sufficient quantity of each reference sample be available to complete all optimisation experiments. Changing reference samples during the course of optimisation is not recommended as this will result in the addition of an uncontrolled variable and a disruption in the continuity of optimisation evidence.

Assessment of repeatability should begin during assay development and optimisation stages. Repeatability is further verified during Stage 1 of assay validation (Section B.1.1). The same reference samples should be used for both processes, again to provide continuity of evidence.

4. Calibration and process controls (WOAH Validation Standard, Section A.2.6)

4.1. International, national or in-house analyte reference standards

International reference standards are highly characterised, contain defined concentrations of analyte, and are usually prepared and held by international reference laboratories. They are the reagents to which all assays and/or other reference materials should be standardised. National reference standards are calibrated by comparison with an international standard reagent whenever possible. In the absence of an international standard, a national reference standard may be selected or prepared and it then becomes the standard of comparison for the candidate assay. In the absence of both of the above, an in-house standard should be selected or prepared by the development laboratory within the responsible organisation. All of the standard reagents, whether natural or prepared, must be highly characterised through extensive analysis, and preferably the methods for their characterisation, preparation, and storage have been published in peer-reviewed publications. These reference standards should also be both stable and innocuous.

Reference standards, especially antibody, are usually provided in one of two formats. They may be provided as a single positive reagent of given titre with the expectation that the candidate assay will be standardised to give an equivalent titre. This is a straight forward analytical approach but many of these ‘single’ standards have been prepared from highly positive samples as a pre-dilution in a negative matrix in order to maximise the number of aliquots available. The drawback here is that there is no accounting for any potential matrix effect in the candidate assay as there is no matrix control

provided. The other approach is to provide a negative and a low and high positive set of reference standards that are of known concentrations or reactivities and are within the operating range of the standard method that was used to prepare them. This compensates for any potentially hidden matrix effect. In addition, this set of three acts as a template for the selection and/or preparation of process controls (discussed below).

Classically, the above standards usually have been polyclonal antibody standards and to a lesser extent, conventional antigen standards used for calibration of serological assays. However, today, reference standards could also be monoclonal antibodies or recombinant/expressed proteins or genomic constructs, if they are to be used to calibrate assays to a single performance standard.

4.2. Working standards or process controls

Working standard reagent(s), commonly known as quality or process controls, are calibrated to international, national, or in-house standard reagents. They are selected or prepared in the local matrix which is found in the population for which the assay is intended. Ideally, negative and both low and high positive working standards should be selected or prepared. Concentrations and/or reactivities should be within the normal operating range of the assay. Large quantities should be prepared, aliquoted and stored for routine use in each diagnostic run of the assay. The intent is that these controls should mimic, as closely as possible, field samples and should be handled and tested like routine samples. They are used to establish upper and lower control limits of assay performance and to monitor random and/or systematic variability using various control charting methods. Their daily performance will determine whether or not an assay is in control and if individual runs may be accepted. As such, these working reference samples are critically important from a quality management standpoint.

5. Technical modifications (WOAH Validation Standard, Section B.5.2.1)

Technical modifications to a validated assay such as changes in instrumentation, extraction protocols, and conversion of an assay to a semi-automated or fully automated system using robotics will typically not necessitate full revalidation of the assay. Rather, a methods comparison study may be done to determine if these minor modifications to the assay protocol will affect the test results. Consult Chapter 2.2.8 *Comparability of assays after minor changes in a validated test method* for statistical approaches to assay precision in the face of technical modifications.

In general, these approaches require the use of three reference samples, a negative and a low and high positive. Again these samples may be either natural or prepared. The important point to re-iterate here is that the same reference samples that were used in the developmental stages of the assay may be used to assess modifications after the method has been put into routine diagnostic use. This provides a higher level of confidence assessing potential impacts because the performance characteristics of these reference samples have been well characterised. At the very least, if new reference samples are to be used, they should be selected or prepared using the same criteria or preparation procedures established for previous materials. Again this enhances the continuity of evidence.

6. Reagent replacement (WOAH Validation Standard, Section B.5.2.3)

When a reagent such as a process control sample is nearing depletion, it is essential to prepare and repeatedly test a replacement before such a control is depleted. The prospective replacement should be included in multiple runs of the assay in parallel with the original control to establish their proportional relationship. It is important to change only one control reagent at a time to avoid the compound problem of evaluating more than one variable.

Again, it cannot be over-emphasised that any replacement reference reagent should be selected or prepared using the same criteria or preparation procedures established for previous materials. Again this enhances the continuity of evidence and confidence in the assay.

B. GROUP B

1. Analytical specificity (WOAH Validation Standard, Section B.1.2)

Analytical specificity (ASp) is the degree to which the assay distinguishes between the target analyte and other components that may be detected in the assay. This is a relatively broad definition that is often not well understood. ASp may be broken down into different elements as described below.

The choice of reference samples that are required to assess ASp is highly dependent on the specific purpose or application that was originally envisaged at the development stage of the assay. Assessment of ASp is a crucial element in proof of concept and verification of fitness for purpose.

An important element is the extent to which a method can accurately detect and or quantify the targeted analyte in the milieu of nucleic acids, proteins and/or antibodies in the test matrix. This is sometimes termed 'selectivity'. An example is the use of reference samples for tests that are designed to differentiate infected from vaccinated animals (DIVA tests).

Reference samples need to be selected and tested from i) non-infected/non-vaccinated, ii) non-infected/vaccinated, iii) infected/non-vaccinated, and iv) infected/vaccinated animals. These samples may be collected under field conditions but it is important that an accurate history be collected, ideally with respect to the animals, but at least to the herds involved, including vaccination practices and disease occurrences. Alternatively, it may be necessary to produce this material in experiments like those described in Section A.2.2 of this chapter, but including a combination of experimentally vaccinated and challenged animals. It is important to avoid use of the vaccine as capture antigen in the assay (e.g. indirect enzyme-linked immunosorbent assay [I-ELISA]), because carrier proteins in the vaccine may stimulate non-specific antibody responses in vaccinated animals that may be detected in ELISA leading to false positives in the assay. Similarly to the comparative approach described above with respect to ASe, at least five animals in each group should be considered. For smaller host species, this number may need to be increased in order to collect sufficient reference material. Depending on the DIVA test, a single experiment could be designed to assess aspects of both ASe and ASp.

A second element, sometimes termed 'exclusivity', is the capacity of the assay to detect an analyte or genomic sequence that is unique to a targeted organism, and excludes all other other known organisms that are potentially cross-reactive. This is especially true in serological assays where there are many examples of antigens expressed by other organisms that are capable of eliciting cross-reacting antibody. An attempt should be made to obtain reference samples from documented cases of infections and/or organisms that may be cross-reactive. Depending on the type of assay, these reference materials may represent the organism itself, host-derived samples, or genomic sequences. A profile for the exclusivity of the assay should be established, and expanded on a continual basis as potentially cross-reactive organisms arise.

Thirdly, a critical design consideration relates to the capacity of an assay to detect one or several strains or serovars of a species, several species of a genus, or a similar grouping of closely related organisms or antibodies. This defines the scope of detection and thus the fitness for purpose. Reference samples are required to define the scope of the assay. If for example an assay is developed as a screening test to detect all known genotypes or serotypes of a virus, then reference samples from each representative type should be tested. As new lineages or serotype variants arise, they too should be tested as part of the test profile, which should be updated on an ongoing basis.

2. Analytical accuracy of adjunct tests (WOAH Validation Standard, Section B.1.4)

Some test methods or procedures are solely analytical tools and are usually applied to further characterise an analyte that has been detected in a primary assay, for example assays like virus neutralisation tests used to type an isolated virus or characterise an antibody response. Such adjunct tests must be validated for analytical performance characteristics, but differ from routine diagnostic tests because they do not require validation for diagnostic performance characteristics. The analytical accuracy of these tests is often dependant on the use of reference reagents. These reagents, whether they are antibody for typing strains of organisms or reference strains of the organism, etc., should be thoroughly documented, as required for any other reference material, with respect to their source, identity and performance characteristics.

C. GROUP C

Reference samples in Group C may be used for a number of purposes. In the initial development stages, they may be used in the assessment of assay repeatability and both preliminary reproducibility in Stage 1 and the more in depth assessment of reproducibility in Stage 3 of the Validation Pathway. However, these samples have a number of other potential uses once the assay is transferred to the diagnostic laboratory. They may be used as panels for training and qualifying of analysts, and for assessing laboratory proficiency in external ring testing programmes. Ideally, 20 or more individual samples should be prepared in large volumes. About a quarter (25%) should be negative samples and the remainder (75%) should represent a collection of positives spanning the operating range of the assay. They should be aliquoted into individual tubes in sufficient volumes for single use only and stored for long term use. The number of aliquots of each that will be required will depend on how many laboratories will be using the assay on a routine diagnostic basis and how often proficiency testing is anticipated. Ideally, they should be prepared in an inexhaustible quantity, but this is seldom feasible. At a minimum, several hundred or more aliquots of each should be prepared at a time if the assay is intended for use in multiple laboratories. This allows assessment of laboratory proficiency by testing the same sample over many testing intervals – a useful means of detecting systematic error (bias) that may creep into long term use of an assay.

These samples may be natural or prepared from either single or pooled starting material. The intent is that they should mimic as closely as possible a true test sample. Because mass storage is always a problem, it may be necessary to store these materials in bulk and prepare working aliquots from time to time. However, if storage space is available, it is preferable to prepare and store large numbers of aliquots at one time because bulk quantities of analyte, undergoing freeze–thaw cycles to prepare a few aliquots at a time, may be subject to degradation. Because this type of reference material is consumed at a fairly high rate, they will need to be replaced or replenished on a continual basis. As potential replacement material is identified during routine testing or during outbreaks, it is advisable to work with field counterparts to obtain bulk reference material and store it for future use. Alternatively, it may be necessary to produce this material in experiments like those described in Section A.2.2 of this chapter. Similar to the comparative approach described above with respect to ASe, at least five animals in each group should be considered. For smaller host species, this number may need to be increased in order to collect sufficient reference material.

1. Repeatability (WOAH Validation Standard, Section B.1.1) and Preliminary reproducibility (WOAH Validation Standard, Section B.2.6)

Repeatability is the level of agreement between results of replicates of a sample both within and between runs of the same test method in a given laboratory. Repeatability is estimated by evaluating variation in results of replicates from a minimum of three (preferably five) samples representing analyte activity within the operating range of the assay. Consult Chapter 2.2.4 *Measurement uncertainty* for statistical approaches for measures of uncertainty for assessments of repeatability.

Reproducibility is the ability of a test method to provide consistent results, as determined by estimates of precision, when applied to aliquots of the same samples tested in different laboratories. However, preliminary reproducibility estimates of the candidate assay should be determined during developmental stages. A small panel of three (but preferably five) representing negative and both low and high positives, like those described above, would be adequate. This type of panel could also be used for a limited evaluation of reproducibility to enhance provisional acceptance status for the assay. The test method is usually assessed in one or more laboratories with a high level experience and proficiency in assays similar to the candidate assay. The panel of 'blind' samples is evaluated using the candidate assay in each of these laboratories, using the same protocol, same reagents and comparable equipment. This is a scaled-down version of Stage 3 of assay validation. Consult Chapter 2.2.4 for further explanation of the topic and its application.

2. Reproducibility (WOAH Validation Standard, Section B.3)

Reproducibility is an important measure of the precision of an assay when used in a cross-section of laboratories located in distinct or different regions or countries using the identical assay (protocol, reagents and controls). As the number of laboratories increases, so does the number of variables encountered with respect to laboratory environments, equipment differences and technical expertise. These studies are a measure of an assay's capacity to remain unaffected by substantial changes or substitutions in test conditions anticipated in multi-laboratory use (e.g. shipping conditions, technology transfer, reagents batches, equipment, testing platforms and/or environments). Each of at least three laboratories should test the same panel of 'blind' samples containing a minimum of 20 samples, representing negative and a range of positive samples. If selected negative and/or

positive samples in the panel are duplicated, within-laboratory repeatability estimates may be augmented by replicate testing of these samples when used in the reproducibility studies.

3. Proficiency testing (WOAH Validation Standard, Section B.5.1)

A validated assay in routine use in multiple laboratories needs to be continually monitored to ensure uniform performance and provide overall confidence in test results. This is assessed through external quality assurance programmes. Proficiency testing is one measure of laboratory competence derived by means of an inter-laboratory comparison; implied is that participating laboratories are using the same (or similar) test methods, reagents and controls. Results are usually expressed qualitatively, i.e. either negative or positive to determine pass/fail criteria. However, for single dilution assays, semi-quantitative results provide additional data for assessment of non-random error among the participating laboratories.

Proficiency testing programmes are varied depending on the type of assay in use. For single dilution type assays, panel sizes also vary but a minimum of five samples, representing negative and both low and high positives, like those described above, would be adequate. Proficiency testing is not unlike a continuous form of reproducibility assessment. However, reproducibility, by definition, is a measure of the assay's performance in multiple laboratories; whereas proficiency testing is an assessment of laboratory competence in the performance of an established and validated assay. Measurements of precision can be estimated for both the reproducibility and repeatability data if replicates of the same reference sample are included in this 'blind' panel. Consult Chapter 2.2.4 for further explanation of the topic and its application.

D. GROUP D

Reference samples in Group D differ from the previous Groups in that each sample in the panel should be from a different individual animal. As indicated in Chapter 2.2.8, experimental challenge studies often include repeated sampling of individual animals to determine the progression of disease, but this is a different objective than comparing performance characteristics that would be associated with diagnostic sensitivity (DSe) and diagnostic specificity (DSP) of a test method. Serially drawn samples, taken on different days from the same animal, cannot be used as representative of individual animals in populations targeted by the assay, because such samples violate the rule of independence of samples required for such studies.

Care must be taken in choosing the reference samples and the standard (independent) method used in this type of comparison to ensure that the analytes being detected (if different) demonstrate the same type of pathogenic profile in terms of time of appearance after exposure to the infectious agent, and relative abundance in the test samples chosen.

1. Standard method comparison and provisional recognition (WOAH Validation Standard, Section B.2.6)

There are situations where it is not possible or desirable to fulfil Stage 2 of the Validation Pathway because appropriate samples from the target population are scarce and animals are difficult to access (such as for exotic diseases). However, a small but select panel of highly characterised test samples representing the range of analyte concentration should be run in parallel in the candidate assay method and by an WOAH standard method, as published in the WOAH *Manuals*. If the methods are deemed to be comparable (Chapter 2.2.8), and depending on the intended application of the assay, the choice may be made that further diagnostic validation is not required. For example, if the intended application is for screening of imported animals or animal products for exotic pathogens or confirmation of clinical signs, full validation beyond a test method comparison may not be feasible or warranted.

Experience has shown that the greatest obstacle to continuing through Stage 2 of the Validation Pathway is the number of defined samples required to estimate diagnostic performance parameters with a high degree of certainty (WOAH Validation Standard, Section B.2). In some cases, provisional recognition by international, national or local authorities may be granted for an assay that has not been completely evaluated past analytical stages. The different rationales for provisional acceptance are well explained in the WOAH Validation Standard. In all cases however, sound evidence must exist for comparative estimates of DSp and DSe based on a small select panel of well-characterised samples containing the targeted analyte.

Ideally, for both comparison with a standard method or provisional recognition, a panel of, for example, 60 samples could be assembled to ensure sufficient sample size for statistical analysis of the resulting data. This would include 30 'true' negatives and 30 'true' positives. Wherever possible, the positives should reflect the range of analyte concentrations or activities expected in the target population. As mentioned above, each sample in this panel must represent an individual animal. Consult Chapter 2.2.5 for statistical approaches to determining methods comparability using diagnostic samples.

2. Biological modifications (WOAH Validation Standard, Section B.5.2.2)

There may be situations where changes to some of the biologicals used in the assay may be necessary and/or warranted. This may include changes to reagents themselves or a change to a different type of specimen which contains the same analyte as targeted in the original validated assay (e.g. from serum to saliva). At the very least, all of the analytical criteria of the validation pathway must be re-assessed before proceeding. If the analytical requisites are met, the remaining question relates to whether or not a full diagnostic validation is required. A similar approach to the above using a panel of 60 individual reference samples may be considered. However, in this case the original test method would be considered as the standard (independent) test and the modified method would be considered the candidate. Consult Chapter.2.2.5 for statistical approaches to determining methods comparability using diagnostic samples.

E. GROUP E

Reference animals and reference samples in this Group E are well described in the WOAH Validation Standard, Section B.2.1). However, there are a few points that are worth re-iterating here.

1. 'Gold standard'[2] – diagnostic specificity and diagnostic sensitivity (WOAH Validation Standard, Section B.2.1)

For conventional estimates of DSp, negative reference samples refer to true negative samples, from animals that have had no possible infection or exposure to the agent. In some situations, where the disease has never been reported in a country or limited to certain regions of a country, identification of true negative reference samples is usually not a problem. However, where the disease is endemic, samples such as these may be difficult to locate. It is often possible to obtain these samples from regions within a large country or perhaps different countries where the disease in question has either been eradicated or has never had the disease in question.

Again for conventional estimates of DSe, positive reference samples refer to true positives. Care must be taken to ensure that the sample population is representative of the population that will be the target of the validated assay. It is generally problematic to find sufficient numbers of true positive reference animals, as determined by isolation of the organism. It may be necessary to resort to samples from animals that have been tested by a combination of methods that unequivocally classify animals as infected/exposed as discussed in the WOAH Validation Standard.

The important point here is that all samples, irrespective of origin, must be documented as they would for any other reference sample so as unequivocally to classify animals as infected or exposed dependent on the fitness for purpose and proposed use of the test. As mentioned in Section A of this chapter, all reference samples should be well characterised. This includes documentation on both the pathogen and donor host. For pathogens, this may include details related to strain, serotype, genotype, lineage, etc. The source of the host material should be well described with respect to species, breed, age, sex, reproductive status, vaccination history, herd history, etc. Wherever possible, the phase of infection should be noted. This could include details related clinical signs, antibody profiles, pathogen load or shedding, etc. In some cases, experimental infection/exposure may be the only viable option for the production of reference material (see the WOAH Validation Standard, Section B.2.3). In this case, all of the above and the experimental protocol should be detailed.

Particularly relevant to these reference samples, the tests that are used to determine their so called 'true' disease/infection status need to be well documented in order to assess potential errors in estimates that may be carried over into the estimates for the candidate assay. Indeed, when using imperfect standard assays to define

2 The term "Gold Standard" is limited to a perfect reference standard as described in the WOAH Validation Standard, Section B.2.1.2, and Chapter 2.2.5, Introduction and Figure 1.

reference animal or sample status, the DSe and DSp performance estimates of the candidate assay may be flawed and often overestimated. Consult Chapter 2.2.5 for statistical considerations.

F. GROUP F

1. Animals of unknown status – diagnostic specificity and diagnostic sensitivity (WOAH Validation Standard, Section B.2.2)

Latent-class models are introduced in the WOAH Validation Standard. They do not rely on the assumption of a perfect reference (standard or independent) test but rather estimate the accuracy of the candidate test and the reference standard with the combined test results. Because these statistical models are complex and require critical assumptions, statistical assistance should be sought to help guide the analysis and describe the sampling from the target population(s), the characteristics of other tests included in the analysis, the appropriate choice of model and the estimation methods based on peer-reviewed literature. Consult Chapter 2.2.5 for statistical considerations.

Reference populations, not individual reference samples, used in latent-class studies need to be well described. This includes documentation on both the pathogen and donor host. For pathogens, this may include details related to strain, serotype, genotype, lineage, etc., that may be circulating in the population. The source of the host material should be well described with respect to species, breed, age, sex, reproductive status, vaccination history, herd history, etc. Wherever possible, the phase of infection in the populations should be noted with respect to morbidity or mortality events, recovery, etc.

As a special note, if latent class models are to be used to ascribe estimates of DSe and DSP and include multiple laboratories in the design, it is possible to incorporate an assessment of reproducibility into the assessment. As stated above, statistical advice should be sought in this respect.

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NB: FIRST ADOPTED IN 2014.

CHAPTER 2.2.8.

COMPARABILITY OF ASSAYS AFTER CHANGES IN A VALIDATED TEST METHOD

INTRODUCTION

There are many reasons why validated assays undergo changes over time. Replacement of depleted reagents is probably the most common example of a minor change to a validated assay (see C*hapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals*, Figure 1, Assay development and validation pathways*). *Small changes may be driven by: the availability of less expensive or better reagents, e.g. nucleic acid extraction reagents for molecular tests; the need for improved standardisation, e.g. changing from operator-coated to pre-coated enzyme-linked immunosorbent assay* (*ELISA*) *plates; increased throughput requirements, e.g. manual versus robotic handling, etc. Such minor changes usually require an experimental study to assess whether the performance characteristics of the validated assay are still comparable with the new procedure* (*Table 1, Figure 1*). *There are other important variables outside of the assay that may require verification, such as the nature of the target population, the species and the specimen. For example, experienced laboratory diagnosticians would be cautious about applying a competitive brucellosis antibody ELISA to cattle in Latin America if it had been validated specifically for cattle in Canada* (*Gall* et al., *1998*). *Assays are often applied to a species other than that for which they had been originally validated, e.g. domestic chickens versus wild birds or beef versus dairy cattle. Other changes included the use of different test specimens, e.g. tracheal swabs versus cloacal swabs from birds for diagnosis of avian influenza using molecular assays. Under these circumstances, a verification study would be necessary to validate the performance characteristics of the test under the new circumstances.*

Controversy exists about what constitutes a "minor" and a "major" change for a diagnostic assay. There are some changes that are regarded as major because the biological basis of the assay is fundamentally altered, for example, evolutionary changes or mutations in the nucleic acid make-up of a pathogen will require adjustments to be primers and probes. Similarly, a change from an indirect to a competitive ELISA format using a highly specific monoclonal antibody is considered a major change that would warrant complete re-validation of the assay. Table 1 provides some examples of minor and major changes that are found frequently in the use of antibody and nucleic acid detection tests. Rigorous and well designed comparability studies provide an objective assessment of whether the assay, when used with a minor change, is as comparable to, and fit for the intended use, as the validated assay. The outcome of the experimental study will determine whether or not the candidate assay requires full re-validation and consequently whether or not it can be used with confidence.

Validation is a process that determines the fitness of an assay, which has been properly developed, optimised and standardised, for an intended purpose.

Verification represents evidence that the performance characteristics, e.g. accuracy and precision of a validated assay, are comparable when used in another laboratory.

Comparability is the preferred term when performance characteristics of a new test, which has undergone a minor change, are as good as those of a validated test within statistically defined limits.

Equivalence or equivalency has historically been used in some diagnostic laboratories for comparability studies. However, the term implies a more stringent requirement than fitness for intended use and also has a specific statistical meaning. For these reasons, the term is not used in this chapter.

A. SETTING UP COMPARISON EXPERIMENTS

Table 1. Examples of change in diagnostic tests

Type of change	Changes in assay	Changes in target population or specimens
Minor	Replacement of depleted reagent, e.g. positive control sample, new batch of antigen, plates, conjugate (ELISA) Change of instrument/platform, e.g. ELISA reader, incubator/shaker, thermocycler (PCR) Change from individually coated to pre-coated ELISA plates Change from manual to robotic handling (ELISA, NAD) Change in nucleic acid extraction procedure (NAD) Use of modified primer(s) or probe (partial substitution of sequences, e.g. degeneracy) Modified PCR reaction conditions using the same primers(s) and probe(s) Addition of an extra probe within the amplified region Change in probe chemistry (NAD)	
Major	Substitution of a recombinant antigen for a cell culture-derived antigen in an ELISA Change from an indirect to a competitive ELISA using a specific monoclonal antibody Change of primers and probes for different targets in different regions of the same or different gens (NAD)	Different species, e.g. cattle versus buffalo, domestic chicken versus wild birds Different specimen types, e.g. tracheal versus cloacal swabs, blood versus semen, different tissues or organs

ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; NAD = nucleic acid detection (tests);

When setting up a comparison experiment, the procedure should be guided by the purpose of the assay (Figure 1, step 1). For example, screening assays require high diagnostic sensitivity, and it is important to compare the limit of detection. In such a case a suitable dilution range and the number of replicates of a control sample have to be determined. A sufficient quantity of well characterised control sample material needs to be produced, aliquoted and stored appropriately.

If the objective is to assess and compare repeatability it is necessary to run well characterised replicates of control samples of different analyte concentrations spanning the expected operating range of the assay, e.g. a high, medium and low analyte concentration. For practical purposes, the example given in this chapter only uses a weak-positive control sample. It is good practice to visually inspect correlation of results between both methods. For example, a scatter diagram and a histogram are easily performed and provide immediate information about the type of correlation and distribution of data (Figures 2 and 3). Basic statistics help to set upper and lower limits and evaluate results, for example for limit of detection or repeatability (Tables 2 and 3). A more sophisticated approach to comparing and analysing results from a comparison study is a Bland–Altman plot (Figure 4 and Table 4).

Figure 5 is an example of how to increase the efficiency of comparison experiments by assessing different parameters simultaneously on a single plate, e.g. assessing analytical sensitivity using dilution steps of a target analyte in triplicate, followed by assessing analytical specificity using a number of negative samples (which do not carry the target analyte), and finally assessing analytical sensitivity using a number of samples from infected animals with different analyte concentrations. The use of replicates in the same run and between runs allows estimation of repeatability (Figure 5 and 6).

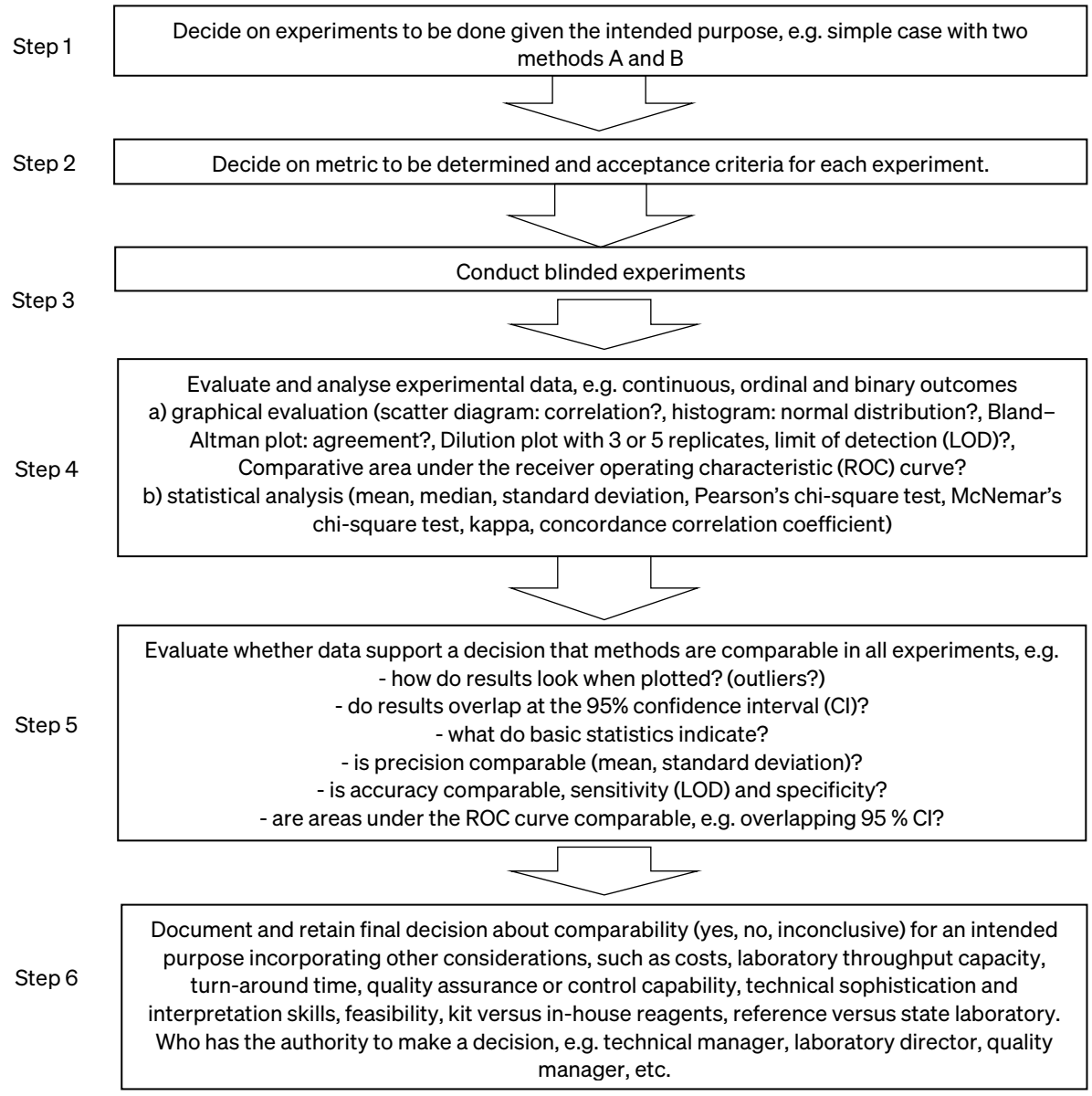
It is important to agree on acceptance criteria to evaluate the outcome of the experiment (Figure 1, step 2), e.g. confidence intervals can be asymmetric to allow for better performance of a novel assay. In this chapter a conservative 95% confidence estimate is regarded as acceptable for a limit of detection experiment (Figures 6 and 7). For comparison of repeatability, the mean and standard deviation (SD) or direct test results plus and minus

a given range can be used as acceptance criteria (Tables 3 and 4 and Figure 4). Results from a panel of infected and non-infected individuals provide information about comparative diagnostic sensitivity and specificity (Figure 8 and Tables 5 and 6).

Data used in Figures 2, 3 and 4 and Tables 2, 3 and 4 were produced using results from a repeatedly tested weak positive control sample in two TaqMan assays that target the M (M1 assay) and N (N1 assay) genes of Hendra virus.

Data for LOD experiments and plate layout in Figure 5, 6 and 7 are fictive. Data for ROC curves in Figure 8 and Tables 5 and 6 are taken from comparison experiments of different Influenza ELISAs in pigs.

Fig. 1. Factors to be considered for comparability studies of diagnostic tests.

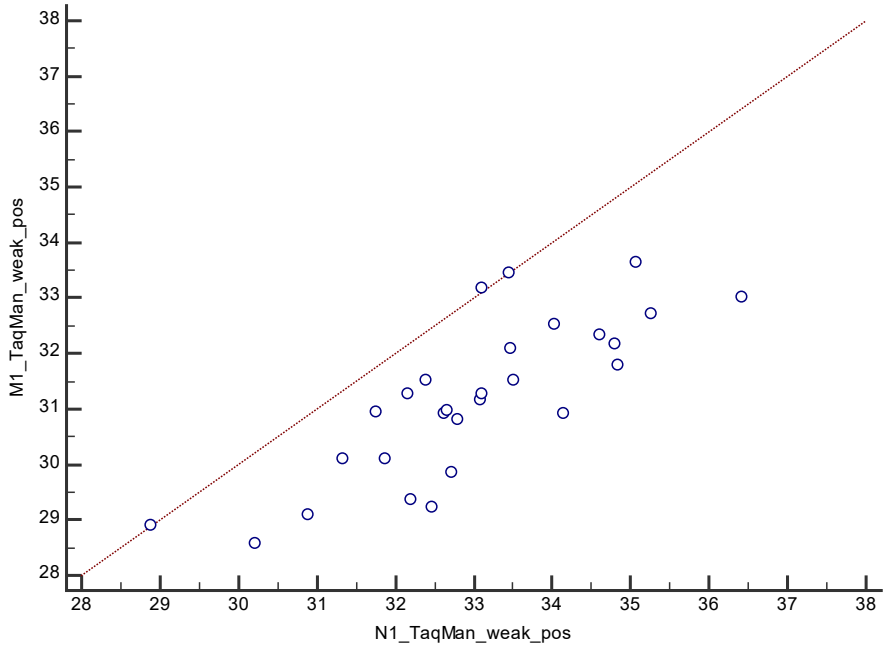


This chapter provides an overview of different approaches to the design of experiments and interpretation of results from assay comparison studies, e.g. analytical (limit of detection) and diagnostic sensitivity, analytical and diagnostic specificity, and repeatability. There are many fundamentally different tests but the examples provided stem from or refer to experiments with nucleic acid detection (NAD) and enzyme-linked immunosorbent assays (ELISAs). It can be assumed that the principles provided in this chapter are equally applicable to other tests.

B. VISUAL INSPECTION

A **Scatter diagram** is useful for visually evaluating the correlation between both methods initially, e.g. is the relationship linear or logarithmic? Are there outliers or missing values or artefacts? The example in Figure 2 uses results from a repeatedly tested weak positive control sample from two TaqMan assays that target the M (M1 assay) and N (N1 assay) genes of Hendra virus. Results show that data for the N1 assay are shifted to the lower right compared with the M1 assay, which indicates consistently higher values for N1 than for M1 assay. However the scatter diagram does not provide information about the agreement of the two tests. There is one exception to this rule, if all results for both tests would fall along the 45% diagonal line, the agreement would be 100%. In Figure 2 only three results fall along the diagonal line. In this paper we define agreement as a set of values of a candidate test that fall within the 95% CI of the results of the established test after repeated runs of the same well characterised control sample. Correlation measures the strength of the relation between measurements from these tests and is expressed as p value.

Fig. 2. Scatter diagram of a weak positive control sample after being tested 28 times in two different Hendra TaqMan assays, M1 and N1 (results expressed as cycle threshold [Ct] values).



Further analysis of results is given in Table 2 below, where r (correlation coefficient) = 0.8 and indicates a strong, positive correlation between the two methods. $p < 0.0001$ indicates that the probability that this association is due to chance is very low and the 95%CI (confidence interval) indicates that when these methods are used on a similar subject and under similar conditions, we are 95% confident that the true unknown value of r lies between 0.61 and 0.9.

Table 2. Statistical analysis of a weak positive control sample after being tested 28 times in two Hendra TaqMan assays, M1 and N1

Variable Y	M1 TaqMan weak positive
Variable X	N1 TaqMan weak positive
Sample size	28
Correlation coefficient r	0.8015
Significance level	$p<0.0001$
95% confidence interval for r	0.6112 to 0.9042

A **histogram**, where skewing to the left or to the right or other relevant characteristics such as a bimodal distribution would be detected is shown Figure 3.

Fig. 3. Histogram of a weak positive control sample after being tested 28 times in two Hendra TaqMan assays, M1 and N1 (results expressed as cycle threshold [Ct] values).

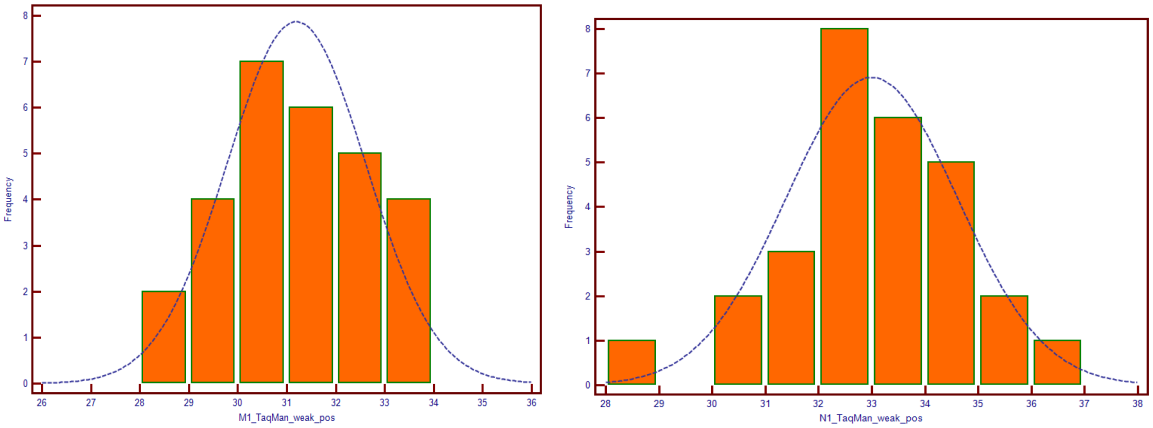


Table 3. Statistical analysis of a weak positive control sample after being tested 28 times in two Hendra TaqMan assays, M1 and N1 (results expressed as cycle threshold [Ct] values)

	M1 Taqman weak positive	**N1 Taqman weak positive**
Sample size	28	28
Lowest value	28.58	28.88
Highest value	33.63	36.42
Mean	31.19	33.00
Median	31.22	32.94
Standard deviation (SD)	1.42	1.62

C. REPEATABILITY

Assay variation can be assessed using replicates of an internal control sample when used in sequential runs over time. In this example, repeatability was compared for two different Hendra TaqMan assays targeting the N and M genes using a weak-positive internal control sample after 28 runs by the same operator on 14 days and during an 18-day period. Cycle threshold (Ct) results were summarised as the mean and standard deviation (SD) in Table 3.

Because the estimates are based on a single control sample, no formal comparison is necessary. Rather, if the predefined acceptance criteria for both assays were, e.g. 2 to 3 SDs or ± 2 to 3 Ct values then both assays would be considered comparable.

D. BLAND–ALTMAN PLOT

A very efficient way to simultaneously display and analyse results from a comparison study where 2 different measurements are made on the each sample (so called matched-pairs design) is a Tukey mean difference plot (Bland & Altman, 1999, 2007; Kozk & Wnuk, 2014). The plot is useful for revealing a relationship between the differences and the averages, evaluating any systematic biases and identifying possible outliers. Average Ct values obtained with methods A (M1 TaqMan assay) and B (N1 TaqMan assay) across a range of results are plotted along the x-axis and differences in the mean values, e.g. A minus B, are displayed on the y-axis (Figure 4, Table 4). In the example, the M1 TaqMan is compared with N1 TaqMan using results from a weak positive control sample after 28 runs. All differences are negative because the mean values for N1 TaqMan (Ct 33) are higher than M1

TaqMan (31.99) (Table 3). Subtracting N1 from M1 reveals that almost all values are negative, which indicates a systematic bias, e.g. the average difference between N1 and M1 was –1.81 (bias). Horizontal lines are drawn at the mean difference (–1.81), and at the limits of agreement, which are defined as the mean difference plus and minus 1.96 times the standard deviation of the differences, which, expressed in average Ct differences, are 0.1 to –3.71. Results in Table 4 show that the 95% CI for the mean difference (–2.18 to –1.43) exclude zero and hence, it cannot be concluded that the two methods are comparable. Adjustment of the cut-off could help to counterbalance this apparent systematic bias between both methods after corroborating these findings with larger sample numbers and over the range of expected results.

One measurement is made by each method on each sample (matched-pairs design) to compare repeatability between the two methods over the measurement range, e.g. for competitive antibody ELISAs it is known that variation increases with the decrease of the analyte concentration in the sample. A negative or weak-positive control sample may have significantly higher variation than high-positive control samples. Figure 4 shows three results that lie along the line for the 0 value, e.g. the line where results for both tests are identical because the difference is zero. These are the same results that fall on the diagonal line of equality in the scatter diagram in Figure 2 at values of 28.9, 33.10 and 33.45 for both assays.

Fig. 4. Bland–Altman plot showing differences of cycle threshold (Ct) values in two Hendra TaqMan assays, M1 and N1 for a weak-positive control sample after being tested 28 times.

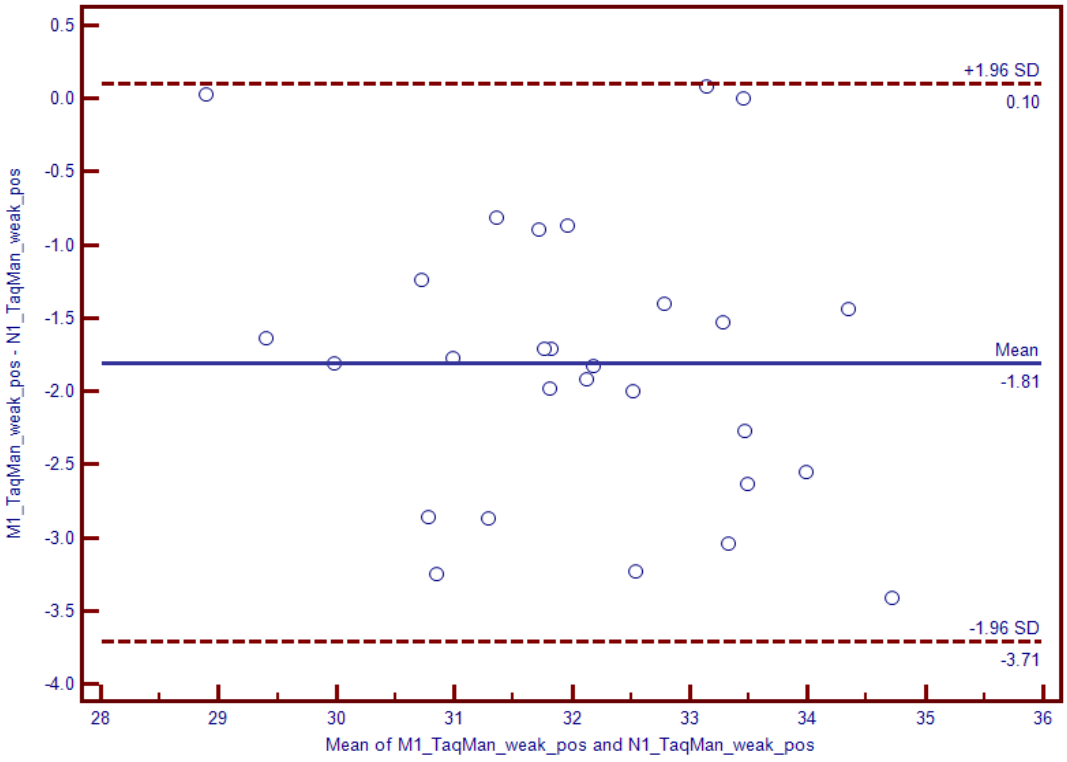


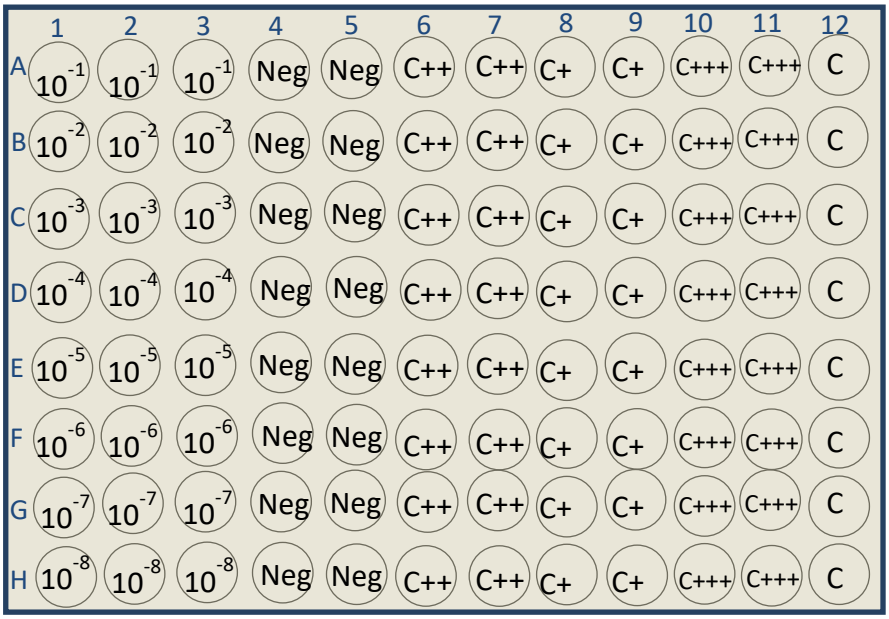
Table 4. Statistical analysis for Bland–Altman plot

Method A	M1 TaqMan weak positive
Method B	N1 TaqMan weak positive
Differences	
Sample size	28
Arithmetic mean	–1.8054
95% CI	–2.1831 to –1.4276
Standard deviation	0.9741

E. LIMIT OF DETECTION (LOD) EXPERIMENT

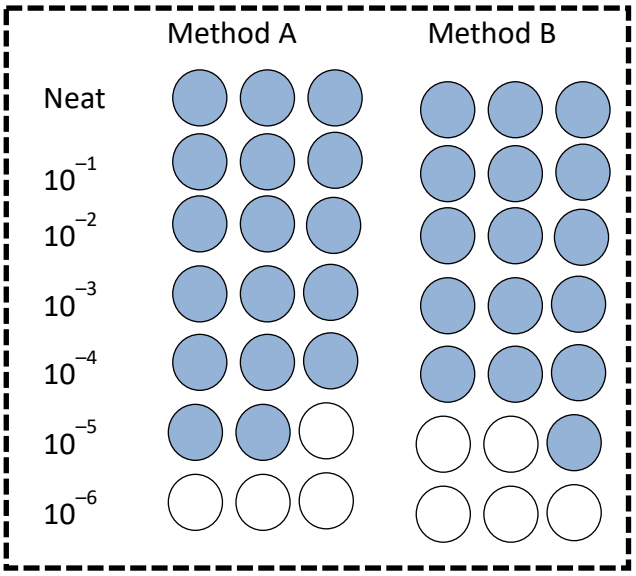
An example of a plate layout for a comparability study of a molecular test is given in Figure 5. Limit of detection (three replicates of positive samples in a dilution series from 10^{-1} to 10^{-8} [analytical sensitivity]), diagnostic specificity (negative diagnostic samples from non-infected animals or animals that have been infected with a non-target pathogen [Neg] tested in duplicate), diagnostic sensitivity (samples from field infected animals of different activity, e.g. extremely high positive [C+++], very high positive [C++], high positive [C+] and positive [C] tested in duplicate), and repeatability are assessed. Cross-contamination can be also evaluated as strong positive samples are placed next to negative samples.

Fig. 5. Layout of 96-well plate to assess analytical Se, diagnostic Se and Sp and repeatability.



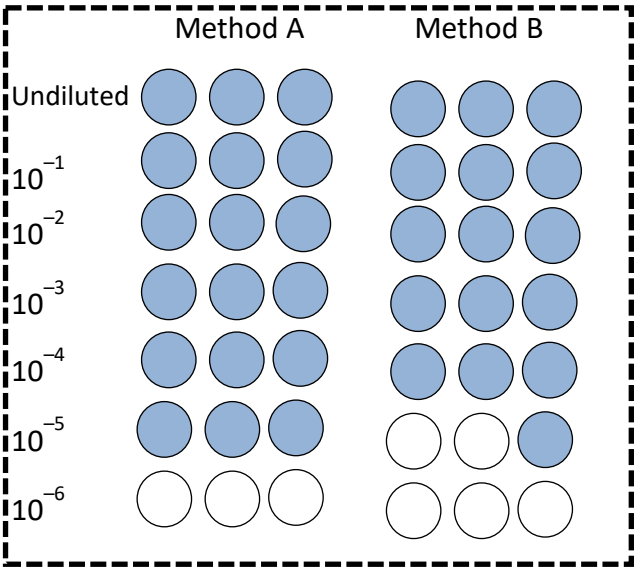
The limit of detection (LOD) is a measure of the analytical sensitivity (ASe) of an assay. The LOD is the estimated amount of analyte in a specified matrix that would produce a positive result at least a specified per cent of the time. Figures 6 and 7 represent hypothetical results of a LOD experiment. For example, in a titration using tenfold dilutions all replicates at all dilutions might show either 100% or 0% response. There are two choices at that point. The last dilution showing 100% response may be accepted as a conservative estimate of the lower limit of detection. A more accurate estimate may be obtained by a second stage experiment using narrower intervals in the dilution scheme focusing on the region between 100% and 0%. The first step is to produce, aliquot and blind a sufficient number of samples to carry out the experiment. The second step is to produce a set of analyte dilutions, preferably using sample matrix as diluent, rather than buffer, which reflect the measurement range of the method, e.g. in a tenfold dilution series, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. Practical examples often use 3–5 replicates per dilution step. Using a conservative estimate of 100% (all 3 wells at the highest dilution dark = positive) Figures 6 and 7 show comparable and non-comparable outcomes, respectively. Method A represents the validated and method B the new method.

Fig. 6. Example of a limit of detection (LOD) experiment with an acceptable outcome.



In Figure 6 at the 10^{-4} dilution all replicates from method A and method B are positive (blue). At the 10^{-5} dilution only two out of three wells are positive for method A and one out of three is positive for method B. As the limit of detection is defined as the dilution where all wells must be positive results from the 10^{-5} dilution downwards are not considered for comparability. Applying these criteria the limit of detection in Figure 6 is the same for methods A and B and therefore the two methods can be regarded as comparable.

Fig. 7. Example of a limit of detection (LOD) experiment with a non-acceptable outcome.



In Figure 7 the highest dilution of all three replicates is at 10^{-5} for method A. In contrast the highest dilution where all three replicates are still positive for method B is at 10^{-4}. Consequently, the limits of detection of methods A and B cannot be considered comparable if a single log dilution is not acceptable. It is advisable to repeat the experiment several times before making a final decision on comparability.

F. COMPARISON OF ROC CURVES

Receiver operating characteristic (ROC) analysis is a powerful method to assess and compare the overall accuracy of a diagnostic test, e.g. diagnostic sensitivity (DSe) and diagnostic specificity (DSp) at different cut-offs of one or more different or modified tests (Greiner *et al.,* 2000). The central measurement is the area under the curve (AUC), e.g. a value of 1 indicates a test with 100% DSe and 100% DSp. In this case there is perfect separation

of the values of the two groups, i.e. there is no overlapping of the distributions and the ROC curve will reach the upper left corner of the plot. In contrast a value of 0.5 indicates no discrimination between infected and non-infected individuals beyond chance; the ROC curve coincides with the diagonal indicating that the test is useless. Values between 0.5 and ≤0.7 can be considered less accurate, from 0.7 to ≤0.9 moderately accurate and from 0.9 to <1 as highly accurate (Greiner *et al.,* 2000).

Figure 8 shows results from four antibody ELISAs for influenza virus in pigs. The serum panel consisted of 168 positive and 77 negative sera using the haemaglutination inhibition as a reference test (*n*=245). When compared on these diagnostic samples the following ranking was established: test 1 (AUC = 0.988), test 4 (AUC = 0.988), test 3 (AUC = 0.929) and test 2 (AUC = 0.814) (Table 5). For this matched pair design experiment the 95% CI for the differences of the Area Under the ROC curve (AUC) can be used as an indicator for statistical significance (Table 6). In summary, the test with the best DSe and DSp and the highest area under the curve (0.988) is test 1. The result of 0.988 means that a randomly selected individual from the positive group has a test value larger than that for a randomly chosen individual from the negative group 99% of the time (Zweig & Campbell, 1993).

Another way to compare results is to evaluate AUC differences. For example for test 1 and 2 the difference between the AUC was 0.0173, for test 1 and test 3 the result was 0.058, for test 1 and 4 the result was 0.00019, for test 2 and 3 the result was 0.1150, for test 2 and 4 the result was 0.174 and for test 3 and 4 the result was 0.0586 (Table 6). The tests with the highest AUCs and smallest difference of AUCs were test 1 and test 4, e.g. both tests had an AUC of 0.988, overlapping 95% CI, the difference between the AUCs was as low as 0.00019 and a *p* value of 0.98 indicated no statistically significant difference at the 5% significance level. Lower AUC values, lack of overlapping 95%CI, significantly increased AUCs and *p* values <0.05 indicated lack of agreement of other test combinations, e.g. 1 vs 3, 1 vs 2, 2 vs 3, 2 vs 4 and 3 vs 4. Test 1 0.964 to 0.997, test 2 0.760 to 0.861, test 3 0.889 to 0.958, test 4 0.965 to 0.997.

More complex comparison studies with tests based on similar diagnostic and biological principles using frequentist and classical statistical approaches have been published (Brocchi *et al.,* 2006; Engel *et al.,* 2008). See also Chapter 2.2.5. *Statistical approaches to validation*.

Fig. 8. Comparison of receiver operating characteristic (ROC) area under the curve (AUC) for four different ELISAs to detect antibodies against influenza virus in pigs.

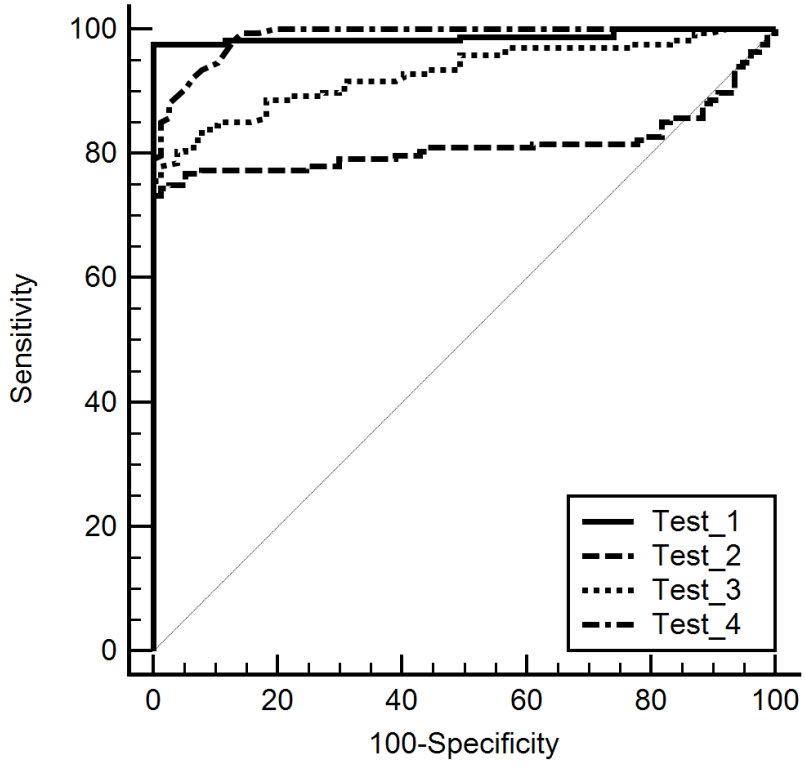


Table 5. Receiver operating characteristic (ROC) comparison of area under the curve (AUC) and p-values for four different ELISAs to detect antibodies against influenza in pigs

Parameter	Test 1	Test 2	Test 3	Test 4
Area under ROC curve	0.988	0.814	0.929	0.988

Table 6. Pairwise comparison of ROC curves

	Test 1 vs 4	Test 1 vs 3	Test 3 vs 4	Test 2 vs 3	Test 1 vs 2	Tetst 2 vs 4
Difference between AUC	0.0002	0.055	0.0589	0.115	0.173	0.174
95% CI	–0.016 to 0.016	0.025 to 0.092	0.027 to 0.090	0.069 to 0.161	0.117 to 0.230	0.118 to 0.229
Significance level	p=0.9808	p =0.0007	p =0.0003	p <0.0001	p <0.0001	p <0.0001

G. DISCUSSION AND CONCLUSIONS

Results from comparison experiments must be evaluated to reach a conclusion as to whether both methods are comparable using statistical analyses and objective assessments to assist with a final decision (NATA, 2013). However, often other criteria such as costs/equipment, throughput capacity, turn-around time, quality assurance capability, technical sophistication, acceptance in regulatory or scientific community and interpretative skills also need to be considered in decision-making (Figure 1, Step 6). It is important to have a process in place, which clarifies who has the authority to make the final decision whether methods are comparable, e.g. technical manager, laboratory director, quality manager.

For example, comparison of two TaqMan assays indicated strong and positive correlation. Repeatability of both methods was comparable when applying 2–3 SD or ± 2–3 Ct. Ct values from a weak positive-control sample were consistently lower for the M1 than for the N1 TaqMan, indicating a slightly superior Se of the M1 assay. If used as a screening test the M1 would be more suitable due to its higher Se. Results were corroborated by testing diagnostic samples from Hendra virus outbreaks between 2011 and 2013 (data not shown). On the other hand having two tests, which target different genes increases the chance of not missing a new variant. In the case of a deadly zoonosis such as Hendra virus this is an important consideration.

Results from the Bland–Altman Plot in Figure 4 and Table 4 show that statistical results sometimes are difficult to interpret, e.g. the 95% CI for the mean difference excludes zero. This might be interpreted as a lack of comparability but change in the cut-off value can be used to compensate for this outcome.

ROC analysis of 4 different swine influenza ELISAs (Figure 8 and Tables 5 and 6) indicated two tests of almost identical DSe and DSp (Tests 1 and 4). At the same time it helps to rank the performance of the other tests. Under these circumstances cost, availability and other criteria will determine the final decision as to which test is the most suitable for a designated purpose.

On the other hand, not all parameters need to be addressed in every method comparison study. For example, an assessment of cross-contamination would be necessary for changes in equipment such as manual vs robotic nucleic acid extraction procedure but not for changing a key reagent, e.g. a different primer or probe. It is good practice to decide on relevant parameters and acceptance/rejection criteria before the experiment. The most relevant question is whether the new test is fit-for-purpose.

It is in the nature of veterinary diagnostic testing to allow for flexibility when it comes to the specification of acceptance limits, e.g. when comparing two different molecular tests it could be that the difference is no more than 1, 2 or 3 Ct in 95% (99%) of tested samples, no more than 10% of the average of the 2 samples in at least 95% (99%) of tested samples, no more than 1, 2, or 3 SD of the samples. It is recommended to consider relevant parameters and limits up front although the qualitative considerations (e.g. cost, ease of testing, rapidity of

results) probably come in at the end. Whatever parameter is chosen as a basic rule the candidate test should on average not be significantly underperforming the validated test.

The data generated and the decision making process for the acceptability of the change should be clearly documented and retained to show an audit trail.

H. DATA ANALYSIS

Data were stored and grouped in Microsoft Excel. Analysis and plotting for scatter diagrams, histograms, data distribution and plotting, Bland Altman Plots, comparison graphs and ROC analysis were performed using MedCalc (MedCalc®, Version 12.4.0.0, 64 bit, Window XP/Vista 7/8, *www.medcalc.org*, Copyright 1993–2013, MedCalc software bvba).

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NB: FIRST ADOPTED IN 2016.

CHAPTER 2.2.8.

COMPARABILITY OF ASSAYS AFTER CHANGES IN A VALIDATED TEST METHOD

INTRODUCTION

There are many reasons why validated assays undergo changes over time. Replacement of depleted reagents is probably the most common example of a minor change to a validated assay (see C*hapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals*, Figure 1, Assay development and validation pathways*). *Small changes may be driven by: the availability of less expensive or better reagents, e.g. nucleic acid extraction reagents for molecular tests; the need for improved standardisation, e.g. changing from operator-coated to pre-coated enzyme-linked immunosorbent assay* (*ELISA*) *plates; increased throughput requirements, e.g. manual versus robotic handling, etc. Such minor changes usually require an experimental study to assess whether the performance characteristics of the validated assay are still comparable with the new procedure* (*Table 1, Figure 1*). *There are other important variables outside of the assay that may require verification, such as the nature of the target population, the species and the specimen. For example, experienced laboratory diagnosticians would be cautious about applying a competitive brucellosis antibody ELISA to cattle in Latin America if it had been validated specifically for cattle in Canada* (*Gall* et al., *1998*). *Assays are often applied to a species other than that for which they had been originally validated, e.g. domestic chickens versus wild birds or beef versus dairy cattle. Other changes included the use of different test specimens, e.g. tracheal swabs versus cloacal swabs from birds for diagnosis of avian influenza using molecular assays. Under these circumstances, a verification study would be necessary to validate the performance characteristics of the test under the new circumstances.*

Controversy exists about what constitutes a "minor" and a "major" change for a diagnostic assay. There are some changes that are regarded as major because the biological basis of the assay is fundamentally altered, for example, evolutionary changes or mutations in the nucleic acid make-up of a pathogen will require adjustments to be primers and probes. Similarly, a change from an indirect to a competitive ELISA format using a highly specific monoclonal antibody is considered a major change that would warrant complete re-validation of the assay. Table 1 provides some examples of minor and major changes that are found frequently in the use of antibody and nucleic acid detection tests. Rigorous and well designed comparability studies provide an objective assessment of whether the assay, when used with a minor change, is as comparable to, and fit for the intended use, as the validated assay. The outcome of the experimental study will determine whether or not the candidate assay requires full re-validation and consequently whether or not it can be used with confidence.

Validation is a process that determines the fitness of an assay, which has been properly developed, optimised and standardised, for an intended purpose.

Verification represents evidence that the performance characteristics, e.g. accuracy and precision of a validated assay, are comparable when used in another laboratory.

Comparability is the preferred term when performance characteristics of a new test, which has undergone a minor change, are as good as those of a validated test within statistically defined limits.

Equivalence or equivalency has historically been used in some diagnostic laboratories for comparability studies. However, the term implies a more stringent requirement than fitness for intended use and also has a specific statistical meaning. For these reasons, the term is not used in this chapter.

A. SETTING UP COMPARISON EXPERIMENTS

Table 1. Examples of change in diagnostic tests

Type of change	Changes in assay	Changes in target population or specimens
Minor	Replacement of depleted reagent, e.g. positive control sample, new batch of antigen, plates, conjugate (ELISA) Change of instrument/platform, e.g. ELISA reader, incubator/shaker, thermocycler (PCR) Change from individually coated to pre-coated ELISA plates Change from manual to robotic handling (ELISA, NAD) Change in nucleic acid extraction procedure (NAD) Use of modified primer(s) or probe (partial substitution of sequences, e.g. degeneracy) Modified PCR reaction conditions using the same primers(s) and probe(s) Addition of an extra probe within the amplified region Change in probe chemistry (NAD)	
Major	Substitution of a recombinant antigen for a cell culture-derived antigen in an ELISA Change from an indirect to a competitive ELISA using a specific monoclonal antibody Change of primers and probes for different targets in different regions of the same or different gens (NAD)	Different species, e.g. cattle versus buffalo, domestic chicken versus wild birds Different specimen types, e.g. tracheal versus cloacal swabs, blood versus semen, different tissues or organs

ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; NAD = nucleic acid detection (tests);

When setting up a comparison experiment, the procedure should be guided by the purpose of the assay (Figure 1, step 1). For example, screening assays require high diagnostic sensitivity, and it is important to compare the limit of detection. In such a case a suitable dilution range and the number of replicates of a control sample have to be determined. A sufficient quantity of well characterised control sample material needs to be produced, aliquoted and stored appropriately.

If the objective is to assess and compare repeatability it is necessary to run well characterised replicates of control samples of different analyte concentrations spanning the expected operating range of the assay, e.g. a high, medium and low analyte concentration. For practical purposes, the example given in this chapter only uses a weak-positive control sample. It is good practice to visually inspect correlation of results between both methods. For example, a scatter diagram and a histogram are easily performed and provide immediate information about the type of correlation and distribution of data (Figures 2 and 3). Basic statistics help to set upper and lower limits and evaluate results, for example for limit of detection or repeatability (Tables 2 and 3). A more sophisticated approach to comparing and analysing results from a comparison study is a Bland–Altman plot (Figure 4 and Table 4).

Figure 5 is an example of how to increase the efficiency of comparison experiments by assessing different parameters simultaneously on a single plate, e.g. assessing analytical sensitivity using dilution steps of a target analyte in triplicate, followed by assessing analytical specificity using a number of negative samples (which do not carry the target analyte), and finally assessing analytical sensitivity using a number of samples from infected animals with different analyte concentrations. The use of replicates in the same run and between runs allows estimation of repeatability (Figure 5 and 6).

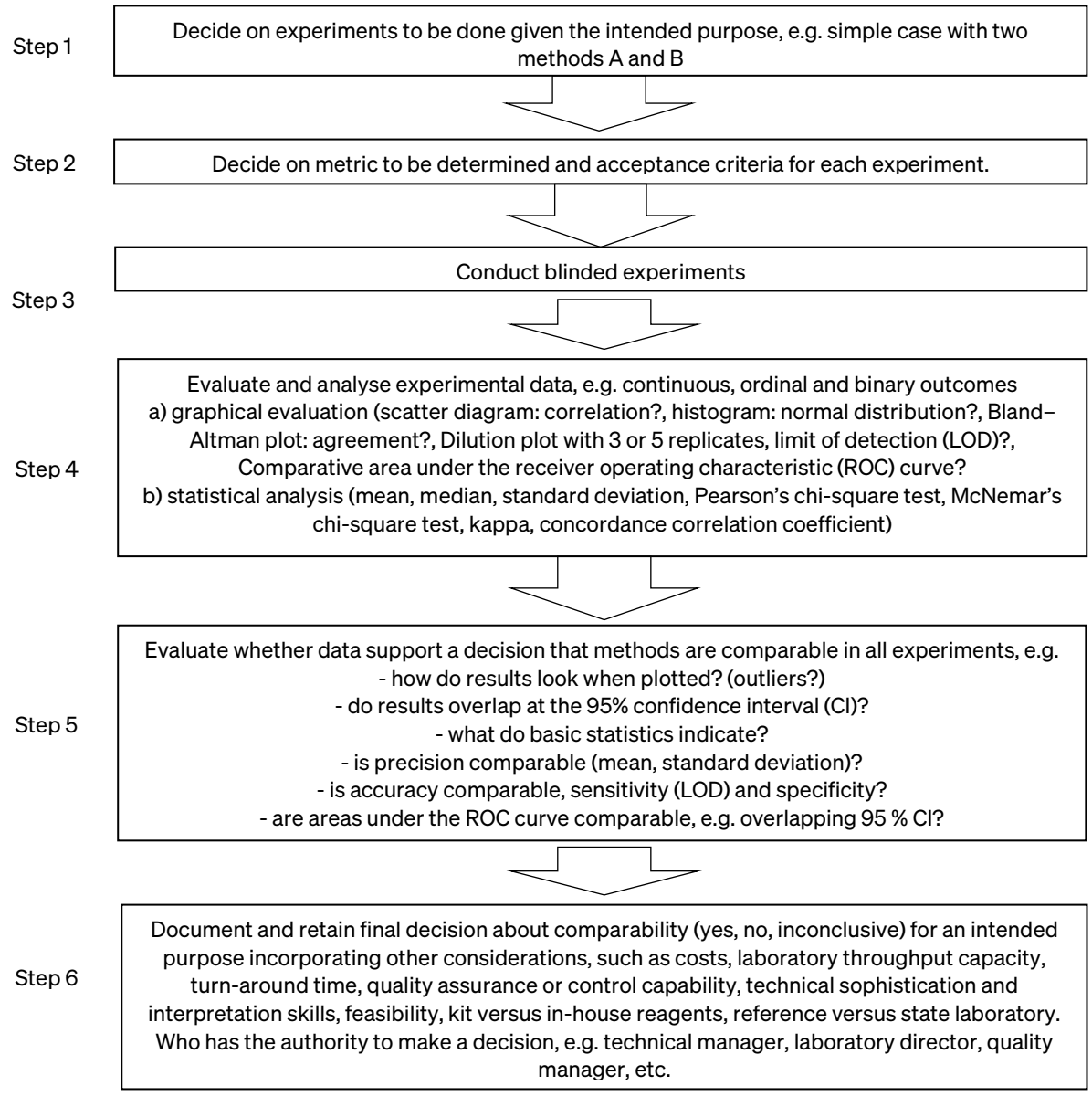
It is important to agree on acceptance criteria to evaluate the outcome of the experiment (Figure 1, step 2), e.g. confidence intervals can be asymmetric to allow for better performance of a novel assay. In this chapter a conservative 95% confidence estimate is regarded as acceptable for a limit of detection experiment (Figures 6 and 7). For comparison of repeatability, the mean and standard deviation (SD) or direct test results plus and minus

a given range can be used as acceptance criteria (Tables 3 and 4 and Figure 4). Results from a panel of infected and non-infected individuals provide information about comparative diagnostic sensitivity and specificity (Figure 8 and Tables 5 and 6).

Data used in Figures 2, 3 and 4 and Tables 2, 3 and 4 were produced using results from a repeatedly tested weak positive control sample in two TaqMan assays that target the M (M1 assay) and N (N1 assay) genes of Hendra virus.

Data for LOD experiments and plate layout in Figure 5, 6 and 7 are fictive. Data for ROC curves in Figure 8 and Tables 5 and 6 are taken from comparison experiments of different Influenza ELISAs in pigs.

Fig. 1. Factors to be considered for comparability studies of diagnostic tests.

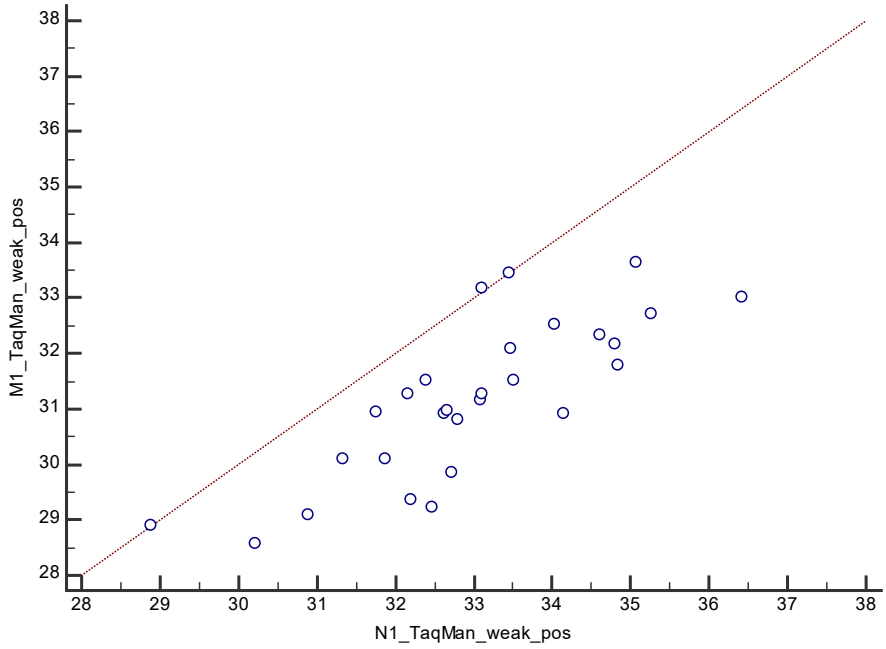


This chapter provides an overview of different approaches to the design of experiments and interpretation of results from assay comparison studies, e.g. analytical (limit of detection) and diagnostic sensitivity, analytical and diagnostic specificity, and repeatability. There are many fundamentally different tests but the examples provided stem from or refer to experiments with nucleic acid detection (NAD) and enzyme-linked immunosorbent assays (ELISAs). It can be assumed that the principles provided in this chapter are equally applicable to other tests.

B. VISUAL INSPECTION

A **Scatter diagram** is useful for visually evaluating the correlation between both methods initially, e.g. is the relationship linear or logarithmic? Are there outliers or missing values or artefacts? The example in Figure 2 uses results from a repeatedly tested weak positive control sample from two TaqMan assays that target the M (M1 assay) and N (N1 assay) genes of Hendra virus. Results show that data for the N1 assay are shifted to the lower right compared with the M1 assay, which indicates consistently higher values for N1 than for M1 assay. However the scatter diagram does not provide information about the agreement of the two tests. There is one exception to this rule, if all results for both tests would fall along the 45% diagonal line, the agreement would be 100%. In Figure 2 only three results fall along the diagonal line. In this paper we define agreement as a set of values of a candidate test that fall within the 95% CI of the results of the established test after repeated runs of the same well characterised control sample. Correlation measures the strength of the relation between measurements from these tests and is expressed as p value.

Fig. 2. Scatter diagram of a weak positive control sample after being tested 28 times in two different Hendra TaqMan assays, M1 and N1 (results expressed as cycle threshold [Ct] values).



Further analysis of results is given in Table 2 below, where r (correlation coefficient) = 0.8 and indicates a strong, positive correlation between the two methods. $p < 0.0001$ indicates that the probability that this association is due to chance is very low and the 95%CI (confidence interval) indicates that when these methods are used on a similar subject and under similar conditions, we are 95% confident that the true unknown value of r lies between 0.61 and 0.9.

Table 2. Statistical analysis of a weak positive control sample after being tested 28 times in two Hendra TaqMan assays, M1 and N1

Variable Y	M1 TaqMan weak positive
Variable X	N1 TaqMan weak positive
Sample size	28
Correlation coefficient r	0.8015
Significance level	$p<0.0001$
95% confidence interval for r	0.6112 to 0.9042

A **histogram**, where skewing to the left or to the right or other relevant characteristics such as a bimodal distribution would be detected is shown Figure 3.

Fig. 3. Histogram of a weak positive control sample after being tested 28 times in two Hendra TaqMan assays, M1 and N1 (results expressed as cycle threshold [Ct] values).

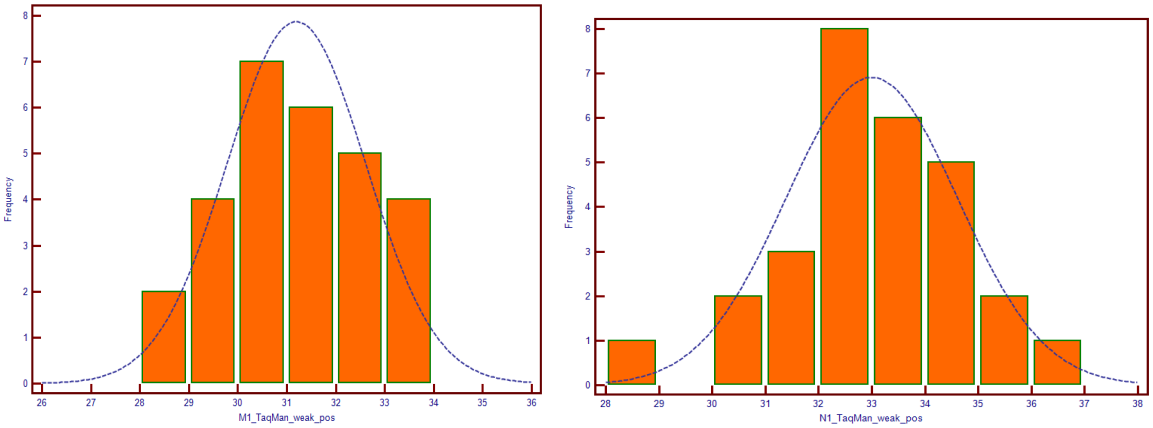


Table 3. Statistical analysis of a weak positive control sample after being tested 28 times in two Hendra TaqMan assays, M1 and N1 (results expressed as cycle threshold [Ct] values)

	M1 Taqman weak positive	**N1 Taqman weak positive**
Sample size	28	28
Lowest value	28.58	28.88
Highest value	33.63	36.42
Mean	31.19	33.00
Median	31.22	32.94
Standard deviation (SD)	1.42	1.62

C. REPEATABILITY

Assay variation can be assessed using replicates of an internal control sample when used in sequential runs over time. In this example, repeatability was compared for two different Hendra TaqMan assays targeting the N and M genes using a weak-positive internal control sample after 28 runs by the same operator on 14 days and during an 18-day period. Cycle threshold (Ct) results were summarised as the mean and standard deviation (SD) in Table 3.

Because the estimates are based on a single control sample, no formal comparison is necessary. Rather, if the predefined acceptance criteria for both assays were, e.g. 2 to 3 SDs or ± 2 to 3 Ct values then both assays would be considered comparable.

D. BLAND–ALTMAN PLOT

A very efficient way to simultaneously display and analyse results from a comparison study where 2 different measurements are made on the each sample (so called matched-pairs design) is a Tukey mean difference plot (Bland & Altman, 1999, 2007; Kozk & Wnuk, 2014). The plot is useful for revealing a relationship between the differences and the averages, evaluating any systematic biases and identifying possible outliers. Average Ct values obtained with methods A (M1 TaqMan assay) and B (N1 TaqMan assay) across a range of results are plotted along the x-axis and differences in the mean values, e.g. A minus B, are displayed on the y-axis (Figure 4, Table 4). In the example, the M1 TaqMan is compared with N1 TaqMan using results from a weak positive control sample after 28 runs. All differences are negative because the mean values for N1 TaqMan (Ct 33) are higher than M1

TaqMan (31.99) (Table 3). Subtracting N1 from M1 reveals that almost all values are negative, which indicates a systematic bias, e.g. the average difference between N1 and M1 was –1.81 (bias). Horizontal lines are drawn at the mean difference (–1.81), and at the limits of agreement, which are defined as the mean difference plus and minus 1.96 times the standard deviation of the differences, which, expressed in average Ct differences, are 0.1 to –3.71. Results in Table 4 show that the 95% CI for the mean difference (–2.18 to –1.43) exclude zero and hence, it cannot be concluded that the two methods are comparable. Adjustment of the cut-off could help to counterbalance this apparent systematic bias between both methods after corroborating these findings with larger sample numbers and over the range of expected results.

One measurement is made by each method on each sample (matched-pairs design) to compare repeatability between the two methods over the measurement range, e.g. for competitive antibody ELISAs it is known that variation increases with the decrease of the analyte concentration in the sample. A negative or weak-positive control sample may have significantly higher variation than high-positive control samples. Figure 4 shows three results that lie along the line for the 0 value, e.g. the line where results for both tests are identical because the difference is zero. These are the same results that fall on the diagonal line of equality in the scatter diagram in Figure 2 at values of 28.9, 33.10 and 33.45 for both assays.

Fig. 4. Bland–Altman plot showing differences of cycle threshold (Ct) values in two Hendra TaqMan assays, M1 and N1 for a weak-positive control sample after being tested 28 times.

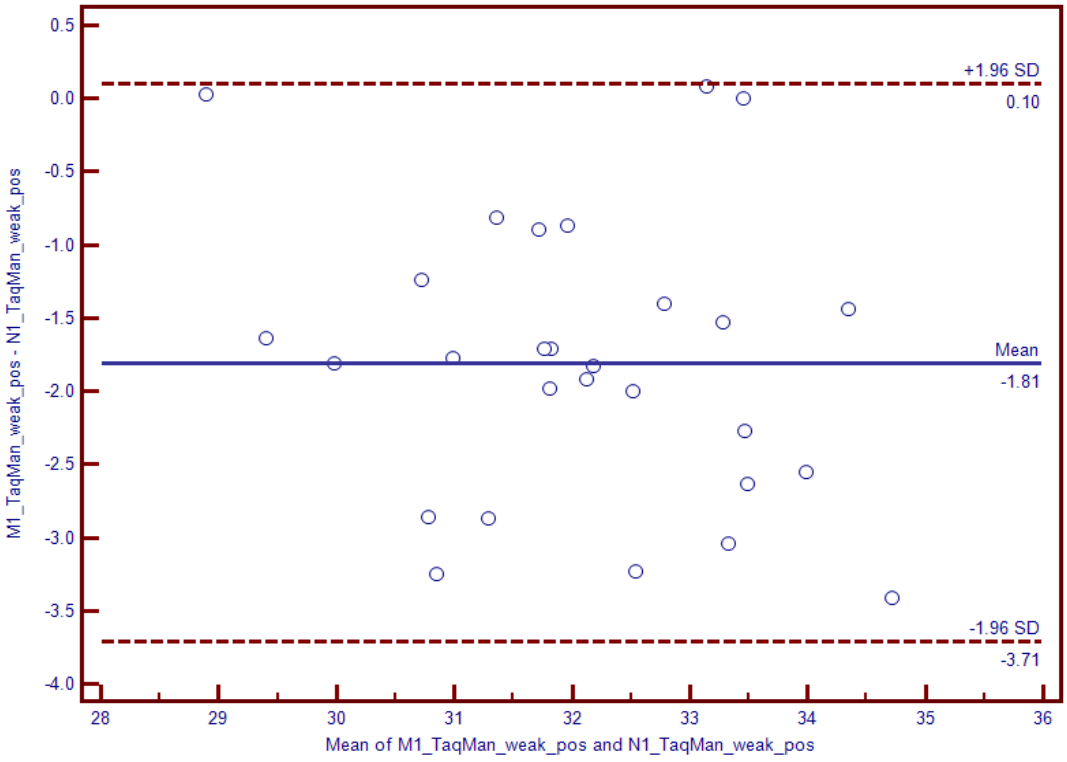


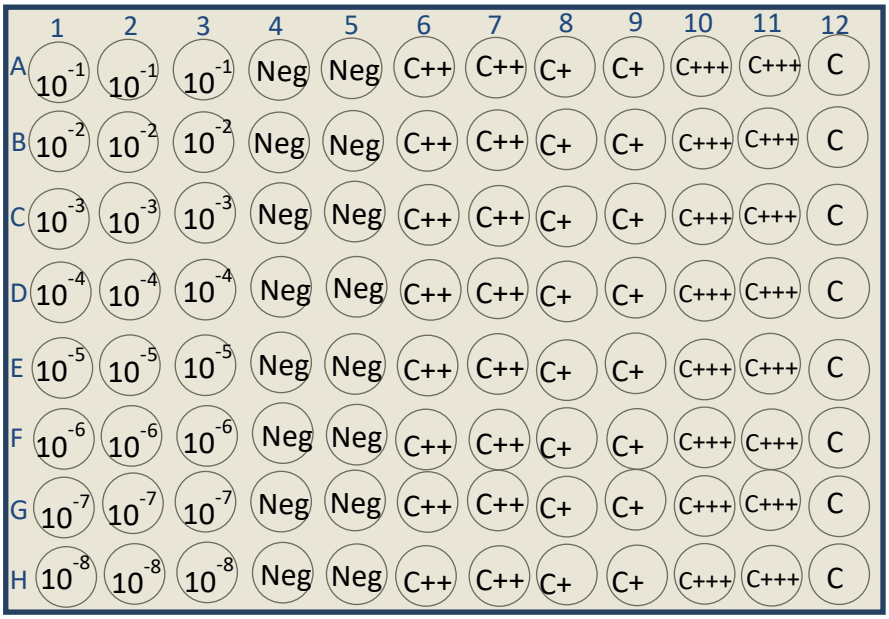
Table 4. Statistical analysis for Bland–Altman plot

Method A	M1 TaqMan weak positive
Method B	N1 TaqMan weak positive
Differences	
Sample size	28
Arithmetic mean	–1.8054
95% CI	–2.1831 to –1.4276
Standard deviation	0.9741

E. LIMIT OF DETECTION (LOD) EXPERIMENT

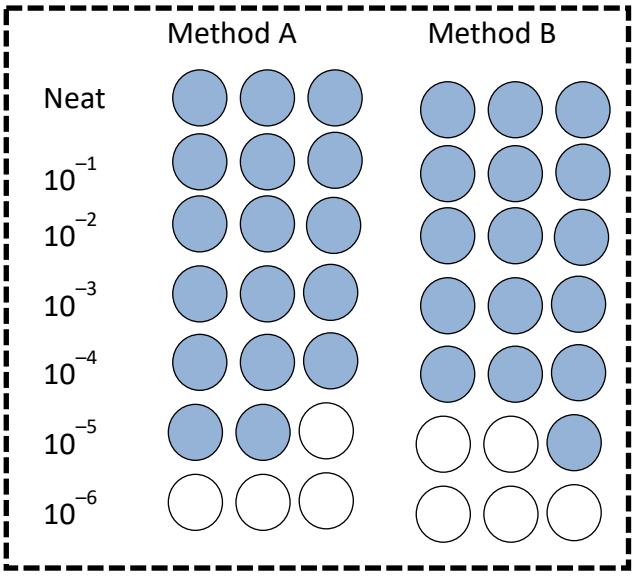
An example of a plate layout for a comparability study of a molecular test is given in Figure 5. Limit of detection (three replicates of positive samples in a dilution series from 10^{-1} to 10^{-8} [analytical sensitivity]), diagnostic specificity (negative diagnostic samples from non-infected animals or animals that have been infected with a non-target pathogen [Neg] tested in duplicate), diagnostic sensitivity (samples from field infected animals of different activity, e.g. extremely high positive [C+++], very high positive [C++], high positive [C+] and positive [C] tested in duplicate), and repeatability are assessed. Cross-contamination can be also evaluated as strong positive samples are placed next to negative samples.

Fig. 5. Layout of 96-well plate to assess analytical Se, diagnostic Se and Sp and repeatability.



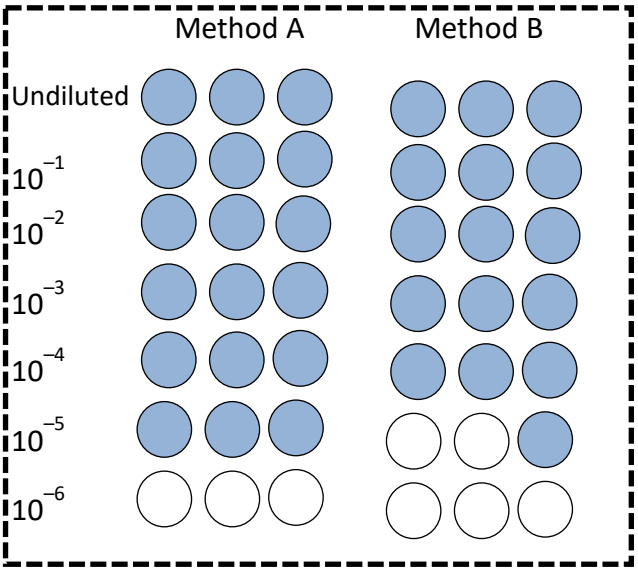
The limit of detection (LOD) is a measure of the analytical sensitivity (ASe) of an assay. The LOD is the estimated amount of analyte in a specified matrix that would produce a positive result at least a specified per cent of the time. Figures 6 and 7 represent hypothetical results of a LOD experiment. For example, in a titration using tenfold dilutions all replicates at all dilutions might show either 100% or 0% response. There are two choices at that point. The last dilution showing 100% response may be accepted as a conservative estimate of the lower limit of detection. A more accurate estimate may be obtained by a second stage experiment using narrower intervals in the dilution scheme focusing on the region between 100% and 0%. The first step is to produce, aliquot and blind a sufficient number of samples to carry out the experiment. The second step is to produce a set of analyte dilutions, preferably using sample matrix as diluent, rather than buffer, which reflect the measurement range of the method, e.g. in a tenfold dilution series, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. Practical examples often use 3–5 replicates per dilution step. Using a conservative estimate of 100% (all 3 wells at the highest dilution dark = positive) Figures 6 and 7 show comparable and non-comparable outcomes, respectively. Method A represents the validated and method B the new method.

Fig. 6. Example of a limit of detection (LOD) experiment with an acceptable outcome.



In Figure 6 at the 10^{-4} dilution all replicates from method A and method B are positive (blue). At the 10^{-5} dilution only two out of three wells are positive for method A and one out of three is positive for method B. As the limit of detection is defined as the dilution where all wells must be positive results from the 10^{-5} dilution downwards are not considered for comparability. Applying these criteria the limit of detection in Figure 6 is the same for methods A and B and therefore the two methods can be regarded as comparable.

Fig. 7. Example of a limit of detection (LOD) experiment with a non-acceptable outcome.



In Figure 7 the highest dilution of all three replicates is at 10^{-5} for method A. In contrast the highest dilution where all three replicates are still positive for method B is at 10^{-4}. Consequently, the limits of detection of methods A and B cannot be considered comparable if a single log dilution is not acceptable. It is advisable to repeat the experiment several times before making a final decision on comparability.

F. COMPARISON OF ROC CURVES

Receiver operating characteristic (ROC) analysis is a powerful method to assess and compare the overall accuracy of a diagnostic test, e.g. diagnostic sensitivity (DSe) and diagnostic specificity (DSp) at different cut-offs of one or more different or modified tests (Greiner *et al.,* 2000). The central measurement is the area under the curve (AUC), e.g. a value of 1 indicates a test with 100% DSe and 100% DSp. In this case there is perfect separation

of the values of the two groups, i.e. there is no overlapping of the distributions and the ROC curve will reach the upper left corner of the plot. In contrast a value of 0.5 indicates no discrimination between infected and non-infected individuals beyond chance; the ROC curve coincides with the diagonal indicating that the test is useless. Values between 0.5 and ≤0.7 can be considered less accurate, from 0.7 to ≤0.9 moderately accurate and from 0.9 to <1 as highly accurate (Greiner *et al.,* 2000).

Figure 8 shows results from four antibody ELISAs for influenza virus in pigs. The serum panel consisted of 168 positive and 77 negative sera using the haemaglutination inhibition as a reference test (*n*=245). When compared on these diagnostic samples the following ranking was established: test 1 (AUC = 0.988), test 4 (AUC = 0.988), test 3 (AUC = 0.929) and test 2 (AUC = 0.814) (Table 5). For this matched pair design experiment the 95% CI for the differences of the Area Under the ROC curve (AUC) can be used as an indicator for statistical significance (Table 6). In summary, the test with the best DSe and DSp and the highest area under the curve (0.988) is test 1. The result of 0.988 means that a randomly selected individual from the positive group has a test value larger than that for a randomly chosen individual from the negative group 99% of the time (Zweig & Campbell, 1993).

Another way to compare results is to evaluate AUC differences. For example for test 1 and 2 the difference between the AUC was 0.0173, for test 1 and test 3 the result was 0.058, for test 1 and 4 the result was 0.00019, for test 2 and 3 the result was 0.1150, for test 2 and 4 the result was 0.174 and for test 3 and 4 the result was 0.0586 (Table 6). The tests with the highest AUCs and smallest difference of AUCs were test 1 and test 4, e.g. both tests had an AUC of 0.988, overlapping 95% CI, the difference between the AUCs was as low as 0.00019 and a *p* value of 0.98 indicated no statistically significant difference at the 5% significance level. Lower AUC values, lack of overlapping 95%CI, significantly increased AUCs and *p* values <0.05 indicated lack of agreement of other test combinations, e.g. 1 vs 3, 1 vs 2, 2 vs 3, 2 vs 4 and 3 vs 4. Test 1 0.964 to 0.997, test 2 0.760 to 0.861, test 3 0.889 to 0.958, test 4 0.965 to 0.997.

More complex comparison studies with tests based on similar diagnostic and biological principles using frequentist and classical statistical approaches have been published (Brocchi *et al.,* 2006; Engel *et al.,* 2008). See also Chapter 2.2.5. *Statistical approaches to validation*.

Fig. 8. Comparison of receiver operating characteristic (ROC) area under the curve (AUC) for four different ELISAs to detect antibodies against influenza virus in pigs.

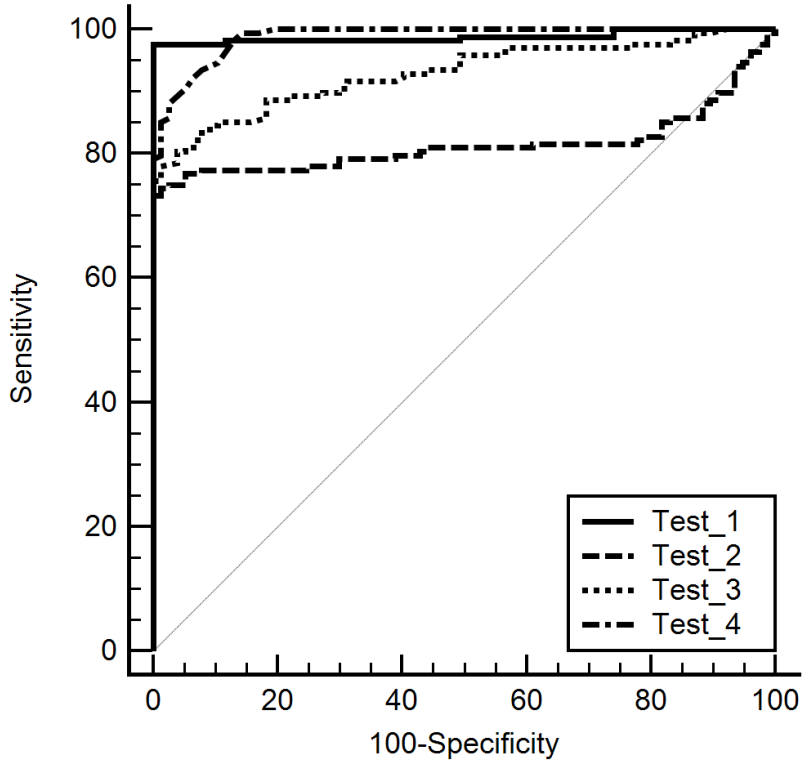


Table 5. Receiver operating characteristic (ROC) comparison of area under the curve (AUC) and p-values for four different ELISAs to detect antibodies against influenza in pigs

Parameter	Test 1	Test 2	Test 3	Test 4
Area under ROC curve	0.988	0.814	0.929	0.988

Table 6. Pairwise comparison of ROC curves

	Test 1 vs 4	Test 1 vs 3	Test 3 vs 4	Test 2 vs 3	Test 1 vs 2	Tetst 2 vs 4
Difference between AUC	0.0002	0.055	0.0589	0.115	0.173	0.174
95% CI	–0.016 to 0.016	0.025 to 0.092	0.027 to 0.090	0.069 to 0.161	0.117 to 0.230	0.118 to 0.229
Significance level	p=0.9808	p =0.0007	p =0.0003	p <0.0001	p <0.0001	p <0.0001

G. DISCUSSION AND CONCLUSIONS

Results from comparison experiments must be evaluated to reach a conclusion as to whether both methods are comparable using statistical analyses and objective assessments to assist with a final decision (NATA, 2013). However, often other criteria such as costs/equipment, throughput capacity, turn-around time, quality assurance capability, technical sophistication, acceptance in regulatory or scientific community and interpretative skills also need to be considered in decision-making (Figure 1, Step 6). It is important to have a process in place, which clarifies who has the authority to make the final decision whether methods are comparable, e.g. technical manager, laboratory director, quality manager.

For example, comparison of two TaqMan assays indicated strong and positive correlation. Repeatability of both methods was comparable when applying 2–3 SD or ± 2–3 Ct. Ct values from a weak positive-control sample were consistently lower for the M1 than for the N1 TaqMan, indicating a slightly superior Se of the M1 assay. If used as a screening test the M1 would be more suitable due to its higher Se. Results were corroborated by testing diagnostic samples from Hendra virus outbreaks between 2011 and 2013 (data not shown). On the other hand having two tests, which target different genes increases the chance of not missing a new variant. In the case of a deadly zoonosis such as Hendra virus this is an important consideration.

Results from the Bland–Altman Plot in Figure 4 and Table 4 show that statistical results sometimes are difficult to interpret, e.g. the 95% CI for the mean difference excludes zero. This might be interpreted as a lack of comparability but change in the cut-off value can be used to compensate for this outcome.

ROC analysis of 4 different swine influenza ELISAs (Figure 8 and Tables 5 and 6) indicated two tests of almost identical DSe and DSp (Tests 1 and 4). At the same time it helps to rank the performance of the other tests. Under these circumstances cost, availability and other criteria will determine the final decision as to which test is the most suitable for a designated purpose.

On the other hand, not all parameters need to be addressed in every method comparison study. For example, an assessment of cross-contamination would be necessary for changes in equipment such as manual vs robotic nucleic acid extraction procedure but not for changing a key reagent, e.g. a different primer or probe. It is good practice to decide on relevant parameters and acceptance/rejection criteria before the experiment. The most relevant question is whether the new test is fit-for-purpose.

It is in the nature of veterinary diagnostic testing to allow for flexibility when it comes to the specification of acceptance limits, e.g. when comparing two different molecular tests it could be that the difference is no more than 1, 2 or 3 Ct in 95% (99%) of tested samples, no more than 10% of the average of the 2 samples in at least 95% (99%) of tested samples, no more than 1, 2, or 3 SD of the samples. It is recommended to consider relevant parameters and limits up front although the qualitative considerations (e.g. cost, ease of testing, rapidity of

results) probably come in at the end. Whatever parameter is chosen as a basic rule the candidate test should on average not be significantly underperforming the validated test.

The data generated and the decision making process for the acceptability of the change should be clearly documented and retained to show an audit trail.

H. DATA ANALYSIS

Data were stored and grouped in Microsoft Excel. Analysis and plotting for scatter diagrams, histograms, data distribution and plotting, Bland Altman Plots, comparison graphs and ROC analysis were performed using MedCalc (MedCalc®, Version 12.4.0.0, 64 bit, Window XP/Vista 7/8, *www.medcalc.org*, Copyright 1993–2013, MedCalc software bvba).

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NB: FIRST ADOPTED IN 2016.

SECTION 2.3.

VETERINARY VACCINES

INTRODUCTORY NOTE ON WOAH RECOMMENDATIONS FOR VETERINARY VACCINES/BIOLOGICALS

WOAH has adopted a formal standard entitled Principles of Veterinary Vaccine Production*. Its most recent version, adopted in May 2022, may be found in this* Terrestrial Manual *as chapter 1.1.8. Further chapters deal with sterility and freedom from contamination of biological materials* (*Chapter 1.1.9*) *and the management of vaccine banks* (*Chapter 1.1.10*)*. In addition, many of the general principles of laboratory management set out in Chapter 1.1.1* Management of veterinary diagnostic laboratories *are applicable to vaccine production, including such areas as accountability, executive management, infrastructure, human resources and compliance.*

Section 2.3 Veterinary Vaccines *provides more detailed information for vaccine design, manufacture and regulation in accordance with the principles set out in the standards. All of the chapters have been written by experts in the respective fields, and have been subjected to an extensive process of consultation in arriving at the final texts.*

None of these chapters should be used in isolation*. Each is designed to complement and inform the application of Chapter 1.1.8* Principles of Veterinary Vaccine Production *in specific situations.*

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CHAPTER 2.3.1.

THE APPLICATION OF BIOTECHNOLOGY TO THE DEVELOPMENT OF VETERINARY VACCINES

INTRODUCTION

The practice of vaccination for the prevention of animal disease has been used for centuries and has proven to be a powerful tool for the alleviation of animal suffering as well as the economic well being of producers of animal products. Up until 15–20 years ago, vaccines had changed little from those originally pioneered by Jenner and Pasteur. Since that time there have been significant changes in the types of vaccines available owing to a number of factors, including compatibility with eradication programmes and international trade policies as well as cost-effectiveness of production. The first recombinant vaccines were introduced in the late 1980s to control Aujeszky's disease and rabies in wild-life (Pastoret et al., 1988) and are the forerunners of similar products that will be available in the future.

The approaches used in the development of vaccines have expanded rapidly as the result of increased knowledge of the mechanisms by which protective immunity is induced, and the explosion of genomic data on both pathogens and their hosts. The associated evolution of new technology in the field of molecular biology and immunology has furthermore had a large impact on the development of new vaccine strategies and the quality of the products that are produced. It has enabled the design of vaccines targeted for the control and eradication of specific pathogens within the framework of regional, national and international requirements. Use of recombinant technologies bring with it the need for the application of a risk–benefit assessment that takes into account the specific aspects that need to be considered, particularly with respect to safety (see Appendix 1.1.8.1 Risk analysis for biologicals for veterinary use*, to Chapter 1.1.8* Principles of veterinary vaccine production *of this* Terrestrial Manual*).*

This chapter describes a range of technologies that are used to produce vaccines engineered for a specific purpose. The categorisation is aimed to assist the reader to understand the technologies employed, but it should be recognised that the categories are not mutually exclusive (i.e. reverse genetics may be used to produce a chimeric vaccine). In principle, the technologies can be used to change the target pathogen itself to alter its properties by deletion, insertion, other genetic modifications, or they can be used to modify the isolated genes or coding sequences of pathogens to produce specific immunogens associated with protective immunity.

A. REVERSE GENETICS

The development of a reverse genetics system for a range of different RNA and DNA viruses has revolutionised the field of virology by making it possible to introduce designed mutations, insertions and deletions into the viral genome of live viruses. It has by now been used in a range of applications that include the attenuation of viruses, the modification of host specificity and the generation of replication-deficient viruses. These strategies have also been applied to the development of new vaccine strategies and are widely used in the characterisation of the structure and function of individual viral genes and coding sequences.

The technology of reverse genetics involves the generation of a cloned copy of complementary DNA (cDNA) from RNA by reverse transcription *in vitro*, manipulating DNA *in vitro* followed by generating the modified live virus by transfection of permissive cells with the cloned DNA(s).The technology was first demonstrated using the bacteriophage Q-Beta, a positive-strand RNA virus (Taniguchi, 1978). Subsequently, a large number of positive-strand RNA viruses including severe acute respiratory syndrome (SARS) coronavirus, with large genomes have

been rescued, which has helped in the study of the biology of these viruses and the development of new live attenuated viral vaccines. For example, reverse genetics was used to develop an infectious clone of transmissible gastroenteritis virus (TGEV), which induced lactogenic immunity in immunised pigs (Sola *et al.,* 2003). This novel technique has also been used to develop a modified porcine respiratory and reproductive syndrome virus, which can be used as a DIVA (differentiating infected and vaccinated animals) vaccine to help differentiate between vaccinated and infected pigs (de Lima *et al*., 2008).

Owing to the inherent characteristics of negative-strand RNA viruses, it took years of work before this technique could be developed and used for generating engineered viruses containing negative-strand RNA genomes. Reverse genetics was first developed for influenza virus, a segmented negative-strand RNA virus. Since then, this technique has been successfully used for the generation of a number of RNA viruses containing either unsegmented or segmented negative-strand genomes. For example, the use of this technique has led to the development of a vaccine for avian influenza virus in which the engineered virus contained a haemagglutinin (HA) gene from an H5N1 virus and a neuraminidase (NA) gene from a H2N3 virus, using a H1N1 backbone (Meeusen *et al*., 2007). The resultant inactivated H5N3 virus vaccine induced complete protection in birds against highly pathogenic H5N1 challenge. A reverse genetics strategy has also been used in the development of Foot and mouth disease, Classical swine fever, and Newcastle disease vaccines (see chapters 3.1.8, 3.8.3 and 3.3.14, respectively). More recently, reverse genetics systems have been developed for segmented double-stranded RNA viruses including bluetongue virus (BTV) introducing the possibility of new vaccine development strategies for these viruses (Boyce *et al*., 2008).

Disabled infectious single-cycle (DISC) vaccine involve the deletion of an open reading frame coding for a key protein involved in the viral replication or viral capsid formation (Widman *et al*., 2008). The DISC virus is isolated in cells expressing the key protein, thus providing the missing protein in *trans*. Such virus, when injected in animals, can complete only one round of replication without producing a progeny virus. Vaccines based on DISC viruses are more stimulatory than a killed virus vaccine and are devoid of problems associated with live vaccines.

B. RECOMBINANT VECTOR TECHNOLOGY

Advances in reverse genetics, genomics, and proteomics have facilitated the identification of mechanisms of virulence, host-pathogen interactions, and protective antigens from many pathogenic microorganisms and also the development of suitable vehicles/vectors for delivery of these antigens to the host. The availability of bacterial and viral genome sequences has facilitated the rapid construction of defined deletions in the genomes of a wide variety of pathogens, which not only results in attenuation, but also creates space for the insertion of foreign genes coding for antigens from heterologous microbes. In general, live bacterial or viral vectors share several characteristics including ease and economy of production, non-integration into the host genome, stability and a reasonable capacity to insert genes coding for heterologous antigens. In addition, as with any live vaccine, the vector should be avirulent and the impact of immunity to the vector should be evaluated.

1. Bacterial vectors

In general bacterial vectors are attenuated by deletion of genes required for key metabolic processes or genes associated for virulence. Although they are not used routinely in animals, rapid progress is being made in developing and evaluating different bacteria as vectors. For several years, BCG (Bacillus Calmette–Guerin) and *Salmonella* have been developed as vectors for delivering vaccine antigens to animals and the latter has been used for the generation of live vaccine strains for poultry. There are currently a number of other bacterial vectors being developed based on commensal microorganisms (*Lactococcus, Streptococcus, Lactobacillus* and *Staphylococcus*) or attenuated pathogenic organisms (*Shigella, Bacillus, Yersinia, Vibrio, Cornebacteria,* and *Bordetella*), all of which are being evaluated for their ability to induce protective immunity.

2. Viral vectors

Most viral vectors are developed using viruses that are associated with mild or no disease or using viruses that are pathogenic but attenuated by deletion of virulence genes. Replication competent virus vectors, which can produce progeny virus, as well as replication-defective virus vectors, which do not produce progeny virus, have been developed and evaluated as vaccine delivery vehicles. A number of commercial vaccines based on DNA virus vectors, including poxviruses and herpesviruses, have been successfully licensed for use in veterinary medicine (reviewed in Gerdts *et al*., 2006). These include vectors based on vaccinia virus, canarypox virus, fowlpox

virus and turkey herpesvirus. A number of viral vectors have been developed or are in the process of being developed, improved and evaluated. These include RNA viruses such as Venezuelan equine encephalitis virus, Newcastle disease virus and feline foamy virus as well as DNA viruses such as adenoviruses, herpesviruses and pox viruses, Fowl pox and canary pox vectors have been used in a wide range of applications (MacLachlan *et al.,* 2007; Swayne, 2009) whereas replication-deficient human adenovirus vectors have been used very successfully in the development of FMDV vaccines (Rodriguez & Grubman, 2009). Licensed canary pox vaccines include vaccines against equine influenza and feline leukaemia. Other licensed vector vaccines include the herpesvirus of turkeys vectored with an infectious bursal disease insert.

C. GENE-DELETED VACCINES

The knowledge of specific virulence factor(s) of a pathogen and the availability of recombinant DNA technology has facilitated the creation of specific gene-deleted pathogens for use as live vaccines. The approach of creating and testing defined gene deletions ultimately aids in reducing the pathogenicity/virulence of the organism without affecting the immunogenicity. Such gene-deleted organisms can be used as vaccines as they retain the immunogenic features of the wild-type organism but cannot cause disease. However, to be effective as viable vaccine(s), these organisms should be genetically stable, easy to grow and easy to administer. So far, genes involved either in determining virulence or regulating key metabolic pathways of the organism(s) have been targeted for such deletions.

This approach has been successfully used to create several live attenuated vaccine strains of bacterial pathogens that are genetically stable, safe to use and induce better protection than killed vaccines. Gene-deleted *Salmonella enterica* serovar *typhimurium* and serovar *enteritidis* vaccines have been licensed for use in poultry (Babu *et al*., 2004; Meesun *et al*., 2007) and similarly, an *aroA* gene-deleted *Streptococcus equi* vaccine has been licensed for use in horses (Jakobs *et al*., 2000; Meesun *et al*., 2007).

This technology has also been successfully used to create live attenuated vaccine strains of viral pathogens that are genetically stable and can be used as marker vaccines to differentiate between vaccinated and infected animals. A double gene (gE and TK) deleted pseudorabies virus marker vaccine has been licensed for use in pigs (Ferrari *et al*., 2000; Meesun *et al*., 2007) and similarly, gE deleted a bovine herpesvirus-1 marker vaccine has been licensed for use in cattle (Meesun *et al*., 2007; Van Oirschot *et al*., 1996).

D. CHIMERIC VIRUSES

Chimeric viruses are defined as recombinant viruses that may contain parts of two closely related viral genomes. For example, a chimeric virus could be one that contains structural genes of one viral serotype and nonstructural genes of another serotype of the same virus. Alternatively, a chimeric virus would be one that contains part of the genome from different members belonging to the same virus family. In principle, chimeric viruses display the biological characteristics of both the parent viruses. One of the main advantages of this approach is that a single dose of chimeric virus delivers the complete repertoire of antigens closely resembling the pathogen(s), which can induce protective immune response against multiple viral pathogens belonging to or different serotypes of the same viral pathogen.

The availability of infectious full-length complementary DNA (cDNA) clones of different RNA viruses using reverse genetics technologies has led to novel vaccine development strategies. Chimeric pestiviruses have been constructed using an infectious cDNA clone containing the classical swine fever virus (CSFV) genome or the bovine viral diarrhoea virus (BVDV) genome backbones. In one instance, a chimeric pestivirus was constructed by replacing the BVDV E2 coding sequence in the infectious DNA copy of BVDV strain CP7 with the corresponding E2 coding sequence of CSFV strain Alfort 187 (Reimann *et al.,* 2004). Another chimeric virus was constructed by replacing the CSFV E2 coding sequence in the infectious DNA copy of CSFV vaccine strain C with the corresponding E2 coding sequence from BVDV (van Gennip *et al.,* 2000). These chimeric viruses appeared to be attenuated in pigs, induced complete protection against CSFV challenge and helped to discriminate between vaccinated and infected pigs (Reimann *et al.,* 2004; van Gennip *et al.,* 2000).

In another application, chimeric porcine circoviruses (PCVs) have been isolated using infectious cDNA clones of porcine circovirus PCV1 in which the capsid protein from pathogenic PCV2 was used to replace the corresponding gene in the nonpathogenic PCV1 strain (PCV1-2). Likewise, the capsid gene in PCV2 has been replaced with the

gene from PCV1 (PCV2-1). The chimeric PCV1-2 virus appeared to be attenuated in pigs and induced protective immunity against wildtype PCV2 challenge in pigs (Fenaux *et al.,* 2004).

This platform technology has also been used to generate chimeric flaviviruses. In one example, a chimeric virus was generated by replacing the coding sequences for structural proteins of yellow fever YF-17D virus with those of West Nile virus (WNV). A single dose of this chimeric flavivirus vaccine induced both cell-mediated and humoral immune responses in horses, and provided protection against WNV challenge without causing any clinical illness (Meeusen *et al*., 2007). The same platform technology has also been used to develop human vaccines for Japanese encephalitis virus, WNV and Dengue virus. Although chimeric flavivirus vaccines have shown satisfactory safety profiles and protective efficacies, caution should be used in evaluating chimeric viruses for the change in virulence.

E. SUBUNIT VACCINES

Subunit vaccines composed of semi-pure or purified proteins have been commercially available since the early 1980s, with subunit components produced by recombinant DNA technology available since the 1990s (Cohen, 1993; Rhodes *et al.,* 1994; Ulmer *et al*., 1993; 1995). The latter have attracted growing interest and activity since that time. Subunit vaccines do not include live recombinant vector technologies, which provide the delivery of recombinant proteins *in vivo.* The field of genomics and related areas has revolutionised the manner in which microbial antigens are identified. Since the first bacterial genome was sequenced in 1995, there has been a huge increase in the number of bacterial, viral, and parasite genomes for which genome sequences are available. Indeed, virtually all pathogens of animals are represented and those pathogens that are not can readily be obtained in less than a day. More importantly, the development of the bioinformatics resources and tools that are required to analyse these genomes has proceeded in parallel and it is now relatively easy to identify surface-exposed antigens, specific B- and T-cell epitopes, etc. There is no requirement to have the ability to grow the organism in culture: for example subunit vaccines for *Piscirickettsia salmonis,* a salmonid pathogen, have been developed even though the organism could not be readily grown (Kuzyk *et al.,* 2001).

The production of subunit antigens can be achieved by both conventional biochemical or recombinant DNA technologies. The latter involves a range of prokaryotic and eukaryotic expression systems including yeast, insect cell and plants (Chichester & Yusibov, 2007) by means of a variety of integrated or transient expression strategies. Biochemical techniques remain useful in some cases where recombinant expression is not appropriate, such as antigens requiring complex assembly (e.g. fimbriae), or when post-translational modification is necessary. For example, *Campylobacter jejuni* is one bacterial species that glycosylates many surface proteins and, as such, they are best produced in *C. jejuni* rather than heterologous expression systems, although *Escherichia coli* strains have been engineered to carry out the same function (Wacker *et al.,* 2002). An excellent example of a subunit vaccine composed of an authentic antigen that retained three dimensional structure was the original *E. coli* K99 vaccine for calf scours, which was tested three decades ago (Acres *et al.,* 1979). This product was based on the K99 fimbrial antigen, which could readily be extracted from cells by heat treatment, thus retaining the three dimensional fimbrial structure. Another example includes a baculovirus expressed vaccine against porcine circovirus type 2 (Fachinger *et al.,* 2008). In a number of cases the expressed subunit vaccine protein spontaneously assembles into well defined particles that may resemble virus particles. These virus-like particles (VLPs) are a sub-class of subunit vaccines (Roy & Noad, 2008) and their application in vaccine development is reviewed in section F. Vaccines containing ORF 2 protein of PCV-2 expressed in baculovirus has been commercialised.

Subunit vaccines could have some advantages over live attenuated and inactivated vaccines, including the ability to induce strong humoral and cell-mediated immune response. The vaccines furthermore have an excellent safety profile, and can be used in combination with other subunit vaccines. However, efficacy is dependent on the protective immunity being induced by inoculation of a single or set of defined recombinant proteins. Experience has shown this may be affected by the gene expression system used. In addition, subunits vaccines may be expensive to produce for some glycoproteins and may require the use of adjuvants to enhance immune responses.

One of the biggest advantages of subunit vaccines is that they are generally compatible with DIVA strategies as long as the antigen is not being used as a marker. In the case of bovine herpesvirus, glycoprotein gD has been successfully used in subunit vaccine formulations. Although immunisation with gD has proven to be protective at an individual animal level (Harland *et al.,* 1992; van Drunen Littel-van den Hurk *et al*., 1994), it has not reduced the prevalence of the virus in the field, thus limiting its use. Subunit vaccines against a variety of other respiratory and

enteric viruses, including BVDV, BRSV, PI3, rotavirus, and coronavirus have been successfully tested, although none of these is used on a commercial basis. Bacterial subunits have arguably proven more successful than their viral counterparts. This is because of the cost-effectiveness of growth of both conventional and recombinant organisms and a general requirement for a Th_2-biased immune response in many cases. Recombinant vaccines are commercially available for respiratory pathogens such as *Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae* based upon the leukotoxins produced by these organisms, as well as transferrin-binding proteins. *Actinobacillus pleuropneumoniae* is an excellent example of a vaccine composed of subunits selected on cross-serotype reactivity, thus providing broad-spectrum protection against disease. Likewise a vaccine against atrophic rhinitis containing a non-toxic derivative of *Pasteurella multocida* dermonecrotic toxin produced by a genetically modified *Escherichia coli* strain together with a conventional *B. bronchiseptica* bacterin has been commercialised.

Vaccines against CSF demonstrate well the need to target recombinant technology to a particular purpose. Conventional, live attenuated CSF vaccines have a rapid onset of immunity and are effective at preventing transmission of infection (Van Oirschot, 2003) but have the disadvantage that it is not possible to differentiate infected pigs from those that have merely been vaccinated. Commercial E_2 subunit vaccines have a slower onset of immunity and reduce but do not prevent viral shedding. However, they enable a DIVA strategy to be followed thereby facilitating a 'vaccination to live' strategy. Their use is therefore likely to be of particular benefit in high value breeder pigs where the vaccine can be used to limit the clinical impact of the disease whilst allowing individual infected pigs to be identified and eliminated.

F. VIRUS-LIKE PARTICLES

Virus-like particles (VLPs) are supra-molecular structures composed of one or more recombinant proteins. The particles form through self-assembly and typically range from 20 to 100 nm in size. Depending on the origin they can be icosahedral or rod-like in structure (reviewed in Jennings & Bachmann, 2008). VLPs offer the advantage of formulating the vaccine antigen in a particulate structure, thereby increasing the immunogenicity of the vaccine. VLPs can be used as either vaccine itself or as carrier for genetically fused (chimeric), incorporated or covalently linked antigens. VLPs have been extensively studied for the past 20 years, with human vaccines against hepatitis B virus (Zuckerman, 2006) and human papillomavirus (Stanley, 2008) commercially available and several vaccines for veterinary application in development. These include vaccine for bluetongue virus, rota- and parvovirus.

VLPs offer several advantages for the use as vaccine including a high safety profile, the similarity to viral and bacterial structures, the ability for large-scale production and the possibility of combining the VLPs with other adjuvants. Typically, immunisation with VLP induces rapid and strong antibody responses. Similar to viruses and bacteria multiple copies of the vaccine antigens are displayed in a highly repetitive and ordered, quasi crystalline-structure (Bachmann & Zinkernagel, 1996), which can cross-link the B cell receptor resulting in activation of the B cell and subsequent induction of T-independent IgM responses (Thyagarajan *et al*., 2003).). Furthermore, this enables interaction with the complement system resulting in increased phagocytosis. Moreover, the particulate structure of VLPs also enhances uptake by dendritic cells and subsequent cross-presentation of the antigen. It was demonstrated by Lenz *et al.* (2001) that cross-presentation of particulate antigens was more effective than presentations of soluble antigens. However, the induction of T cell responses overall is still not as effective as those induced by live vaccines. To overcome this, VLPs have been successfully combined with molecular adjuvants such as CpG ODN and single-stranded RNA. Other VLPs have been demonstrated to directly stimulate dendritic cells (DC.) For example, L1 protein-VLPs of papillomavirus have been shown to directly activate DC.

VLPs can either be used as vaccine itself or be used as carrier for recombinant antigens, either incorporated, directly, genetically fused or covalently linked. For example, the bovine rotavirus virus protein 6 (VP6) forms VLPs that are highly immunogenic and already confer protection against challenge infection (Redmond *et al.,* 1993). However, using the VP4 and VP7, other antigens can be covalently linked to the VP6 particles and used for immunisation (Redmond *et al.,* 1993). Other prominent examples include the hepatitis B surface antigen VLPs (HBsAg-VLP), human immunodeficiency virus 1, dengue virus VLPs, norovirus VLPs, and influenza A VLPs. Examples of VLPs used as carriers include the well characterised hepatitis B core antigen VLPs (HBcAg VLPs; [Blanchet & Sureau, 2006; Pumpens & Grens, 2001]) as carrier for the influenza a M2 protein (M2-HBcAg [Jegerlehner *et al.,* 2002]), or malaria B- and T-cell epitopes (Nardin *et al.,* 2004). While mostly administered systemically, some VLPs-based vaccines already have been tested for mucosal administration.

G. DNA VACCINES

Immunisation with DNA represents a relatively new vaccination strategy that is based on a simple concept. DNA vaccines can be defined as antigen-encoding bacterial plasmids that are capable of inducing specific immune responses upon inoculation into a suitable host. Immunisation is accomplished by the uptake of purified plasmid in the host cells, where it persists extrachromosomally in the nuclei. Subsequent expression of protein results in the presentation of normally processed or modified forms of the protein to the immune system. In the host, native forms of the proteins have access to presentation pathways by class I major histocompatibility (MHC) antigens in addition to class II MHC presentation, which results in a balanced immune response. The use of pure plasmid DNA offers many advantages over other vaccine delivery vehicles. One of the greatest advantages is the ability of DNA vaccines to induce both humoral and cell-mediated immune responses, which is critical for protection from many diseases. There is also evidence that DNA vaccines can induce long-term immunity, which is a further requirement for vaccine efficacy. As the vector itself does not induce immune responses, DNA vaccines can be repeatedly administered without the interference of antibodies. From a technical viewpoint, DNA vaccines are easy to engineer, produce and purify, so new DNA vaccines can be constructed and evaluated in animal models within months. DNA vaccines are very stable and therefore have a long shelf life and can be transported without a cold chain. The safety of DNA vaccines has been established in various trials in several species including humans (Bagarazzi *et al.,* 1998; Kim *et al.,* 2001).

As soon as the concept of DNA immunisation began to be explored, this technology was found to be very effective in rodents, but initially did not perform as well in larger species. However, recent progress has resulted in the development of DNA vaccines in a number of target species (Carvalho *et al*., 2009; Redding & Weiner, 2009). Currently, four veterinary DNA vaccines have been licensed, against growth hormone releasing hormone for swine in Australia, infectious haematopoietic necrosis virus for salmon in Canada, WNV for horses and melanoma for dogs in the USA (Kutzler & Weiner, 2008). To achieve higher efficacy in large animal species, optimisation at various levels has been required, including (i) vector modifications; (ii) protein engineering to modify subcellular localisation; (iii) improvements in DNA delivery routes and methods; (iv) inclusion of adjuvants, as a gene or co-administered agent, and (v) antigen targeting to antigen-presenting cells (APCs). It is likely that the often unsatisfactory efficacy of DNA vaccines in large animals was caused by inefficient transfection, as well as 'immunological blandness', of the administered plasmids. The use of a needle-free vaccine delivery device was shown to reduce the effective dose of an experimental polyvalent DNA vaccine for avian influenza, and to rapidly deliver repeated injections in poultry (Rao *et al.,* 2009).

H. ANTIGEN DELIVERY AND MOLECULAR ADJUVANTS

Adjuvants are substances that enhance immune responses when co-administered with antigens. They are a critical component of killed (recombinant and subunit) vaccines, which are often poorly immunogenic. Adjuvants can be classified into two broad categories based on their presumed mechanism of action: i) delivery systems, and ii) immunostimulatory adjuvants. Delivery systems include many conventional adjuvants and numerous particulate adjuvants and will be discussed separately below.

Despite the importance of adjuvants in vaccines, their mechanisms of action remains poorly understood. Recent advances in the understanding of innate immunity have provided important clues on the molecular mechanisms of action of immunostimulatory adjuvants. In this regard, immune cells express a variety of receptors, collectively termed pattern recognition receptors (PRR) that broadly detect conserved microbial components referred to as pathogen-associated molecular patterns (PAMPs). A number of PRR have been described including toll-like receptors (TLR); e.g. TLR 9 recognises bacterial CpG nucleic acid motifs, natural agonist of TLR7/8, single-stranded viral RNA (oligoribonucleotides, ORN) strongly activate innate immune responses in mice, humans and are particularly potent in large animals; TLR4 agonist such as lipolysaccharide (LPS) that is known for its powerful immunostimulatory and adjuvant properties, but unfortunately this molecule is highly toxic; nucleotide oligomerisation domain (NOD)-like receptor (NLL), retinoic acid inducible gene (RIG)-like receptors (RLL), and C-type lectin receptors (CLR), all of which detect microbial components. Engagement of these receptors by their agonists leads to a cascade of molecular and cellular events that result in activation of innate immunity, which directs antigen-specific adaptive immunity. Of these receptors, TLR agonists are most widely explored and have shown great promise as adjuvants. Interestingly, the live-attenuated yellow fever vaccine 17D (YF-17D), one of the most successful vaccines available, activates TLR2, 7, 8 and 9 (Querec *et al.,* 2006), suggesting that the success of at least some of the live vaccines may be the result of their ability to activate TLRs. This has generated a great deal of interest in TLR agonist as adjuvants.

The existing paradigm in the veterinary vaccine industry of "one adjuvant-one vaccine," is driven partly by costs associated with including more than one adjuvant in a vaccine; however, it may severely limit the efficacy of potentially safe vaccine candidates, and may explain, at least in part, why some vaccines or adjuvants have only achieved suboptimal efficacy. Evidence is slowly accumulating to indicate that multiple adjuvants may offer more than can be achieved with a single adjuvant. For example, although CpG ODNs are a good adjuvant, they can have even greater adjuvant activity if formulated or coadministered with other compounds, such as particulates, mineral salts, saponins, liposomes, cationic peptides, polysaccharides and bacterial toxins and the synthetic polymers, polyphosphazenes (Wack *et al.,* 2008).

The adjuvant effect of microparticles has been known for some time and has been previously reviewed (Mutwiri *et al.,* 2005). Particulate delivery systems are thought to promote trapping and retention of antigens in local lymph nodes. In addition, microparticles facilitate antigen presentation by APCs via both MHC class I and MHC class II restricted processing and presentation pathways. One of the main advantages of microparticles for targeted antigen delivery is that they can be a flexible delivery platform that can be used to deliver both antigens and immunostimulatory molecules.

Other potential antigen delivery systems include polyphosphazenes, a class of synthetic polymers consisting of a backbone with alternating phosphorus and nitrogen atoms and organic side groups attached to each phosphorus (Mutwiri *et al.,* 2007). Immune stimulating complex (ISCOM), which is a small 40 nm nanoparticle composed of saponin (adjuvant), lipids and antigen, and has been described as an antigen delivery system because it not only has adjuvant activity and but also the ability to target APC (Morein *et al.,* 2004). A commercial ISCOM-based vaccine against equine influenza has been licensed for many years (Heldens *et al*., 2009).

I. VACCINE DELIVERY

Vaccine delivery comprises a diverse range of approaches with the overall goal of providing vaccines for mass vaccination during disease outbreaks and delivery of vaccines to wildlife. Oral vaccines used in rabies vaccination of wildlife such as foxes were initially based on attenuated rabies vaccine viruses such as the ERA strain, but concerns that these vaccines could rarely cause rabies (Fehlner-Gardiner *et al*., 2008) have largely lead to their replacement (http://ec.europa.eu/food/animal/diseases/eradication/rabies_pres_19.pdf). In Canada, a live adenovirus-vectored rabies vaccine with a good safety profile (Knowles *et al.,* 2009) is currently being used in rabies vaccination campaigns directed at controlling skunk and raccoon rabies (Rosatte *et al*., 2009). The live oral vaccinia-rabies glycoprotein (V-RG) vaccine is widely used elsewhere, and attempts are being made to optimise the vaccine baits for efficacy for other species including dogs (Cliquet *et al.,* 2008). Rabies infection in stray dogs and wildlife represents a serious problem for humans globally, and research for safer, more stable and efficacious live oral rabies vaccines continue (Faber *et al.,* 2009). Other possibilities for mass vaccination using edible plant-made vaccines have been actively investigated, but in spite of biotechnological advances in plant expression of vaccine antigens, no commercial products for oral use have been identified to date (Rice *et al.,* 2005).

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CHAPTER 2.3.2.

THE ROLE OF OFFICIAL BODIES IN THE INTERNATIONAL REGULATION OF VETERINARY BIOLOGICALS

SUMMARY

The official control of veterinary biologicals is vested in various national and regional organisations that differ in their approach to ensuring the quality, safety and efficacy of the products. International harmonisation of regulations concerning biological products did not begin until well after those concerning chemically defined products. The first biological products for veterinary use were not manufactured and distributed until the end of the nineteenth century. They were often produced under unsophisticated conditions, and distributed or sold without any control other than those of their manufacturers. Later, each manufacturer developed its own standards. In Europe, these were subject to State controls as early as 1895 for certain diagnostic products (e.g. mallein, tuberculin) or vaccines. Gradually the conditions for international harmonisation of standards evolved, beginning with the comparative testing of products being issued by different European laboratories. It was only in the second half of the twentieth century that national laws covering veterinary biologicals were imposed. These demanded that precisely defined techniques be followed before biological products for veterinary use could receive relevant regulatory approval. This was followed by considerable efforts to harmonise these national regulations, first at the regional level (notably in Europe and the Americas) then at the global level, notably by the World Organisation for Animal Health (WOAH, founded as OIE) with the publication of the first edition of the WOAH Manual of Standards for Diagnostic Tests and Vaccines *in 1989.*

World-wide harmonisation of standards for veterinary biologicals will be of help to Chief Veterinary Officers who must follow the instructions given in the WOAH Terrestrial Animal Health Code*, as they apply to all biological products for use in international trade. It will also be of assistance to vaccine producers, who have expressed their wish for world-wide harmonisation of rules for relevant regulatory approvals so as to simplify and facilitate marketing of their products. Evidently, it will also be of interest to farmers and to consumers, who would benefit from the fact that the quality, safety and efficacy of the products that they use would have been assured to a uniformly high level.*

The different sections of this chapter will review and compare regulations from the regions of the world that have made most progress in this field and will describe current attempts at harmonising these regulations on an international scale.

Note: *In this chapter the term 'veterinary biological' will be taken to include vaccines for use in animals, antisera for use in animals, and* in-vivo *diagnostic preparations.*

A. REGULATION OF VETERINARY BIOLOGICALS: PRESENT SITUATION

1. In Japan

1.1. Introduction

Medicinal products that are exclusively used for animals, including veterinary biologicals, are under the jurisdiction of the Ministry of Agriculture, Forestry and Fisheries, and securing their quality, efficacy and safety is stipulated in the Law on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical devices (the Pharmaceutical and Medical Device Law) (Government of

Japan, Act No.145 of 10 August, 1960). Since 1972, registration procedures have been developed with the aim of rationalising the examination procedure and facilitating the acquisition of approval. These procedures are stipulated in the Pharmaceutical and Medical Device Law and other related regulations. Consequently, a speedy and simple examination procedure has been achieved with emphasis on the assurance of quality, safety and efficacy. The Food Safety Commission was established in the Cabinet Office, Government of Japan, in July 2003. In the case of approval examination, re-examination and re-evaluation, all veterinary vaccines, except products of dogs and cats, must comply with the Food Safety Basic Law.

1.2. Regulations governing the approval and quality assurance of veterinary biologicals

1.2.1. Application for relevant regulatory approval

A person intending to distribute veterinary biologicals shall obtain the license for marketing approval holders and the marketing approval for each biological from the Minister of Agriculture, Forestry and Fisheries after an intensive reviewing process by the National Veterinary Assay Laboratory (NVAL). The application for the marketing approval should be submitted with designated appended documents, such as those on clinical studies. Of the latter, the safety studies and clinical studies using the target animal species should have been performed in compliance with GLP (Good Laboratory Practice) and GCP (Good Clinical Practice). It is also required, as a condition of approval, that the manufacturing and quality control of the product complies with GMP (Good Manufacturing Practice). A marketing approval holder shall comply with the standard of GQP (Good Quality Practice) and the GVP (Good Vigilance Practice).

Regulatory approval to manufacture veterinary biologicals is issued by the Minister of Agriculture, Forestry and Fisheries and must be renewed every 5 years.

1.2.2. National assay

After receiving the marketing approval, each batch of the veterinary biological must be examined by the NVAL according to the procedures of the Assay Standard for Veterinary Biological Products, except for those approved as “seed-lot system”-based products (Anon, 2002b; Makie, 1998). A marketing approval holder must apply it to the national assay.

1.2.3. Re-examination and re-evaluation

Re-examination is performed on newly approved veterinary biologicals. Usually a field assessment of the veterinary vaccines is conducted over a period of 6 years following initial approval of the veterinary vaccines. During this investigation, the efficacy and the safety are re-examined.

Re-evaluation is performed on availability of approved products after marketing by order of the Minister of Agriculture, Forestry and Fisheries. This may happen when it is suspected that a veterinary biological does not conform to the latest standards of veterinary biological products.

1.2.4. Minimum requirement of veterinary biological products

The examinations provide information about the consistency of the manufacturing process and the quality of the product: manufacturing methods, properties of strains used for manufacturing, methods of quality control, methods of storage and shelf life, according to the standards given in the ‘Minimum Requirement of Veterinary Biological Products’ (Anon, 2002a). Any product that does not conform to these product standards cannot be manufactured, imported or marketed.

1.2.5. Cases of rejection of approval

When the quality of the veterinary biological that has been submitted for approval is found to be unsatisfactory, or its adverse effects are marked as compared with its indications, the product is judged to be of little value and approval is not given.

1.2.6. Cancellation of approvals

At the time of granting approval to market, the quality, safety and efficacy of the product are carefully examined with reference to the latest available technology. However, if scientific

knowledge acquired since the granting of approval indicates that there could be a health hazard associated with the product, re-examination and re-evaluation are performed and an order of 'cancellation of approval' may be made.

1.3. Procedure for marketing approval

When a person intends to market veterinary biologicals, an application for approval to market the veterinary drug must be submitted on a designated form to an official in charge of veterinary biologicals at the Ministry of Agriculture, Forestry and Fisheries. If the documentation is satisfactory, the application for approval to market, together with appended documents, are sent to and reviewed by the Secretariat of the Ministry of Agriculture, Forestry and Fisheries. At that time, a hearing may be conducted if necessary. The application is then discussed in the Pharmaceutical Affairs Sub-council, Pharmaceutical Affairs and Food Sanitation Council, and if any problems are not found, notice of approval to market the veterinary product is sent to the applicant.

2. In the European Union

2.1. Introduction

The pharmaceutical legislation of the European Union (EU), which has evolved over a 45-year period, covers both medicinal products for human and veterinary use. Harmonisation of requirements in the area of veterinary medicines began in 1981 with the adoption of Directives 81/851/EEC and 81/852/EEC, laying down common requirements for manufacturing and marketing authorisations, based on the evaluation of the quality, safety, and efficacy of the product. These Directives, and subsequent veterinary and human pharmaceutical legislation, were consolidated into Directive 2001/82/EC and 2001/83/EC for veterinary and human products, respectively. A series of detailed guidelines were first published in 1994 entitled 'Rules Governing Medicinal Products in the EU' (European Union, 1999). These have since been updated and describe in detail the legal basis for obtaining marketing authorisations, how dossiers should be compiled and how they should be assessed. These rules serve as extremely useful reference publications for any authority that is setting up a system for authorisation of veterinary biologicals. The rules were formally adopted and applied specifically to veterinary biologicals from 1993. Many additional measures were taken to further harmonise the procedures and the criteria for the evaluation of veterinary medicinal products, such as framework requirements and interpretive guidelines for their testing, principles and guidelines of GMP, and a Community procedure for the evaluation of high-technology products. However, granting of authorisations remained at the national level. As a consequence, although applications were evaluated on the basis of these harmonised criteria and procedures, and in some cases simultaneously by the authorities of the Member States, there were differences in the decisions reached by the Member States on individual products. This was why in 1990 the Commission proposed a new system for marketing authorisation for medicinal products, which was adopted by the Council of Ministers in 1993 and entered into force on 1 January 1995.

One of the first consequences was the creation of the European Medicines Evaluation Agency (EMEA), now the European Medicines Agency (EMA).

Legislation for veterinary products (Regulation 726/2004 and Directive 2004/28/EC) was published in May 2004. This legislation, for the main part, came into force in 2005 and resulted in a number of changes aimed at strengthening public and animal health, and environment protection by reinforcing requirements and controls. Directive 2001/82/EC already stated that the competent authorities cannot grant a marketing authorisation (MA) without having conducted a benefit–risk analysis. The document in the MA dossier must "demonstrate that the benefit bound to efficacy outweighs potential risk". But the relation between benefit and risk was not defined in that Directive. The new Directive gives the definition of the "risk–benefit balance": an evaluation of the positive therapeutic effects of the veterinary medicinal product in relation to the risk".

The risks concern:

i) The animal;

ii) The user of medicinal product;

iii) The consumer liable to ingest animal food containing medicinal products residues;

iv) The environment.

With the revision of the Directive, if no medicinal product is available for 3 consecutive years, its MA is secluded. Before the revision, the MA was renewed every 5 years. Now, a single renewal is required. The pharmacovigilance is reinforced.

Title IV of regulation 726/2004 related to responsibilities and administrative structure of the European Agency came into force in April 2004 in order to face the consequences of the enlargement of the EU.

Annex I of Directive 2001/82/EC was further amended to take account of scientific and technical progress. In particular, two new systems were introduced for particular immunological veterinary medicinal products and by derogation from the provisions of Title II, Part 2 Section C on active substances:

i) The first was based on the concept of a master file (vaccine antigen master file, VAMF), becoming a stand-alone part of the marketing authorisation application dossier for a vaccine, which contains all relevant information on quality concerning each of the active substances that are part of the veterinary medicinal product. The stand-alone part may be common to one or more monovalent or combined vaccines presented by the same applicant or marketing authorisation holder.

ii) The second was aimed at permitting authorisation of vaccines against antigenically variable viruses in a way that ensures that the most effective measures can be taken swiftly by the Community against the incursion or spread of three epizootic diseases (foot and mouth disease, avian influenza and bluetongue), leading to the concept of a multi-strain dossier. A multi-strain dossier means a single dossier containing the relevant data for a unique and thorough scientific assessment of the different options of strains or combinations of strains permitting the authorisation of vaccines against antigenically variable viruses.

In September 2015 the European Commission launched a revision of the legal framework for veterinary medicinal products in EU (i.e. directive 2001/82/EC). The purpose of this revision was to increase the availability of veterinary medicinal products, to reduce the administrative burden on commercial producers, to improve the functioning of the internal market for veterinary medicinal products and to assess the possibilities of having an improved response to antimicrobial resistance related to the use of veterinary medicines. The revision will contribute to the realisation of certain actions in the Animal Health Strategy.

2.2. The role of the European Medicines Agency

In 1995, a new European system for the authorisation of medicinal products came into force. After 10 years of cooperation between national registration authorities at the EU level and 4 years of negotiations, the Council of the EU adopted, in June 1993, three directives and one regulation, which together form the legal basis of the system (Brunko, 1997).

The EMEA was established by Council Regulation 2309/93/EEC of 22 July 1993 (*Official Journal* No. L214, 24.8.1993). In December 2009, the EMEA officially launched a new organisational structure and visual identity, and became the European Medicines Agency (EMA). This was the second major reorganisation of the Agency's services since it was established in 1995, and resulted from the expansion of the Agency's responsibilities and tasks, giving it a reinforced role in the protection of public and animal health in Europe.

This agency formulates opinions and, apart from the administrative staff and the management board, is composed of two scientific committees: the CHMP (Committee for Human Medicinal Products) in charge of medicinal products for humans and the CVMP (Committee for Veterinary Medicinal Products) in charge of medicinal products for use in animals.

The CVMP is responsible for the evaluation of applications for marketing authorisation for products derived from biotechnology, for productivity enhancers, new chemical entities and other innovative new products. In addition, the CVMP makes recommendations regarding MRLs (maximum residue limits) for substances used in food-producing animals, and manages referrals when EU issues such as

concerns over the safety or benefit–risk balance of a medicine or a class of medicines need to be resolved. To support its activities, the CVMP relies on a pool of experts put at the disposal of the agency by the EU Member States. These experts may participate in any of the CVMP working parties. Among the working parties, the Immunologicals Working Party (CVMP/IWP) advises the CVMP on general policy issues such as the elaboration and revision of guidelines on immunological veterinary medicinal products (IVMPs). A scientific advice working party foreseen in regulation 726/2004 has been created. The aim of this working party is to advise applicants during the development phase of a veterinary medicinal product. The CVMP prepares scientific guidelines in consultation with the national competent authorities of the EU Member States, to help applicants prepare marketing authorisation applications for medicinal products for veterinary use.

Guidelines are intended to provide a basis for the practical harmonisation of the manner in which the EU Member States and the Agency interpret and apply the detailed requirements for the demonstration of quality, safety and efficacy contained in the Community directives. They also help to ensure that applications for marketing authorisation are prepared in a manner that will be recognised as valid by the Agency.

Immunological guidelines are provided, for instance, for general items, quality, stability, efficacy, summary of product characteristics (SPC), multi-strain dossiers and availability (minor uses or minor species), and are available on the EMA website at: www.ema.europa.eu

2.3. Current European procedures for marketing authorisation

At the time of the revision of the Community Code in 2004, the difficulties encountered in practice as well as jurisprudentially precise details were taken into account and modifications to various Community procedures were adopted: tightening of the conditions of examination and the safeguard clause, official recognition of the coordination group for mutual recognition and decentralised procedures (veterinary), arbitration and creation of a new procedure (decentralised procedure, see below). These provisions were introduced by the Directive 2004/28/EC amending Directive 2001/82/EC.

Four registration procedures for human and veterinary medicinal products have become available through this new legislation:

1. The centralised procedure allows a unique marketing authorisation (MA) to be obtained and made available in all the Member States. This applies to high technology products defined in the annex to the Regulation. It is optional for innovative medicinal products. This procedure was extended to veterinary vaccines covering animal diseases that are subject to Community prophylactic measures.
2. The national procedure allows an MA to be obtained for a medicinal product in a single country or in a country that will be the origin of a mutual recognition procedure.
3. The mutual recognition procedure: applications for authorisation of a product may still be obtained in a single Member State (the 'Reference Member State') by means of a national procedure. Following approval in the Reference Member State, applications may be made, if desired, to other 'Concerned' Member States for identical authorisations to be granted on the basis of '*mutual recognition*'.
4. The decentralised procedure, which was introduced in Directive 2004/28/EC, is the addition of the national one and the mutual recognition procedure, i.e. it is based upon the principle of mutual recognition of national authorisations. At the beginning of this procedure, all Member States are associated, but assessment is conducted by one reference Member State chosen by the applicant.

The most important change is the compulsory aspect of *arbitrage* in the case of a disagreement between Member States during the mutual recognition or the decentralised procedures. If a Member State considers that there is a serious risk to public health, a pre-arbitrage procedure must be carried out. In such a situation, an MA holder cannot remove his/her demand. The arbitrage allows a decision to be made on whether there is a "serious risk" with the use of the medicinal product. Finally, the decision (to grant or refuse the MA) is harmonised throughout the community.

2.4. Manufacturing authorisation and batch release control

In accordance with Directive 2001/82/EC, authorisation is also required for the manufacture of veterinary medicinal products, including immunologicals. This directive provides for regular inspections and stipulates that manufacture must be supervised by a 'qualified person', who certifies that each batch is in conformity with the approved specifications for the product. For the implementation of these requirements, the Commission has adopted Directive 91/412/EEC relating to the principle and guidelines of GMP, and published a detailed guide on GMP developed by a group of pharmaceutical inspectors from the Member States (European Union, 1999: Volume 4).

The directive also establishes GMP for active starting materials for medicinal products. This provision is reinforced through the provision of the opportunity for Member States to carry out inspections of active materials destined for the manufacturers of veterinary medicinal products.

Manufacturers are required to have the services of a qualified person at their disposal to certify that each batch of product has been manufactured and checked in accordance with the conditions for marketing authorisation. This is a basic requirement of the pharmaceutical legislation. In the case of batches imported from third countries, each batch has to undergo a full qualitative and a quantitative analysis of at least the active ingredients in the first Member State of import into the EU, under the supervision of a qualified person. Until this control by the qualified person has been carried out, a batch cannot circulate within the EU without further control. When the certificate is released, no more controls are necessary. In the special case of immunological veterinary medicinal products, an additional step may be introduced. Article 82 of Directive 2001/82/EC, as amended by Directive 2004/28/EC, of the European Parliament and the Council allows, for reasons of human or animal health, a Member State to request samples of each batch of a given IVMP to be submitted to a Competent Authority (CA) for official testing by an Official Medicine Control laboratory (OMCL) before it is placed on the market and establishes the conditions under which a restricted test list can be applied. This is referred to as 'Official Control Authority Batch Release'. OCABR performed by any given Member State must be mutually recognised by all other member states requiring OCABR for that product.

Article 81 of Directive 2001/82/EC allows a Member State, where appropriate, to ask a MA holder to provide documentation to a control authority or an OMCL proving that control tests were carried out in accordance with the methods laid down in the MA dossier. This is referred to as an 'Official Batch Protocol Review'. A goodwill agreement has been adopted by the Veterinary Batch Release Network (VBRN) to mutually recognise Official Batch Protocol Review (OBPR) certificates between Member States provided the procedure and rules codified by the network are followed. This legislation concerns EU/EEA Member States and is also applied by any state having signed a formal agreement, which includes recognition of OCABR, with the EU. Currently, Switzerland has done so via a Mutual Recognition Agreement (MRA) (source EDQM).

2.5. The role of the European Pharmacopoeia

The Convention on the elaboration of a European Pharmacopoeia (or international treaty) adopted at the Council of Europe in 1964, laid the groundwork for the European Pharmacopoeia as a guarantee of the quality of medicines produced in Europe.

The European Pharmacopoeia Convention has now been signed by 37 Member States[1] and the EU; moreover 27 European and non-European countries[2], and the World Health Organisation (WHO) have observer status. Close relations are maintained with the regulatory authorities of the European

1 Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Montenegro, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey, Ukraine, United Kingdom and the European Union. Member States must apply the standards of the European Pharmacopoeia.

2 Albania, Algeria, Argentina, Armenia, Australia, Azerbaijan, Belarus, Brazil, Canada, China (People's Rep. of), Chinese Taipei (Food and Drug Administration), Georgia, Guinea, Israel, Kazakhstan, Korea (Rep. of), Madagascar, Malaysia, Moldova, Morocco, Russian Federation, Senegal, Singapore, South Africa, Syria, Tunisia, United States of America and the World Health Organization. Observer States do not have to apply the European Pharmacopoeia standards. Some of them apply the standards on a voluntary basis.

Economic Area, where integration is developing through contact with the EMA and the implementation of common directives and guidelines on medicines for human and veterinary use.

The European Pharmacopoeia consists of monographs describing individual quality standards (set of control tests applicable to one ingredient) and general quality standards applicable to families of ingredients or to dosage forms, as well as general methods of analysis. It defines the minimum acceptable standards for products to be authorised within the European Union because compliance with monographs is a mandatory requirement within Directive 2001/82/EC. This requires that products must comply with the relevant specific monograph where one exists or with the general monographs where one does not.

The European Directorate for the Quality of Medicines & Health Care (EDQM) is the administrative entity in the Council of Europe that provides the secretariat services for the European Pharmacopoeia. EDQM creates, maintains and distributes the international standard reagents referred to in monographs of the European Pharmacopoeia, including standards for veterinary biologicals.

In 1990, the European Pharmacopoeia co-founded, with the Japanese Pharmacopoeia and the United States (US) Pharmacopoeia, the Pharmacopoeial Discussion Group (PDG); this group is working assiduously for harmonisation at the global level.

The European Pharmacopoeia Commission adopted several harmonised texts for veterinary vaccines. The safety tests and the tests for increased virulence performed during development of the vaccines were harmonised in the framework of harmonisation with VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products; see below) Guidelines 41 and 44, and to ensure consistency with European regulations.

3. In the United States of America

3.1. Introduction

In the United States of America (USA), veterinary biologics or veterinary biological products are defined as all viruses, sera, toxins (excluding substances that are selectively toxic to microorganisms, e.g. antibiotics), or analogous products at any stage of production, shipment, distribution, or sale, that are intended for use in the treatment (prevention, diagnosis, management, or cure) of diseases of animals and that act primarily through the direct stimulation, supplementation, enhancement, or modulation of the immune system or immune response. The term biological products includes, but is not limited to, vaccines, bacterins, allergens, antibodies, antitoxins, toxoids, immunostimulants, certain cytokines, antigenic or immunising components of live organisms, and diagnostic components that are of natural or synthetic origin or that are derived from synthesising or altering various substances or components of substances such as microorganisms, genes or genetic sequences, carbohydrates, proteins, antigens, allergens, or antibodies.

3.2. Legal basis

The Virus/Serum/Toxin Act of 1913 (the 'VST Act'), as amended, 21 U.S.C. Sections 151 to 159, provides the legal authority for the regulation of immunologicals and biologicals for animal use in the USA. The regulatory programme implementing the requirements of the VST Act is administered by the Center for Veterinary Biologics (CVB), Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA). Administrative regulations, duly promulgated and with effect of law, are published in Title 9, Code of Federal Regulations, Parts 101 to 118 (2006). In addition, APHIS has issued programme guidance in CVB Notices, Veterinary Services Memoranda, Veterinary Biologics General Licensing Considerations, and the Veterinary Biologics Program Manual. These may be accessed on the CVB web site at www.aphis.usda.gov/vs/cvb.

The VST Act requires that products governed by the Act that enter channels of commerce be 'not worthless, contaminated, dangerous or harmful'. The regulatory scheme implementing these standards is structured to require manufacturers of these products to apply for licences prior to marketing, and to place certain evidentiary responsibilities on those applicants, i.e. manufacturers are required to demonstrate through the submission of certain information, research data, and test results that their

products are 'pure, safe, potent and efficacious'. The APHIS programme for immunologicals and biologicals for animal use regulates the manufacture and release of products on to the market through a system of licensing, inspection, testing and post-marketing surveillance that ensures that the statutory and regulatory standards are met.

3.3. Licensing and initial inspection

Any person or firm seeking to manufacture in the USA an immunological or biological for animal use must obtain from APHIS both a licence to manufacture at a specified facility (Establishment Licence), and a licence for every particular product to be manufactured (Product Licence). These licence requirements apply whether the product is to be released on to the US market or is to be exported to markets abroad. Typically, an applicant will request a facility licence at the same time as the licence for the first product. Once the facility licence and one product licence have been obtained, a firm that seeks to manufacture and market new products needs only to apply for additional product licences. A person or firm located overseas that seeks to market its product in the USA must also apply for marketing authorisation. In the case of an imported product, however, the authorisation is termed a 'permit' rather than a 'licence'.

To obtain a facility licence, the applicant must submit for approval the blueprint (that is, the architect's plan of the buildings) and blueprint legends for the facility. APHIS reviews these blueprints and legends to ensure that the facility will operate in a manner consistent with GMP. If the applicant subsequently makes any physical or operational changes to the facility, revised blueprints and legends must be submitted immediately.

To obtain a product licence, the applicant must establish the purity and identity of all master seeds and master cell stocks that will be used in the manufacture of the product, and must submit for approval a detailed outline of production. The outline of production includes not only the details of the method of product manufacture, but also a description of the procedures for collecting and submitting samples and for releasing batches. The applicant must also provide information regarding the professional and technical credentials of company personnel, and must identify a qualified individual (termed under US regulations as the 'government liaison') who acts as the official contact with CVB during the licensing process, and who is subsequently responsible for the submission of the firm's test reports in conjunction with the release of the product on to the market. The applicant is required to submit test data that demonstrate that the product produced in accordance with the outline is pure, safe, potent and efficacious. The applicant must submit to CVB laboratories samples of three consecutive batches of the product so that the results of the applicant's tests of the product can be confirmed.

Finally, before the facility or product licences are issued, the applicant's premises are subject to a comprehensive inspection by APHIS examiners. The inspection ensures that the facility is operating in a manner consistent with GMP by confirming that the establishment is configured in the manner set out in the approved blueprints and legends, that the production line is set up and operating in accordance with the approved outline of production, and that records are adequately kept and maintained for each step in production. The inspection also confirms that the applicant follows procedures consistent with GLPs, that the in-process and final product testing programme is conducted properly and appropriately documented, that the sampling is conducted properly, and that adequate procedures for determining and documenting the release of the product on to the market are in place.

3.4. Post-licensing inspection

Once a firm has been issued facility and product licences, APHIS will routinely conduct thorough follow-up inspections of the facility to ensure that the licensee continues to operate the establishment in accordance with the programme regulations and in the manner represented at the time of licensing. Post-licensing inspections are conducted unannounced periodically. If the licensee proposes any significant changes to the facility or to the method of production of a licensed product, APHIS retains the right to conduct a special inspection prior to approving the changes.

3.5. Testing

Each licensee is responsible for thoroughly testing all of its production processes and each serial (or lot) of every product prior to release on to the market. The type and amount of testing required

depends on the particular product, but is determined and approved by the regulatory authority prior to the issuing of the product licence. A qualified individual employed by the licensee ('government liaison') is responsible for selecting the samples to be tested, for monitoring the licensee's testing programme, and for certifying the test results to the regulatory authority.

At the same time that the firm selects its samples for its own in-house testing, it also selects samples to be submitted to the CVB laboratories. The CVB retains the right to conduct confirmatory testing. CVB then selects a percentage of the samples submitted for confirmatory testing to verify the accuracy and proficiency of the manufacturer's tests. The testing is conducted prior to marketing authorisation for each serial. By regulation, CVB policy stipulates that it is required to put its selected samples on test within 14 days of the date on which the samples are received; ordinarily, samples are put on test sooner than the 14-day limit so that the testing of production by the firm and the CVB proficiency testing programme are effectively being conducted at the same time.

When the firm receives the results of its own tests, the government liaison submits those results to the regulatory authority along with a batch release form, initiating the release procedure. If the batch has not been selected as part of the proficiency testing programme, or if it has been selected but the CVB tests confirm the company's test results, the release form is counter-signed by the regulatory authority completing the release procedure. If either the company tests or the proficiency tests indicate that the batch may be unsatisfactory, the batch is not eligible for release.

If the licensee makes a proposal to modify its facility or its operation in any way that could affect the purity, safety, potency or efficacy of the product, the regulatory authority may require the licensee to provide data demonstrating the purity, safety, potency and efficacy of the product as well as to submit samples of the product to CVB's laboratories for confirmatory testing.

3.6. Post-marketing surveillance

CVB operates a post-marketing surveillance programme to monitor the performance of products on the market. Under this programme, CVB typically learns of any problems relating to product quality through consumer reports or complaints, although the programme regulations also place an obligation on the licensee to inform CVB of any problem that comes to its attention regarding the purity, safety or potency of the product. CVB has the legal authority to intervene in the marketplace where there are serious concerns with respect to the purity, safety, potency or efficacy of the product.

B. COMPARISON OF EUROPEAN UNION AND UNITED STATES REGULATIONS

Veterinary biologicals must meet certain basic criteria, regardless of the Regulatory Agency overseeing their production. These criteria include:

- Safety: the product must be safe in the target species and, if live, in species exposed to shed organisms;
- Efficacy: the product should be effective according to claims indicated on the label;
- Quality: includes purity, potency and consistency;
- Purity: the product must be free from contaminating agents;
- Potency: each batch of product should be formulated, and tested, to ensure effectiveness and reproducibility of activity as demonstrated in the registration data.

Although, on a global basis, agencies and regulations differ, all strive to ensure that products offered to the end-consumer conform to these basic standards.

The EU uses a complete system that is a combination of GMP, including validated processes and specifications of materials, together with production methods that ensure the quality of the final product. In-process and batch controls (tests) constitute additional guarantees of the quality of IVMPs. The safety tests are conducted under GLP and the field efficacy tests under GCP. The USA defines acceptable manufacturing processes in the outline of production and detailed facility description (blueprints and blueprint legends), and relies on inspection and confirmatory testing to achieve the same goal. Although different, both systems are designed to allow only pure, safe, potent, and effective biologicals to be released to the consumer.

In addition to the data provided by the applicant, expert reports have to be included in the EU marketing authorisation application file (dossier). Each main section of the EU dossier, including analytical, safety and efficacy, must be reviewed by an independent expert. The assessment of the expert is included in the marketing authorisation file. No such system of third-party review exists under the USDA registration system with the exception of certain biotechnologically derived products.

There are many procedural differences between the EU and the USA. Harmonisation between the two systems should be established where possible, on the recognition of equivalence for tests and procedures that are performed to assess a vaccine and that ensure quality, safety and efficacy of the product. Mutual recognition agreements (MRAs) covering veterinary biologicals have been signed between the EU and Australia and between the EU and New Zealand. These MRAs are now at an operational stage. Progress on MRAs between the EU and the USA, regarding veterinary biologicals, is likely to take longer to achieve.

C. THE ROLE OF INTERNATIONAL ORGANISATIONS

Most nations have a range of official acts that regulate the sale and use of veterinary biologicals. Almost all of these acts stipulate 'minimum requirements' for quality, safety and efficacy of veterinary biologicals (mostly vaccines), to be tested at independent laboratories, usually under State supervision. These acts and tests may differ from one country to another, and they involve costs and restrictions for producers, users and testers.

Many of the vaccines described in this *Terrestrial Manual* are produced and/or used in countries that do not currently apply regimens of authorisation and testing as stringent as those described in this chapter. Nevertheless, it is useful to be aware of the regulations operating in different regions and, therefore, the testing and inspection that is likely to have been carried out there on a veterinary biological.

The idea of harmonising this testing to simplify and reduce costs on a regional, or even world scale, is not new, and much has been accomplished during the past 20 years. The purpose of this section is to review the current situation by describing the role of international organisations in the regulation of veterinary vaccines.

In this section the term 'international organisation' refers to those concerned with animal health on a world-wide scale (WOAH, the Food and Agriculture Organization of the United Nations [FAO] and the WHO).

1. The role of WOAH (World Organisation for Animal Health)

WOAH was founded, as OIE, in Paris in 1924 as the world organisation for animal health, and comprised 182 Member Countries in the year 2018. The principal aims of WOAH are: to ensure transparency in the global animal disease and zoonosis situation, to collect, analyse and disseminate scientific veterinary information, to provide expertise and encourage international solidarity in the control of animal diseases, within its mandate under the Agreement on Sanitary and Phytosanitary Measures (SPS Agreement) of the World Trade Organization (WTO), to safeguard world trade by publishing health standards for international trade in animals and animal products, to improve the legal framework and resources of national Veterinary Services and to provide a better guarantee of the safety of food of animal origin and to promote animal welfare through a science-based approach (Truszczynski & Blancou, 1992).

Within WOAH there are four Specialist Commissions: the Terrestrial Animal Health Standards Commission, which deals with the *Terrestrial Animal Health Code,* the Biological Standards Commission, the Scientific Commission for Animal Diseases and the Aquatic Animal Health Standards Commission (including diseases of molluscs and crustaceans). In addition, there is one Working Group: the Working Group on Wildlife.

Among the Specialist Commissions, the one most closely connected with standardisation is the Biological Standards Commission. This Commission establishes standards for diagnostic methods (including diagnostic preparations) and for vaccines. Its terms of reference reflect the Commission's obligation to participate in the standardisation of biological products, including vaccines used for prophylactic purposes. The Biological Standards Commission is responsible for the preparation of the WOAH *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, and the organisation of Reference Laboratories for many of the diseases on the WOAH List.

However, full standardisation of vaccine testing can be achieved only when the necessary standards have been devised. It is hoped to reach the goal of standardisation and wide availability of standards through the participation of WOAH Reference Laboratories. In 2018, the WOAH has 182 Member Countries with a global network of 246 Reference Laboratories covering 105 diseases or topics in 35 countries, and 55 Collaborating Centres covering 49 topics in 28 countries. The functions and responsibilities of experts at the WOAH Reference Laboratories include the provision of a centre of excellence in a designated activity; standardisation of methods; preparation, storage and distribution of standard antisera, antigens and other reagents.

Among the WOAH Collaborating Centres, seven may be involved at some stage in veterinary vaccine control or harmonisation: the Collaborating Centre for Veterinary Medicinal Products in Fougères (France), the Collaborating Centre for ELISA (enzyme-linked immunosorbent assay) and Molecular Techniques in Animal Disease Diagnosis in Vienna (Austria), Collaborating Centre for Diagnosis and Control of Animal Diseases and Related Veterinary Product Assessment in Asia in Tokyo (Japan), the Collaborating Centre for the Diagnosis of Animal Diseases and Vaccine Evaluation in the Americas in Ames (USA), the Collaborating Centre for Development and Production of Vaccines, Pharmaceutical Products and Veterinary Diagnostic Systems using Biotechnology, Centro de Ingeniería Genética y Biotecnología, Havana (Cuba), the Collaborating Centre for Quality Control of Veterinary Vaccines, Pan-African Veterinary Vaccine Centre (Ethiopia) and the Collaborating Centre for Validation, Quality Assessment and Quality Control of Diagnostic Assays and Vaccine for Vesicular Diseases in Europe**,** Centre d'Etudes et de Recherches Vétérinaires et Agrochimiques de Ukkel (Belgium).

In 1994, following discussions with the International Technical Consultation on Veterinary Drug Registration (ITCVDR), WOAH set up an *ad hoc* Group on the Harmonisation of Veterinary Medicines, which was the first step towards the creation of the VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products) (see Section C.4 below).

In May 2003, the WOAH International Committee (now World Assembly) adopted a resolution entitled WOAH Procedure for Validation and Certification of Diagnostic Assays (Test Methods) for Infectious Animal Diseases. This resolution required the WOAH Director General to establish a WOAH registry for assays with levels of validation specified. Fitness for purpose is used as a criterion for validation.

2. The role of the Food and Agriculture Organization of the United Nations

FAO, established in 1945, is responsible for agricultural development and food production. The Animal Production and Health Division ('AGA') within the Agriculture Department is concerned with livestock development, and it includes the Animal Health Service ('AGAH'), the main role of which is to assist Member Countries in the control of animal diseases, with the aim of improving livestock production as an integral component of general social, economic and agricultural development. FAO's involvement in testing veterinary biologicals is primarily through its technical assistance system to Member Countries to set up and even execute independent quality control of vaccines and other biologicals. One example is FAO's assistance to the AU (African Union) in setting up a system for continental testing of veterinary vaccines, by the Pan African Veterinary Vaccine Center (PANVAC). FAO also commissions, at the request of Member Countries, initiatives for either quality assurance of vaccines and other biologicals or expert consultations on this subject, or publication of manuals on the production and quality control of vaccines. Furthermore, two auxiliary services can be asked to intervene on matters concerning these products, namely Codex Alimentarius and the Division of Nuclear Techniques in Food and Agriculture. The latter is operated jointly by FAO and the International Atomic Energy Agency (IAEA) based in Vienna (Austria). It has an Animal Production and Health Section, which assists veterinary services and research institutes in developing countries to establish radio-immunoassay (RIA) and ELISA techniques. Linked to this activity is a quality assurance scheme under which laboratories in receipt of FAO/IAEA ELISA kits are required to routinely monitor internal quality controls and to periodically (once or twice a year) test a batch of unknown samples, and to forward the results to IAEA. The overall aim is to provide assurance to all concerned that the results being generated through the use of such internationally standardised and validated kits can be relied upon as correct.

3. The role of the World Health Organization

Currently WHO is not directly involved in preparing international reference preparations (i.e. antigens or antibodies) for purely veterinary use, but has developed and still retains in the National Institute for Biological Standards and Control, Potter's Bar (UK) some materials related to purely veterinary diseases (e.g. Newcastle disease live vaccine, classical swine fever serum). WHO wishes to retain a role in this area in instances where the veterinary reference preparations and guidance documents have a direct relevance to human health (Joint

FAO/WHO Expert Committee on Brucellosis, 1986; Meslin *et al.,* 1996; WHO Expert Committee on Biological Standardisation, 1992; WHO Expert Committee on Rabies, 1992). This involves zoonotic and potentially zoonotic agents and other infectious agents of animal origin that are potential contaminants of biological products, whether these are vaccines produced in cell cultures or organs for xenotransplantation. At the meeting of the Expert Committee on Biological Standardization in October 1998, a review of currently retained international standards and reference preparations for veterinary medicine was carried out and a list of candidates for discontinuation, replacement and revision was suggested (Joint FAO/WHO Expert Committee on Brucellosis, 1986). The Expert Committee however decided to defer taking action on preparation of veterinary reference materials pending an evaluation by WHO with its partners in the veterinary field of the need for these various biological products. In addition, the present day topicality of certain preparations, especially veterinary vaccines against known zoonoses (e.g. anthrax, brucellosis) adopted and/or revised in the 1960s and 1970s, also needs to be evaluated.

The format of the list of Requirements for Biological Substances published as an Appendix to each report of the Expert Committee on Biological Standardization was revised in 1998 and should facilitate the retrieval of information and achieve the aim of improved transparency.

4. The role of VICH

4.1. What is VICH?

4.1.1. Short description of VICH

The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) is an international programme aimed at providing guidance on the technical requirements for registration of veterinary medicinal products. VICH was established in 1996 as a means of collaboration, primarily between the regulatory authorities and the animal health industry of the EU, Japan and the USA. The regulatory authorities and animal health industry of Australia, New Zealand, Canada and South Africa also participate actively as VICH observer members. WOAH participates as an associate member in the VICH process with the goal of supporting and disseminating the outcomes at the global level.

4.1.2. Background and history

The initiative to begin the harmonisation process came in 1983 when the first International Technical Consultation on Veterinary Drug Registration (ITCVDR) was held. Since then a series of government and industry initiatives have been developed, culminating in the formation of the VICH.

The Codex Alimentarius formed a Committee on Residues of Veterinary Drugs in Foods in 1985. Standard requirements for veterinary product registration were adopted in Europe in 1981.

The US Food and Drug Administration and the European Commission have held regular bilateral meetings for the last decade to discuss common areas of interest. This has involved mutual exchange of guidelines for consultation.

The first International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) was held in Brussels in November 1991. The meeting brought together regulators and industry representatives from the USA, the EU and Japan to address quality, safety and efficacy requirements in the three regions.

Meetings on harmonisation of veterinary biologicals were held in Ploufragan, France, in January 1992, in Arlington, USA, in 1994 and in Singapore in 1995.

In January 1993 the GHOST (Global harmonisation of standards) discussion document was published by FEDESA[3]. It set out a programme for the international harmonisation of registration requirements for veterinary pharmaceuticals and biologicals.

Following discussions at ITCVDR and WOAH conferences, WOAH set up an *ad hoc* Group on harmonisation of veterinary medicinal products in 1994.

4.1.3. The creation and scope of VICH

Preparatory work for the establishment of VICH was carried out by this WOAH *ad hoc* Group. During 1994 and 1995, two meetings were held at which the scope of veterinary harmonisation was discussed and the membership and objectives of the VICH proposed.

On the subject of food safety standards, it was decided that the VICH should complement the work of Codex and JECFA[4]. Issues related to GLP and GMP that are already the subject of mutual agreements will not normally come within the remit of the VICH. Issues related to biologicals were considered appropriate to fall within the scope of VICH.

Fundamental to the selection of priority topics for consideration by the VICH was the discussion document prepared by COMISA for the Steering Committee. This report:

- assesses those ICH guidelines which could be adapted to the VICH programme;
- defines in detail areas of non-harmonisation between the EU, the US and Japan and provides a series of 'concept papers' on key topics; and
- puts forward preliminary suggestions for priority topics.

With all the ground-breaking work completed, the Steering Committee of the VICH held its first meeting in April 1996, at which the membership and the working procedures were agreed and a work programme established.

In November 2011, the VICH Outreach Forum was created to provide a basis for wider international harmonisation of registration requirements beyond the VICH member and observer regions and countries. The Forum aims to improve information exchange and raise awareness of VICH and understanding of VICH guidelines, thereby facilitating their wider adoption. The VICH Outreach Forum currently includes representatives from the regulatory authorities of many countries and three regional organisations for Africa, Asia and Americas.

4.1.4. The objectives of VICH

The objectives of the VICH are along the same lines as those of the ICH.

i) Establish and implement harmonised regulatory requirements for veterinary medicinal products in the VICH regions, which meet high quality, safety and efficacy standards and minimise the use of test animals and costs of product development.

ii) Provide a basis for wider international harmonisation of registration requirements.

iii) Monitor and maintain existing VICH guidelines, taking particular note of the International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) work programme and, where necessary, update these VICH Guidelines.

iv) Ensure efficient processes for maintaining and monitoring consistent interpretation of data requirements following the implementation of VICH guidelines.

3 FEDESA: Fédération Européenne de la Santé Animale. In 2002, FEDESA became the International Federation for Animal Health (IFAH) and later HealthforAnimals. Now fulfils the role of VICH Secretariat.

4 JECFA: Joint FAO/WHO Expert Committee on Food Additives.

v) By means of a constructive dialogue between regulatory authorities and industry provide technical guidance enabling response to significant emerging global issues and science that impact on regulatory requirements within the VICH regions.

4.1.5. Progress toward achieving the VICH objectives

i) For veterinary immunologicals there is an ongoing programme of harmonisation in a number of areas including target animal safety studies, reversion to virulence and tests for the presence of *Mycoplasma*. To date only a relatively small number of VICH guidelines have been developed for veterinary biologicals and it is worth emphasising the difficulties in reaching agreement on veterinary biologicals between the three regions.

ii) The first two VICH Guidelines for veterinary biologicals were adopted in May 2003; one concerned the testing of residual formaldehyde and the other the testing of residual moisture. Several other Guidelines that apply to veterinary biologicals and all other veterinary medicinal products have also been adopted.

a) In 2013 and 2015 two new Guidelines were adopted: one concerning the harmonisation of criteria to waive target animal batch safety testing (TABST) for inactivated vaccines for veterinary biologicals (GL50), and one on the harmonisation of criteria to waive TABST for live vaccines (GL55).

b) In 2016, the Steering Committee (SC) adopted a Concept Paper on the development of further guidance on testing of veterinary vaccines for freedom from extraneous viruses. and a list of viruses. These two future VICH guidelines will be developed by taking into account the new relevant revised WOAH Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

Following the "VICH5"conference, held in Tokyo, Japan in 2015, the VICH Priorities Phase 4 document was published providing a vision for 2016–2020. To foster the already close collaboration with WOAH and in view of implementing the wider objective of international harmonisation, VICH supports the strategic activities of WOAH that are targeted at good governance of veterinary medicinal product in WOAH Member Countries.

More information is available at: https://vichsec.org/en/.

CONCLUSION

There is a clear intention to achieve greater international harmonisation of regulatory requirements for veterinary biologicals (Vannier *et al.,* 1997). Progress has already been achieved through international organisations to allow fair competition in the marketing of veterinary products. Although past efforts by international organisations have not resulted in a level of harmonisation sufficient to facilitate international trade, they have laid the groundwork for current efforts. National authorities recognise the advantages of harmonisation and are now committed to working toward this goal.

The efforts of international organisations have made the goal of harmonisation possible and have resulted in an organisation and process for proceeding toward this goal. Success in achieving this goal will depend on the willingness of participating national authorities to work together and accept the compromises that will be necessary to resolve the difficult scientific and policy issues.

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NB: FIRST ADOPTED IN 2000. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 2.3.3.

MINIMUM REQUIREMENTS FOR THE ORGANISATION AND MANAGEMENT OF A VACCINE MANUFACTURING FACILITY

SUMMARY

This chapter sets out the management requirements for the manufacture of veterinary vaccines in accordance with Chapter 1.1.8 Principles of veterinary vaccine production. *Manufacturers should use the chapter as a basis for the elaboration of specific rules adapted to their individual needs. Many of the general principles of laboratory management set out in Chapter 1.1.1* Management of veterinary diagnostic laboratories *are applicable to vaccine production, including accountability, executive management, infrastructure, human resources and compliance.*

There should be sufficient qualified personnel to carry out all the tasks that are the responsibility of the manufacturer. Individual responsibilities should be clearly understood by the individuals and recorded. All personnel should be aware of the principles of Good Manufacturing Practice (GMP) that affect them and receive initial and continuing training, including hygiene instructions, relevant to their needs. Appropriate procedures should be in place for biorisk management, to protect the personnel, to prevent hazardous biological agents from leaving the plant, and to prevent contamination of products within the plant.

Premises and equipment should be located, designed, constructed, adapted and maintained to suit the operations. Their layout and design should aim to minimise the risk of errors and permit effective cleaning and maintenance in order to avoid cross-contamination, build-up of dust or dirt and, in general, any adverse effect on the quality of products.

Good documentation (including electronic records) constitutes an essential part of the quality assurance system and is key to operating in compliance with GMP requirements. The types of documents and electronic media used should be fully defined in the manufacturer's quality management system. The main objective of the system of documentation should be to establish, control, monitor and record all activities that directly or indirectly impact on the quality of products. The quality management system should include sufficient detail to facilitate a common understanding of the requirements, in addition to providing sufficient records of processes and evaluation of observations.

Any outsourced activity should be appropriately defined, agreed and controlled in order to avoid misunderstandings that could result in a product or operation of unsatisfactory quality. Written contracts should cover outsourced activities, products or operations to which they are related, and any connected technical arrangements. All arrangements for the outsourced activities including any proposed changes in technical or other aspects should be in accordance with regulations in force, and the marketing authorisation for the product concerned. Where the marketing authorisation holder and the manufacturer are not the same, appropriate arrangements should be in place to take into account the principles described in this chapter.

All complaints and other information concerning potentially defective products should be reviewed carefully following written procedures. A system should be in place to evaluate complaints and, if necessary, initiate a recall from the market promptly and effectively for products known or suspected to be defective.

Self-inspections should be conducted in order to monitor the implementation and compliance with GMP principles, and to propose necessary corrective measures.

1. Rules for personnel

The establishment and maintenance of a satisfactory system of quality assurance and the correct manufacture of veterinary vaccines relies upon people. For this reason, there must be sufficient qualified personnel to carry out all the tasks that are the responsibility of the manufacturer. Individual responsibilities should be clearly understood by the individuals and recorded. All personnel should be aware of the principles of Good Manufacturing Practices (GMP) that affect them and receive initial and continuing training, including hygiene instructions, relevant to their needs.

1.1. General rules

i) The manufacturer should have an adequate number of personnel with the necessary qualifications and practical experience. The responsibilities placed on any one individual should not be so extensive as to present any risk to quality.

ii) The manufacturer must have an organisation chart. People in responsible positions should have specific duties recorded in written job descriptions and adequate authority to carry out their responsibilities. Their duties may be delegated to designated deputies of a satisfactory qualification level. There should be no gaps or unexplained overlaps in the responsibilities of those personnel concerned with the application of GMP.

1.1.1. Key personnel

i) Key personnel include the head of production, the head of quality control, the head of packaging and distribution, the head of technical services and the person(s) responsible for the batch release. Normally key posts should be occupied by full-time personnel. The heads of production and quality control should be held by different individuals, independent from each other. In large organisations, it may be necessary to delegate some of the functions.

1.1.2. Training

i) The manufacturer should provide training for all the personnel whose duties take them into production areas or into control laboratories (including the technical, maintenance and cleaning personnel), and for other personnel whose activities could affect the quality of the product.

ii) Besides the basic training on the theory and practice of GMP, newly recruited personnel should receive training appropriate to the duties assigned to them. Continuing training should also be given, and its practical effectiveness should be periodically assessed. Training programmes should be available, approved by a qualified person. Training records should be kept.

iii) Personnel working in areas where contamination is a hazard, e.g. clean areas or areas where highly active, toxic, infectious or sensitising materials are handled, should be given specific training (see Section 2.4 *Specific requirements for vaccine production*).

iv) Visitors or untrained personnel should, preferably, not be taken into the production and quality control areas. If this is unavoidable, they should be given information in advance, particularly about personal hygiene and the prescribed protective clothing. They should be closely supervised.

v) The concept of quality assurance and all the measures capable of improving its understanding and implementation should be fully discussed during the training sessions.

1.1.3. Personal hygiene

i) Detailed hygiene programmes should be established and adapted to the different needs within the factory. They should include procedures relating to the health, hygiene practices and clothing of personnel. These procedures should be understood and followed in a very strict way by every person whose duties take him into the production and control areas. Hygiene programmes should be promoted by management and widely discussed during training sessions.

ii) All personnel should receive a medical examination upon recruitment. It must be the manufacturer's responsibility that there are instructions ensuring that health conditions that can be of relevance to the quality of products come to the manufacturer's knowledge. After the first medical examination, examinations should be carried out when necessary for the work and personal health.

iii) Steps should be taken to ensure as far as is practicable that no person affected by an infectious disease or having open lesions on the exposed surface of the body is engaged in the manufacture of vaccines.

iv) Every person entering the manufacturing areas should wear protective garments appropriate to the operations to be carried out.

v) Eating, drinking, chewing or smoking, or the storage of food, drink, smoking materials or personal medication in the production and storage areas should be prohibited. In general, any unhygienic practice within the manufacturing areas or in any other area where the product might be adversely affected should be forbidden.

vi) Direct contact should be avoided between the operator's hands and the exposed product as well as with any part of the equipment that comes into contact with the products.

vii) Personnel should be instructed to use the hand-washing facilities.

viii) Any specific requirements for the manufacture of aseptic or sterile preparations related to production are covered in 2.4 *Specific requirements for vaccine production*.

1.2. Specific rules for personnel involved in the manufacture of vaccines

i) All personnel (including those concerned with cleaning and maintenance) employed in areas where immunological products are manufactured should be given training in and information on hygiene and microbiology. They should receive additional training specific to the products with which they work.

ii) Responsible personnel should be formally trained in some or all of the required fields.

iii) Personnel should be protected against possible infection with the biological agents used in manufacture. In the case of biological agents known to cause disease in humans, adequate measures should be taken to prevent infection of personnel working with the agent or with experimental animals.

iv) Where relevant, the personnel should be vaccinated and subject to medical examination.

v) Adequate measures should be taken to prevent biological agents being taken outside the manufacturing plant by personnel acting as a carrier. Dependent on the type of biological agent, such measures may include complete change of clothes and compulsory showering before leaving the production area.

vi) For immunological products, the risk of contamination or cross-contamination by personnel is particularly important.

vii) Prevention of contamination by personnel should be achieved by a set of measures and procedures to ensure that appropriate protective clothing is used during the different stages of the production process.

viii) Prevention of cross-contamination by personnel involved in production should be achieved by a set of measures and procedures to ensure that they do not pass from one area to another unless they have taken appropriate measures to eliminate the risk of contamination. In the course of a working day, personnel should not pass from areas where contamination with live micro-organisms is likely or where animals are housed to premises where other products or organisms are handled. If such passage is unavoidable, clearly defined decontamination procedures, including change of clothing and shoes, and, where necessary, showering, should be followed by staff involved in any such production.

ix) Personnel entering a contained area where organisms had not been handled in open circuit operations in the previous 12 hours to check on cultures in sealed, surface decontaminated flasks would not be regarded as being at risk of contamination, unless the organism involved was an exotic.

2. Requirements for premises and equipment

Premises and equipment must be located, designed, constructed, adapted and maintained to suit the operations to be carried out. Their layout and design must aim to minimise the risk of errors and permit effective cleaning and maintenance in order to avoid cross-contamination, build-up of dust or dirt and, in general, any adverse effect on the quality of products.

Premises and equipment used for critical operations must be qualified[1].

Added requirements for premises and equipment used in aseptic preparations are given in 2.4 *Specific requirements for vaccine production.*

2.1. General

i) Premises should be situated in an environment which, when considered together with measures to protect the manufacture, presents minimal risk of causing contamination of materials or products. Research and development activities associated with organisms not authorised to be handled in the premises must be carried out in buildings completely separated from the manufacturing facility.

ii) Premises should be carefully maintained, ensuring that repair and maintenance operations do not present any hazard to the quality of products. They should be cleaned and, where applicable, disinfected according to detailed written procedures.

iii) Lighting, temperature, humidity and ventilation should be appropriate and such that they do not adversely affect, directly or indirectly, either the medicinal products during their manufacture and storage, or the accurate functioning of equipment.

iv) Premises should be designed and equipped so as to afford maximum protection against the entry of insects or other animals.

v) Steps should be taken in order to prevent the entry of unauthorised people. Production, storage and quality control areas should not be used as a right of way by personnel who do not work in them.

2.2. Storage areas

i) Storage areas should be of sufficient capacity to allow orderly storage of the various categories of materials and products: starting and packaging materials, intermediate, bulk and finished products, products in quarantine, released, rejected, returned or recalled.

ii) Storage areas should be designed or adapted to ensure good storage conditions. In particular, they should be clean and dry and maintained within acceptable temperature limits. Where special storage conditions are required (e.g. temperature, humidity) these should be validated, checked and monitored.

iii) Receiving and dispatch bays should protect materials and products from the weather. Reception areas should be designed and equipped to allow containers of incoming materials to be cleaned where necessary before storage.

1 This qualification may be done according to the rules laid down in the PIC/S (see References).

iv) Where quarantine status is ensured by storage in separate areas, these areas must be clearly marked and their access restricted to authorised personnel. Any system replacing the physical quarantine should give equivalent security.

v) There should normally be a separate sampling area for starting materials. If sampling is performed in the storage area, it should be conducted in such a way as to prevent contamination or cross-contamination.

vi) Segregated areas should be provided for the storage of rejected, recalled or returned materials or products.

vii) Highly active materials or products should be stored in safe and secure areas.

viii) Printed packaging materials are considered critical to the conformity of the vaccine and special attention should be paid to the safe and secure storage of these materials.

2.3. Production premises – general requirements

i) Premises should preferably be laid out in such a way as to allow the production to take place in areas connected in a logical order corresponding to the sequence of the operations and to the requisite cleanliness levels.

ii) The adequacy of the working and in-process storage space should permit the orderly and logical positioning of equipment and materials so as to minimise the risk of confusion between different medicinal products or their components, to avoid cross-contamination and to minimise the risk of omission or wrong application of any of the manufacturing or control steps.

iii) Where starting and primary packaging materials, intermediate or bulk products are exposed to the environment, interior surfaces (walls, floors and ceilings) should be smooth, free from cracks and open joints, and should not shed particulate matter and should permit easy and effective cleaning and, if necessary, disinfection.

iv) Pipework, light fittings, ventilation points and other services should be designed and sited to avoid the creation of recesses that are difficult to clean. As far as possible, for maintenance purposes, they should be accessible from outside the manufacturing areas.

v) Drains should be of adequate size, and have trapped gullies. Open channels should be avoided where possible, but if necessary, they should be shallow to facilitate cleaning and disinfection.

vi) Production areas should be effectively ventilated, with air control facilities (including temperature and, where necessary, humidity and filtration) appropriate both to the products handled, to the operations undertaken within them and to the external environment.

vii) Weighing of starting materials usually should be carried out in a separate weighing room designed for that use.

viii) In cases where dust is generated (e.g. during sampling, weighing, mixing and processing operations, packaging of dry products), specific provisions should be taken to avoid cross-contamination and facilitate cleaning.

ix) Premises for the packaging of vaccines should be specifically designed and laid out so as to avoid mix-ups or cross-contamination.

x) Production areas should be well lit, particularly where visual on-line controls are carried out.

xi) In-process controls may be carried out within the production area provided they do not carry any risk for the production.

2.3.1. Ancillary areas

i) Rest and refreshment rooms should be separate from other areas.

ii) Facilities for changing clothes and for washing and toilet purposes should be easily accessible and appropriate for the number of users. Toilets should not directly communicate with production or storage areas.

iii) Maintenance workshops should as far as possible be separated from production areas. Whenever parts and tools are stored in the production area, they should be kept in rooms or lockers reserved for that use.

2.3.2. Quality control areas

i) Normally, quality control laboratories should be separated from production areas. This is particularly important for laboratories for the control of biologicals, microbiologicals and radioisotopes, which should also be separated from each other.

ii) Control laboratories should be designed to suit the operations to be carried out in them. Sufficient space should be given to avoid mix-ups and cross-contamination. There should be adequate suitable storage space for samples and records.

iii) Separate rooms may be necessary to protect sensitive instruments from vibration, electrical interference, humidity, etc.

iv) Special requirements are needed in laboratories handling particular substances, such as biological or radioactive samples.

v) Animal houses should be well isolated from other areas, with separate entrance (animal access) and air handling facilities.

2.4. Specific requirements for vaccine production

2.4.1. Production areas

i) Premises should be designed in such a way as to control both the risk to the product and to the environment. This can be achieved by the use of containment, clean, clean or contained or controlled areas[2].

ii) Live biological agents should be handled in contained areas. The level of containment should depend on the pathogenicity of the micro-organism and whether it has been classified as exotic.

iii) Inactivated biological agents should be handled in clean areas. Clean areas should also be used when handling non-infected cells isolated from multicellular organisms and, in some cases, filtration-sterilised media.

iv) Open circuit operations involving products or components not subsequently sterilised should be carried out within a laminar air flow work station according to the rules for aseptic preparation.

v) Other operations where live biological agents are handled (quality control, research and diagnostic services, etc.) should be appropriately contained and separated if production operations are carried out in the same building. The level of containment should depend on the pathogenicity of the biological agent and whether they have been classified as exotic. Whenever diagnostic activities are carried out, there is the risk of introducing highly pathogenic organisms. Therefore, the level of containment should be adequate to cope with all such risks. Containment may also be required if quality control or other activities are carried out in buildings in close proximity to those used for production.

vi) Containment premises should be easily disinfected and should have the following characteristics:

a) the absence of direct venting to the outside;

2 Guidance on the qualification of the clean areas is given in ISO 14644 and described in the PIC/S GMP Guide (see References).

b) a ventilation system with air at negative pressure. Air should be extracted through HEPA (high-efficiency particulate air) filters and not be re-circulated except to the same area, and provided further HEPA filtration is used (normally this condition would be met by routing the re-circulated air through the normal supply HEPAs for that area). However, recycling of air between areas may be permissible provided that it passes through two exhaust HEPAs, the first of which is continuously monitored for integrity, and there are adequate measures for safe venting of exhaust air should this filter fail;

c) air from manufacturing areas used for the handling of exotic organisms should be vented through two sets of HEPA filters in series, and that from production areas not re-circulated;

d) a system for the collection and disinfection of liquid effluents (see Section 2.4.4 *Disinfection – waste disposal*);

e) changing rooms designed and used as air locks, and equipped with washing and showering facilities if appropriate. Air pressure differentials should be such that there is no flow of air between the work area and the external environment or risk of contamination of outer clothing worn outside the area;

f) an air lock system for the passage of equipment, which is constructed so that there is no flow of contaminated air between the work area and the external environment or risk of contamination of equipment within the lock. The air lock should be of a size that enables the effective surface decontamination of materials being passed through it. Consideration should be given to having a timing device on the door interlock to allow sufficient time for the decontamination process to be effective.

g) in many instances, a barrier double-door autoclave for the secure removal of waste materials and introduction of sterile items.

vii) Water treatment plants and distribution systems should be designed, constructed and maintained so as to ensure a reliable source of water of an appropriate quality. They should not be operated beyond their designed capacity. Water for injections should be produced, stored and distributed in a manner that prevents microbial growth, for example by constant circulation at a temperature above 70°C.

viii) Equipment passes and changing rooms should have an interlock mechanism or other appropriate system to prevent the opening of more than one door at a time. Changing rooms should be supplied with air filtered to the same standard as that for the work area, and equipped with air extraction facilities to produce an adequate air circulation independent of that of the work area. Equipment passes should normally be ventilated in the same way, but unventilated passes, or those equipped with supply air only, may be acceptable.

ix) Production operations such as cell maintenance, media preparation, virus culture, etc., likely to cause contamination should be performed in separate areas. Animals and animal products should be handled with appropriate precautions.

x) Production areas where biological agents particularly resistant to disinfection (e.g. spore forming bacteria) are handled should be separated and dedicated to that particular purpose until the biological agents have been inactivated.

xi) With the exception of blending and subsequent filling operations (for example with multi-valent vaccines), one biological agent only should be handled at a time within an area.

xii) Production areas should be designed to permit disinfection between campaigns, using validated methods.

xiii) Production of biological agents may take place in controlled areas provided it is carried out in totally enclosed and heat-sterilised equipment, all connections being also heat sterilised after making and before breaking. It may be acceptable for connections to be made under local laminar air flow provided these are few in number and proper aseptic techniques are used and there is no risk of leakage. The sterilisation parameters used before breaking the connections must be validated for the organisms being used. Different products may be placed in different biogenerators (fermenters), within the same area, provided that there is

no risk of accidental cross-contamination. However, organisms generally subject to special requirements for containment should be in areas dedicated to such products.

xiv) Access to manufacturing areas should be restricted to authorised personnel. Clear and concise written procedures should be posted as appropriate.

xv) The manufacturing site and buildings should be described in sufficient detail (by means of plans and written explanations) so that the designation and conditions of use of all the rooms are correctly identified as well as the biological agents that are handled in them. The flow of people and product should also be clearly marked.

xvi) The activities carried out in the vicinity of the site should also be indicated.

xvii) Plans of contained or clean area premises should describe the ventilation system indicating inlets and outlets, filters and their specifications, the number of air changes per hour, and pressure gradients. They should indicate which pressure gradients are monitored by pressure indicator.

2.4.2. Animals and animal houses

i) Animal houses should be separated from the other production premises and suitably designed.

ii) The sanitary status of the animals used for production should be defined, monitored, and recorded. Some animals should be handled as defined in specific monographs (e.g. specific pathogen free [SPF] flocks).

iii) Animals, biological agents, and tests carried out should be the subject of an identification system so as to prevent any risk of confusion and to control all possible hazards.

iv) Animal houses where animals intended or used for production are accommodated, should be provided with the appropriate containment or clean area measures, and should be separate from other animal accommodation.

v) Animal houses where animals used for quality control, involving the use of pathogenic biological agents, are accommodated, should be adequately contained.

vi) The animal species accommodated in the animal houses or otherwise on the site should be identified.

2.4.3. Equipment

i) The equipment used should be designed and constructed so that it meets the particular requirements for the manufacture of each product.

ii) Before being put into operation the equipment should be qualified and validated and subsequently be regularly maintained and validated.

iii) Where appropriate, the equipment should ensure satisfactory primary containment of the biological agents.

iv) Where appropriate, the equipment should be designed and constructed as to allow easy and effective decontamination or sterilisation.

v) Closed equipment used for the primary containment of the biological agents should be designed and constructed as to prevent any leakage or the formation of droplets and aerosols.

vi) Inlets and outlets for gases should be protected so as to achieve adequate containment, e.g. by the use of sterilising hydrophobic filters.

vii) The introduction or removal of material should take place using a sterilisable closed system, or possibly in an appropriate laminar air flow.

viii) Equipment where necessary should be properly sterilised before use, preferably by pressurised dry steam. Other methods can be accepted if steam sterilisation cannot be used because of the nature of the equipment. It is important not to overlook such individual items as bench centrifuges and water baths.

ix) Equipment used for purification, separation or concentration should be sterilised or disinfected at least between use for different products. The effect of the sterilisation methods on the effectiveness and validity of the equipment should be studied in order to determine the life span of the equipment.

x) All sterilisation procedures should be validated.

xi) Equipment should be designed so as to prevent any mix-up between different organisms or products. Pipes, valves and filters should be identified as to their function.

xii) Separate incubators should be used for infected and non-infected containers and also generally for different organisms or cells. Incubators containing more than one organism or cell type will only be acceptable if adequate steps are taken to seal, surface decontaminate and segregate the containers.

xiii) Culture vessels, etc., should be individually labelled. The cleaning and disinfection of the items can be particularly difficult and should receive special attention.

xiv) Equipment used for the storage of biological agents or products should be designed and used in such a manner as to prevent any possible mix-up. All stored items should be clearly and unambiguously labelled and in leak-proof containers. Items such as cells and organisms seed stock should be stored in dedicated equipment.

xv) Relevant equipment, such as that requiring temperature control, should be fitted with recording or alarm systems.

xvi) To avoid breakdowns, a system of preventive maintenance, together with trend analysis of recorded data, should be implemented.

xvii) The loading of freeze dryers requires an appropriate clean or contained area. Unloading freeze dryers contaminate the immediate environment. Therefore, for single-ended freeze dryers, the clean room should be decontaminated before a further manufacturing batch is introduced into the area, unless this contains the same organisms, and double door freeze dryers should be sterilised after each cycle unless opened in a clean area.

xviii) Sterilisation of freeze dryers should be done before use, preferably by pressurised dry steam. In case of campaign working, they should at least be sterilised after each campaign.

2.4.4. Disinfection – waste disposal

i) Disinfection or wastes and effluents disposal may be particularly important in the case of manufacture of immunological products. Careful consideration should therefore be given to procedures and equipment aiming at avoiding environmental contamination as well as to their validation or qualification.

3. Rules governing documentation

Documentation relating to the premises should be readily available in a **Site Master File**.

3.1. Principle

Good documentation constitutes an essential part of the quality assurance system and is key to operating in compliance with GMP requirements. The various types of documents and media used should be fully defined in the manufacturer's quality management system. Documentation may exist in a variety of forms, including paper-based, electronic or photographic media. The main objective of the system of documentation used must be to establish, control, monitor and record all activities that directly or indirectly impact on all aspects of the quality of products. The quality management system should include sufficient instructional detail to facilitate a common understanding of the requirements, in addition to providing for sufficient recording of the various processes and evaluation of any observations, so that on-going application of the requirements may be demonstrated.

There are two primary types of documentation used to manage and record GMP compliance: instructions (directions, requirements) and records or reports.

Suitable controls should be implemented to ensure the accuracy, integrity, availability and legibility of documents. Instruction documents should be free from errors and available in writing. This includes electronic records from which data may be rendered in a human readable form.

3.2. Required documentation

i) **Site Master File:** a document describing the GMP related activities of the manufacturer.

3.2.1. Instructions

i) **Specifications** describe in detail the requirements with which the products or materials used or obtained during manufacture have to conform. They serve as a basis for quality evaluation.

ii) **Manufacturing composition, processing, packaging and testing instructions** provide detail all the starting materials, equipment and computerised systems (if any) to be used and specify all processing, packaging, sampling and testing instructions. In-process controls and process analytical technologies to be employed should be specified where relevant, together with acceptance criteria.

iii) **Protocols** give instructions for performing and recording certain discreet operations.

iv) **Technical agreements** are agreed between contract givers and acceptors for outsourced activities.

3.2.2. Records or Reports

i) **Records** provide evidence of various actions taken to demonstrate compliance with instructions, including activities, events, investigations, and in the case of manufactured batches a history of each batch of product, from initiation of manufacture to final distribution. Records include the raw data that is used to generate other records. For electronic records regulated users should define which data are to be used as raw data. At least, all data on which quality decisions are based should be defined as raw data

ii) **Certificates of analysis** provide a summary of testing results on samples of products or materials together with the evaluation for compliance to a stated specification.

iii) **Reports** document the conduct of particular exercises, projects or investigations, together with results, conclusions and recommendations.

3.3. Generation and control of documentation

i) All types of document should be defined and adhered to. The requirements apply equally to all forms of document media types. Complex systems need to be understood, well documented, validated, and adequate controls should be in place. Many documents (instructions or records) may exist in hybrid forms, i.e. some elements as electronic and others as paper based. Relationships and control measures for master documents, official copies, data handling and records need to be stated for both hybrid and homogenous systems. Appropriate controls for electronic documents such as templates, forms, and master documents should be implemented. Appropriate controls should be in place to ensure the integrity and authenticity of the record throughout the retention period.

ii) Documents should be designed, prepared, reviewed, and distributed with care. They should comply with the relevant parts of product specification files, manufacturing and marketing authorisation dossiers, as appropriate. The reproduction of working documents from master documents should not allow any error to be introduced through the reproduction process.

iii) Documents containing instructions should be approved, signed and dated by appropriate and authorised persons. Documents should have unambiguous contents and be uniquely identifiable. The effective date should be defined.

iv) Documents containing instructions should be laid out in an orderly fashion and be easy to check. The style and language of documents should fit with their intended use. Standard Operating Procedures (SOPs), work instructions and methods should be written in an imperative mandatory style.

v) Documents within the quality management system should be regularly reviewed and kept up-to-date.

vi) Documents should not be hand-written; although, where documents require the entry of data, sufficient space should be provided for such entries.

3.4. Good documentation practices

i) Handwritten entries should be made in clear, legible, indelible way.

ii) Records should be made or completed at the time each action is taken and in such a way that all significant activities concerning the manufacture of vaccines are traceable.

iii) Any alteration made to the entry on a document should be signed and dated; the alteration should permit the reading of the original information. Where appropriate, the reason for the alteration should be recorded.

3.5. Retention of documents

i) It should be clearly defined which record is related to each manufacturing activity and where this record is located. Secure controls must be in place to ensure the integrity of the record throughout the retention period and validated where appropriate.

ii) Specific requirements apply to batch documentation that must be kept for 1 year after expiry of the batch to which it relates or at least 5 years after certification of the batch by the authorised person, whichever is the longer. For investigational vaccines, the batch documentation must be kept for at least 5 years after the completion or formal discontinuation of the last clinical trial in which the batch was used.

For other types of documentation, the retention period will depend on the business activity that the documentation supports. Critical documentation, including raw data (for example relating to validation or stability), which supports information in the marketing authorisation should be retained whilst the authorisation remains in force. It may be considered acceptable to retire certain documentation (e.g. raw data supporting validation reports or stability reports) where the data has been superseded by a full set of new data.

More information and details can be found on documentation within the PIC/S GMP guide (PIC/S, PE 009-11 [Part I], see References).

4. Rules governing outsourced activities

4.1. Principle

Any activity that is outsourced should be appropriately defined, agreed and controlled in order to avoid misunderstandings that could result in a product or operation of unsatisfactory quality.

4.2. General

i) There should be a written contract covering the outsourced activities, the products or operations to which they are related, and any technical arrangements made in connection with it.

ii) All arrangements for the outsourced activities including any proposed changes in technical or other arrangements should be in accordance with regulations in force, and the marketing authorisation for the product concerned, where applicable.

iii) Where the marketing authorisation holder and the manufacturer are not the same, appropriate arrangements should be in place, taking into account the principles described in this chapter.

4.3. The contract giver

i) The pharmaceutical quality system of the contract giver should include the control and review of any outsourced activities. The contract giver is ultimately responsible to ensure processes are in place to assure the control of outsourced activities. These processes should incorporate quality risk management principles.

ii) Prior to outsourcing activities, the contract giver is responsible for assessing the legality, suitability and the competence of the contract acceptor to carry out successfully the outsourced activities. The contract giver is also responsible for ensuring by means of the contract that the principles and guidelines of GMP are followed.

iii) The contract giver should provide the contract acceptor with all the information and knowledge necessary to carry out the contracted operations correctly in accordance with regulations in force, and the marketing authorisation for the product concerned. The contract giver should ensure that the contract acceptor is fully aware of any problems associated with the product or the work that might pose a hazard to his premises, equipment, personnel, other materials or other products.

iv) The contract giver should audit, monitor and review the performance of the contract acceptor and the identification and implementation of any needed improvement.

v) The contract giver should be responsible for reviewing and assessing the records and the results related to the outsourced activities. He should also ensure, either by himself, or based on the confirmation of the contract acceptor's qualified person, that all products and materials delivered to him by the contract acceptor have been processed in accordance with GMP and the marketing authorisation.

4.4. The contract acceptor

i) The contract acceptor must be able to carry out satisfactorily the work ordered by the contract giver such as having adequate premises, equipment, knowledge, experience, and competent personnel.

ii) The contract acceptor should ensure that all products, materials and knowledge delivered to him are suitable for their intended purpose.

iii) The contract acceptor should not subcontract to a third party any of the work entrusted to him under the contract without the contract giver's prior evaluation and approval of the arrangements. Arrangements made between the contract acceptor and any third party should ensure that information and knowledge, including those from assessments of the suitability of the third party, are made available in the same way as between the original contract giver and contract acceptor.

iv) The contract acceptor should not make unauthorised changes, outside the terms of the contract, that may adversely affect the quality of the outsourced activities for the contract giver.

v) The contract acceptor should understand that outsourced activities, including contract analysis, may be subject to inspection by the competent authorities.

4.5. The contract

i) A contract should be drawn up between the contract giver and the contract acceptor that specifies their respective responsibilities and communication processes relating to the outsourced activities. Technical aspects of the contract should be drawn up by competent persons suitably knowledgeable in related outsourced activities and GMP. All arrangements for outsourced activities must be in accordance with regulations in force and the marketing authorisation for the product concerned and agreed by both parties.

ii) The contract should describe clearly who undertakes each step of the outsourced activity, e.g. knowledge management, technology transfer, supply chain, subcontracting, quality and

purchasing of materials, testing and releasing materials, undertaking production and quality controls (including in-process controls, sampling and analysis).

iii) All records related to the outsourced activities, e.g. manufacturing, analytical and distribution records, and reference samples, should be kept by, or be available to, the contract giver. Any records relevant to assessing the quality of a product in the event of complaints or a suspected defect or to investigating in the case of a suspected falsified product must be accessible and specified in the relevant procedures of the contract giver.

iv) The contract should permit the contract giver to audit outsourced activities, performed by the contract acceptor or his mutually agreed subcontractors

5. Rules governing complaints and products recall

5.1. Principle

All complaints and other information concerning potentially defective products must be reviewed carefully according to written procedures. A system should be designed to evaluate complaints and initiate a recall from the market, if necessary, promptly and effectively for products known or suspected to be defective.

5.2. Complaints

i) A person should be designated responsible for handling the complaints and deciding the measures to be taken together with sufficient supporting staff to assist him.

ii) There should be written procedures describing the risk evaluation action to be taken, including the need to consider a recall, in the case of a complaint concerning a possible product defect.

iii) Any complaint concerning a product defect should be recorded with all the original details and thoroughly investigated. The person responsible for quality control should normally be involved in the study of such problems.

iv) If a product defect is discovered or suspected in a batch, ingredient or equipment used, consideration should be given to checking other batches in order to determine whether they are also affected. In particular, other batches that may contain reworks of the defective batch should be investigated.

v) All the decisions and measures taken as a result of a complaint should be recorded and referenced to the corresponding batch records.

vi) Complaints records should be reviewed regularly for any indication of specific or recurring problems requiring attention and possibly the recall of marketed products.

vii) Special attention should be given to establishing whether a complaint was caused because of counterfeiting.

viii) The competent authorities should be informed as soon as possible if a manufacturer is considering action following possibly faulty manufacture, product deterioration, detection of counterfeiting or any other serious quality problems with a product

5.3. Recalls

i) A person should be designated as responsible for execution and co-ordination of recalls and should be supported by sufficient staff to handle all the aspects of the recalls with the appropriate degree of urgency. This responsible person should normally be independent of the sales and marketing organisation.

ii) There should be established written procedures, regularly checked and updated when necessary, in order to organise any recall activity.

iii) Recall operations should be capable of being initiated promptly, communicated effectively to customers and at any time.

iv) All Competent Authorities of all countries to which products may have been distributed should be informed promptly if products are intended to be recalled because they are, or are suspected of being defective.

v) The distribution records should be readily available to the person(s) responsible for recalls, and should contain sufficient information on wholesalers and directly supplied customers (with addresses, email addresses, phone or fax numbers inside and outside working hours, batches and amounts delivered), including those for exported products and medical samples.

vi) Recalled products should be identified and stored separately in a secure area while awaiting a decision on their fate.

vii) The progress of the recall process should be recorded and a final report issued, including reconciliation between the delivered and recovered quantities of the products.

viii) The effectiveness of the arrangements for recalls should be evaluated regularly.

6. Rules governing self-inspection

6.1. Principle

Self-inspections should be conducted in order to monitor the implementation and compliance with GMP principles laid down in this guide, and to propose necessary corrective measures.

i) Personnel matters, premises, equipment, documentation, production, quality control, distribution of the vaccines, arrangements for dealing with complaints and recalls, and self-inspection, should be examined at intervals following a pre-arranged programme in order to verify their conformity with the principles of quality assurance.

ii) Self-inspections should be conducted in an independent and detailed way by designated competent person(s) from the company. Independent audits by external experts may also be useful.

iii) All self-inspections should be recorded. Reports should contain all the observations made during the inspections and, where applicable, proposals for corrective measures. Statements on the actions subsequently taken should also be recorded.

REFERENCES

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NB: FIRST ADOPTED IN 2016.

CHAPTER 2.3.3.

MINIMUM REQUIREMENTS FOR THE ORGANISATION AND MANAGEMENT OF A VACCINE MANUFACTURING FACILITY

SUMMARY

This chapter sets out the management requirements for the manufacture of veterinary vaccines in accordance with Chapter 1.1.8 Principles of veterinary vaccine production. *Manufacturers should use the chapter as a basis for the elaboration of specific rules adapted to their individual needs. Many of the general principles of laboratory management set out in Chapter 1.1.1* Management of veterinary diagnostic laboratories *are applicable to vaccine production, including accountability, executive management, infrastructure, human resources and compliance.*

There should be sufficient qualified personnel to carry out all the tasks that are the responsibility of the manufacturer. Individual responsibilities should be clearly understood by the individuals and recorded. All personnel should be aware of the principles of Good Manufacturing Practice (GMP) that affect them and receive initial and continuing training, including hygiene instructions, relevant to their needs. Appropriate procedures should be in place for biorisk management, to protect the personnel, to prevent hazardous biological agents from leaving the plant, and to prevent contamination of products within the plant.

Premises and equipment should be located, designed, constructed, adapted and maintained to suit the operations. Their layout and design should aim to minimise the risk of errors and permit effective cleaning and maintenance in order to avoid cross-contamination, build-up of dust or dirt and, in general, any adverse effect on the quality of products.

Good documentation (including electronic records) constitutes an essential part of the quality assurance system and is key to operating in compliance with GMP requirements. The types of documents and electronic media used should be fully defined in the manufacturer's quality management system. The main objective of the system of documentation should be to establish, control, monitor and record all activities that directly or indirectly impact on the quality of products. The quality management system should include sufficient detail to facilitate a common understanding of the requirements, in addition to providing sufficient records of processes and evaluation of observations.

Any outsourced activity should be appropriately defined, agreed and controlled in order to avoid misunderstandings that could result in a product or operation of unsatisfactory quality. Written contracts should cover outsourced activities, products or operations to which they are related, and any connected technical arrangements. All arrangements for the outsourced activities including any proposed changes in technical or other aspects should be in accordance with regulations in force, and the marketing authorisation for the product concerned. Where the marketing authorisation holder and the manufacturer are not the same, appropriate arrangements should be in place to take into account the principles described in this chapter.

All complaints and other information concerning potentially defective products should be reviewed carefully following written procedures. A system should be in place to evaluate complaints and, if necessary, initiate a recall from the market promptly and effectively for products known or suspected to be defective.

Self-inspections should be conducted in order to monitor the implementation and compliance with GMP principles, and to propose necessary corrective measures.

1. Rules for personnel

The establishment and maintenance of a satisfactory system of quality assurance and the correct manufacture of veterinary vaccines relies upon people. For this reason, there must be sufficient qualified personnel to carry out all the tasks that are the responsibility of the manufacturer. Individual responsibilities should be clearly understood by the individuals and recorded. All personnel should be aware of the principles of Good Manufacturing Practices (GMP) that affect them and receive initial and continuing training, including hygiene instructions, relevant to their needs.

1.1. General rules

i) The manufacturer should have an adequate number of personnel with the necessary qualifications and practical experience. The responsibilities placed on any one individual should not be so extensive as to present any risk to quality.

ii) The manufacturer must have an organisation chart. People in responsible positions should have specific duties recorded in written job descriptions and adequate authority to carry out their responsibilities. Their duties may be delegated to designated deputies of a satisfactory qualification level. There should be no gaps or unexplained overlaps in the responsibilities of those personnel concerned with the application of GMP.

1.1.1. Key personnel

i) Key personnel include the head of production, the head of quality control, the head of packaging and distribution, the head of technical services and the person(s) responsible for the batch release. Normally key posts should be occupied by full-time personnel. The heads of production and quality control should be held by different individuals, independent from each other. In large organisations, it may be necessary to delegate some of the functions.

1.1.2. Training

i) The manufacturer should provide training for all the personnel whose duties take them into production areas or into control laboratories (including the technical, maintenance and cleaning personnel), and for other personnel whose activities could affect the quality of the product.

ii) Besides the basic training on the theory and practice of GMP, newly recruited personnel should receive training appropriate to the duties assigned to them. Continuing training should also be given, and its practical effectiveness should be periodically assessed. Training programmes should be available, approved by a qualified person. Training records should be kept.

iii) Personnel working in areas where contamination is a hazard, e.g. clean areas or areas where highly active, toxic, infectious or sensitising materials are handled, should be given specific training (see Section 2.4 *Specific requirements for vaccine production*).

iv) Visitors or untrained personnel should, preferably, not be taken into the production and quality control areas. If this is unavoidable, they should be given information in advance, particularly about personal hygiene and the prescribed protective clothing. They should be closely supervised.

v) The concept of quality assurance and all the measures capable of improving its understanding and implementation should be fully discussed during the training sessions.

1.1.3. Personal hygiene

i) Detailed hygiene programmes should be established and adapted to the different needs within the factory. They should include procedures relating to the health, hygiene practices and clothing of personnel. These procedures should be understood and followed in a very strict way by every person whose duties take him into the production and control areas. Hygiene programmes should be promoted by management and widely discussed during training sessions.

ii) All personnel should receive a medical examination upon recruitment. It must be the manufacturer's responsibility that there are instructions ensuring that health conditions that can be of relevance to the quality of products come to the manufacturer's knowledge. After the first medical examination, examinations should be carried out when necessary for the work and personal health.

iii) Steps should be taken to ensure as far as is practicable that no person affected by an infectious disease or having open lesions on the exposed surface of the body is engaged in the manufacture of vaccines.

iv) Every person entering the manufacturing areas should wear protective garments appropriate to the operations to be carried out.

v) Eating, drinking, chewing or smoking, or the storage of food, drink, smoking materials or personal medication in the production and storage areas should be prohibited. In general, any unhygienic practice within the manufacturing areas or in any other area where the product might be adversely affected should be forbidden.

vi) Direct contact should be avoided between the operator's hands and the exposed product as well as with any part of the equipment that comes into contact with the products.

vii) Personnel should be instructed to use the hand-washing facilities.

viii) Any specific requirements for the manufacture of aseptic or sterile preparations related to production are covered in 2.4 *Specific requirements for vaccine production*.

1.2. Specific rules for personnel involved in the manufacture of vaccines

i) All personnel (including those concerned with cleaning and maintenance) employed in areas where immunological products are manufactured should be given training in and information on hygiene and microbiology. They should receive additional training specific to the products with which they work.

ii) Responsible personnel should be formally trained in some or all of the required fields.

iii) Personnel should be protected against possible infection with the biological agents used in manufacture. In the case of biological agents known to cause disease in humans, adequate measures should be taken to prevent infection of personnel working with the agent or with experimental animals.

iv) Where relevant, the personnel should be vaccinated and subject to medical examination.

v) Adequate measures should be taken to prevent biological agents being taken outside the manufacturing plant by personnel acting as a carrier. Dependent on the type of biological agent, such measures may include complete change of clothes and compulsory showering before leaving the production area.

vi) For immunological products, the risk of contamination or cross-contamination by personnel is particularly important.

vii) Prevention of contamination by personnel should be achieved by a set of measures and procedures to ensure that appropriate protective clothing is used during the different stages of the production process.

viii) Prevention of cross-contamination by personnel involved in production should be achieved by a set of measures and procedures to ensure that they do not pass from one area to another unless they have taken appropriate measures to eliminate the risk of contamination. In the course of a working day, personnel should not pass from areas where contamination with live micro-organisms is likely or where animals are housed to premises where other products or organisms are handled. If such passage is unavoidable, clearly defined decontamination procedures, including change of clothing and shoes, and, where necessary, showering, should be followed by staff involved in any such production.

ix) Personnel entering a contained area where organisms had not been handled in open circuit operations in the previous 12 hours to check on cultures in sealed, surface decontaminated flasks would not be regarded as being at risk of contamination, unless the organism involved was an exotic.

2. Requirements for premises and equipment

Premises and equipment must be located, designed, constructed, adapted and maintained to suit the operations to be carried out. Their layout and design must aim to minimise the risk of errors and permit effective cleaning and maintenance in order to avoid cross-contamination, build-up of dust or dirt and, in general, any adverse effect on the quality of products.

Premises and equipment used for critical operations must be qualified[1].

Added requirements for premises and equipment used in aseptic preparations are given in 2.4 *Specific requirements for vaccine production.*

2.1. General

i) Premises should be situated in an environment which, when considered together with measures to protect the manufacture, presents minimal risk of causing contamination of materials or products. Research and development activities associated with organisms not authorised to be handled in the premises must be carried out in buildings completely separated from the manufacturing facility.

ii) Premises should be carefully maintained, ensuring that repair and maintenance operations do not present any hazard to the quality of products. They should be cleaned and, where applicable, disinfected according to detailed written procedures.

iii) Lighting, temperature, humidity and ventilation should be appropriate and such that they do not adversely affect, directly or indirectly, either the medicinal products during their manufacture and storage, or the accurate functioning of equipment.

iv) Premises should be designed and equipped so as to afford maximum protection against the entry of insects or other animals.

v) Steps should be taken in order to prevent the entry of unauthorised people. Production, storage and quality control areas should not be used as a right of way by personnel who do not work in them.

2.2. Storage areas

i) Storage areas should be of sufficient capacity to allow orderly storage of the various categories of materials and products: starting and packaging materials, intermediate, bulk and finished products, products in quarantine, released, rejected, returned or recalled.

ii) Storage areas should be designed or adapted to ensure good storage conditions. In particular, they should be clean and dry and maintained within acceptable temperature limits. Where special storage conditions are required (e.g. temperature, humidity) these should be validated, checked and monitored.

iii) Receiving and dispatch bays should protect materials and products from the weather. Reception areas should be designed and equipped to allow containers of incoming materials to be cleaned where necessary before storage.

1 This qualification may be done according to the rules laid down in the PIC/S (see References).

iv) Where quarantine status is ensured by storage in separate areas, these areas must be clearly marked and their access restricted to authorised personnel. Any system replacing the physical quarantine should give equivalent security.

v) There should normally be a separate sampling area for starting materials. If sampling is performed in the storage area, it should be conducted in such a way as to prevent contamination or cross-contamination.

vi) Segregated areas should be provided for the storage of rejected, recalled or returned materials or products.

vii) Highly active materials or products should be stored in safe and secure areas.

viii) Printed packaging materials are considered critical to the conformity of the vaccine and special attention should be paid to the safe and secure storage of these materials.

2.3. Production premises – general requirements

i) Premises should preferably be laid out in such a way as to allow the production to take place in areas connected in a logical order corresponding to the sequence of the operations and to the requisite cleanliness levels.

ii) The adequacy of the working and in-process storage space should permit the orderly and logical positioning of equipment and materials so as to minimise the risk of confusion between different medicinal products or their components, to avoid cross-contamination and to minimise the risk of omission or wrong application of any of the manufacturing or control steps.

iii) Where starting and primary packaging materials, intermediate or bulk products are exposed to the environment, interior surfaces (walls, floors and ceilings) should be smooth, free from cracks and open joints, and should not shed particulate matter and should permit easy and effective cleaning and, if necessary, disinfection.

iv) Pipework, light fittings, ventilation points and other services should be designed and sited to avoid the creation of recesses that are difficult to clean. As far as possible, for maintenance purposes, they should be accessible from outside the manufacturing areas.

v) Drains should be of adequate size, and have trapped gullies. Open channels should be avoided where possible, but if necessary, they should be shallow to facilitate cleaning and disinfection.

vi) Production areas should be effectively ventilated, with air control facilities (including temperature and, where necessary, humidity and filtration) appropriate both to the products handled, to the operations undertaken within them and to the external environment.

vii) Weighing of starting materials usually should be carried out in a separate weighing room designed for that use.

viii) In cases where dust is generated (e.g. during sampling, weighing, mixing and processing operations, packaging of dry products), specific provisions should be taken to avoid cross-contamination and facilitate cleaning.

ix) Premises for the packaging of vaccines should be specifically designed and laid out so as to avoid mix-ups or cross-contamination.

x) Production areas should be well lit, particularly where visual on-line controls are carried out.

xi) In-process controls may be carried out within the production area provided they do not carry any risk for the production.

2.3.1. Ancillary areas

i) Rest and refreshment rooms should be separate from other areas.

ii) Facilities for changing clothes and for washing and toilet purposes should be easily accessible and appropriate for the number of users. Toilets should not directly communicate with production or storage areas.

iii) Maintenance workshops should as far as possible be separated from production areas. Whenever parts and tools are stored in the production area, they should be kept in rooms or lockers reserved for that use.

2.3.2. Quality control areas

i) Normally, quality control laboratories should be separated from production areas. This is particularly important for laboratories for the control of biologicals, microbiologicals and radioisotopes, which should also be separated from each other.

ii) Control laboratories should be designed to suit the operations to be carried out in them. Sufficient space should be given to avoid mix-ups and cross-contamination. There should be adequate suitable storage space for samples and records.

iii) Separate rooms may be necessary to protect sensitive instruments from vibration, electrical interference, humidity, etc.

iv) Special requirements are needed in laboratories handling particular substances, such as biological or radioactive samples.

v) Animal houses should be well isolated from other areas, with separate entrance (animal access) and air handling facilities.

2.4. Specific requirements for vaccine production

2.4.1. Production areas

i) Premises should be designed in such a way as to control both the risk to the product and to the environment. This can be achieved by the use of containment, clean, clean or contained or controlled areas[2].

ii) Live biological agents should be handled in contained areas. The level of containment should depend on the pathogenicity of the micro-organism and whether it has been classified as exotic.

iii) Inactivated biological agents should be handled in clean areas. Clean areas should also be used when handling non-infected cells isolated from multicellular organisms and, in some cases, filtration-sterilised media.

iv) Open circuit operations involving products or components not subsequently sterilised should be carried out within a laminar air flow work station according to the rules for aseptic preparation.

v) Other operations where live biological agents are handled (quality control, research and diagnostic services, etc.) should be appropriately contained and separated if production operations are carried out in the same building. The level of containment should depend on the pathogenicity of the biological agent and whether they have been classified as exotic. Whenever diagnostic activities are carried out, there is the risk of introducing highly pathogenic organisms. Therefore, the level of containment should be adequate to cope with all such risks. Containment may also be required if quality control or other activities are carried out in buildings in close proximity to those used for production.

vi) Containment premises should be easily disinfected and should have the following characteristics:

a) the absence of direct venting to the outside;

2 Guidance on the qualification of the clean areas is given in ISO 14644 and described in the PIC/S GMP Guide (see References).

b) a ventilation system with air at negative pressure. Air should be extracted through HEPA (high-efficiency particulate air) filters and not be re-circulated except to the same area, and provided further HEPA filtration is used (normally this condition would be met by routing the re-circulated air through the normal supply HEPAs for that area). However, recycling of air between areas may be permissible provided that it passes through two exhaust HEPAs, the first of which is continuously monitored for integrity, and there are adequate measures for safe venting of exhaust air should this filter fail;

c) air from manufacturing areas used for the handling of exotic organisms should be vented through two sets of HEPA filters in series, and that from production areas not re-circulated;

d) a system for the collection and disinfection of liquid effluents (see Section 2.4.4 *Disinfection – waste disposal*);

e) changing rooms designed and used as air locks, and equipped with washing and showering facilities if appropriate. Air pressure differentials should be such that there is no flow of air between the work area and the external environment or risk of contamination of outer clothing worn outside the area;

f) an air lock system for the passage of equipment, which is constructed so that there is no flow of contaminated air between the work area and the external environment or risk of contamination of equipment within the lock. The air lock should be of a size that enables the effective surface decontamination of materials being passed through it. Consideration should be given to having a timing device on the door interlock to allow sufficient time for the decontamination process to be effective.

g) in many instances, a barrier double-door autoclave for the secure removal of waste materials and introduction of sterile items.

vii) Water treatment plants and distribution systems should be designed, constructed and maintained so as to ensure a reliable source of water of an appropriate quality. They should not be operated beyond their designed capacity. Water for injections should be produced, stored and distributed in a manner that prevents microbial growth, for example by constant circulation at a temperature above 70°C.

viii) Equipment passes and changing rooms should have an interlock mechanism or other appropriate system to prevent the opening of more than one door at a time. Changing rooms should be supplied with air filtered to the same standard as that for the work area, and equipped with air extraction facilities to produce an adequate air circulation independent of that of the work area. Equipment passes should normally be ventilated in the same way, but unventilated passes, or those equipped with supply air only, may be acceptable.

ix) Production operations such as cell maintenance, media preparation, virus culture, etc., likely to cause contamination should be performed in separate areas. Animals and animal products should be handled with appropriate precautions.

x) Production areas where biological agents particularly resistant to disinfection (e.g. spore forming bacteria) are handled should be separated and dedicated to that particular purpose until the biological agents have been inactivated.

xi) With the exception of blending and subsequent filling operations (for example with multi-valent vaccines), one biological agent only should be handled at a time within an area.

xii) Production areas should be designed to permit disinfection between campaigns, using validated methods.

xiii) Production of biological agents may take place in controlled areas provided it is carried out in totally enclosed and heat-sterilised equipment, all connections being also heat sterilised after making and before breaking. It may be acceptable for connections to be made under local laminar air flow provided these are few in number and proper aseptic techniques are used and there is no risk of leakage. The sterilisation parameters used before breaking the connections must be validated for the organisms being used. Different products may be placed in different biogenerators (fermenters), within the same area, provided that there is

no risk of accidental cross-contamination. However, organisms generally subject to special requirements for containment should be in areas dedicated to such products.

xiv) Access to manufacturing areas should be restricted to authorised personnel. Clear and concise written procedures should be posted as appropriate.

xv) The manufacturing site and buildings should be described in sufficient detail (by means of plans and written explanations) so that the designation and conditions of use of all the rooms are correctly identified as well as the biological agents that are handled in them. The flow of people and product should also be clearly marked.

xvi) The activities carried out in the vicinity of the site should also be indicated.

xvii) Plans of contained or clean area premises should describe the ventilation system indicating inlets and outlets, filters and their specifications, the number of air changes per hour, and pressure gradients. They should indicate which pressure gradients are monitored by pressure indicator.

2.4.2. Animals and animal houses

i) Animal houses should be separated from the other production premises and suitably designed.

ii) The sanitary status of the animals used for production should be defined, monitored, and recorded. Some animals should be handled as defined in specific monographs (e.g. specific pathogen free [SPF] flocks).

iii) Animals, biological agents, and tests carried out should be the subject of an identification system so as to prevent any risk of confusion and to control all possible hazards.

iv) Animal houses where animals intended or used for production are accommodated, should be provided with the appropriate containment or clean area measures, and should be separate from other animal accommodation.

v) Animal houses where animals used for quality control, involving the use of pathogenic biological agents, are accommodated, should be adequately contained.

vi) The animal species accommodated in the animal houses or otherwise on the site should be identified.

2.4.3. Equipment

i) The equipment used should be designed and constructed so that it meets the particular requirements for the manufacture of each product.

ii) Before being put into operation the equipment should be qualified and validated and subsequently be regularly maintained and validated.

iii) Where appropriate, the equipment should ensure satisfactory primary containment of the biological agents.

iv) Where appropriate, the equipment should be designed and constructed as to allow easy and effective decontamination or sterilisation.

v) Closed equipment used for the primary containment of the biological agents should be designed and constructed as to prevent any leakage or the formation of droplets and aerosols.

vi) Inlets and outlets for gases should be protected so as to achieve adequate containment, e.g. by the use of sterilising hydrophobic filters.

vii) The introduction or removal of material should take place using a sterilisable closed system, or possibly in an appropriate laminar air flow.

viii) Equipment where necessary should be properly sterilised before use, preferably by pressurised dry steam. Other methods can be accepted if steam sterilisation cannot be used because of the nature of the equipment. It is important not to overlook such individual items as bench centrifuges and water baths.

ix) Equipment used for purification, separation or concentration should be sterilised or disinfected at least between use for different products. The effect of the sterilisation methods on the effectiveness and validity of the equipment should be studied in order to determine the life span of the equipment.

x) All sterilisation procedures should be validated.

xi) Equipment should be designed so as to prevent any mix-up between different organisms or products. Pipes, valves and filters should be identified as to their function.

xii) Separate incubators should be used for infected and non-infected containers and also generally for different organisms or cells. Incubators containing more than one organism or cell type will only be acceptable if adequate steps are taken to seal, surface decontaminate and segregate the containers.

xiii) Culture vessels, etc., should be individually labelled. The cleaning and disinfection of the items can be particularly difficult and should receive special attention.

xiv) Equipment used for the storage of biological agents or products should be designed and used in such a manner as to prevent any possible mix-up. All stored items should be clearly and unambiguously labelled and in leak-proof containers. Items such as cells and organisms seed stock should be stored in dedicated equipment.

xv) Relevant equipment, such as that requiring temperature control, should be fitted with recording or alarm systems.

xvi) To avoid breakdowns, a system of preventive maintenance, together with trend analysis of recorded data, should be implemented.

xvii) The loading of freeze dryers requires an appropriate clean or contained area. Unloading freeze dryers contaminate the immediate environment. Therefore, for single-ended freeze dryers, the clean room should be decontaminated before a further manufacturing batch is introduced into the area, unless this contains the same organisms, and double door freeze dryers should be sterilised after each cycle unless opened in a clean area.

xviii) Sterilisation of freeze dryers should be done before use, preferably by pressurised dry steam. In case of campaign working, they should at least be sterilised after each campaign.

2.4.4. Disinfection – waste disposal

i) Disinfection or wastes and effluents disposal may be particularly important in the case of manufacture of immunological products. Careful consideration should therefore be given to procedures and equipment aiming at avoiding environmental contamination as well as to their validation or qualification.

3. Rules governing documentation

Documentation relating to the premises should be readily available in a **Site Master File**.

3.1. Principle

Good documentation constitutes an essential part of the quality assurance system and is key to operating in compliance with GMP requirements. The various types of documents and media used should be fully defined in the manufacturer's quality management system. Documentation may exist in a variety of forms, including paper-based, electronic or photographic media. The main objective of the system of documentation used must be to establish, control, monitor and record all activities that directly or indirectly impact on all aspects of the quality of products. The quality management system should include sufficient instructional detail to facilitate a common understanding of the requirements, in addition to providing for sufficient recording of the various processes and evaluation of any observations, so that on-going application of the requirements may be demonstrated.

There are two primary types of documentation used to manage and record GMP compliance: instructions (directions, requirements) and records or reports.

Suitable controls should be implemented to ensure the accuracy, integrity, availability and legibility of documents. Instruction documents should be free from errors and available in writing. This includes electronic records from which data may be rendered in a human readable form.

3.2. Required documentation

i) **Site Master File:** a document describing the GMP related activities of the manufacturer.

3.2.1. Instructions

i) **Specifications** describe in detail the requirements with which the products or materials used or obtained during manufacture have to conform. They serve as a basis for quality evaluation.

ii) **Manufacturing composition, processing, packaging and testing instructions** provide detail all the starting materials, equipment and computerised systems (if any) to be used and specify all processing, packaging, sampling and testing instructions. In-process controls and process analytical technologies to be employed should be specified where relevant, together with acceptance criteria.

iii) **Protocols** give instructions for performing and recording certain discreet operations.

iv) **Technical agreements** are agreed between contract givers and acceptors for outsourced activities.

3.2.2. Records or Reports

i) **Records** provide evidence of various actions taken to demonstrate compliance with instructions, including activities, events, investigations, and in the case of manufactured batches a history of each batch of product, from initiation of manufacture to final distribution. Records include the raw data that is used to generate other records. For electronic records regulated users should define which data are to be used as raw data. At least, all data on which quality decisions are based should be defined as raw data

ii) **Certificates of analysis** provide a summary of testing results on samples of products or materials together with the evaluation for compliance to a stated specification.

iii) **Reports** document the conduct of particular exercises, projects or investigations, together with results, conclusions and recommendations.

3.3. Generation and control of documentation

i) All types of document should be defined and adhered to. The requirements apply equally to all forms of document media types. Complex systems need to be understood, well documented, validated, and adequate controls should be in place. Many documents (instructions or records) may exist in hybrid forms, i.e. some elements as electronic and others as paper based. Relationships and control measures for master documents, official copies, data handling and records need to be stated for both hybrid and homogenous systems. Appropriate controls for electronic documents such as templates, forms, and master documents should be implemented. Appropriate controls should be in place to ensure the integrity and authenticity of the record throughout the retention period.

ii) Documents should be designed, prepared, reviewed, and distributed with care. They should comply with the relevant parts of product specification files, manufacturing and marketing authorisation dossiers, as appropriate. The reproduction of working documents from master documents should not allow any error to be introduced through the reproduction process.

iii) Documents containing instructions should be approved, signed and dated by appropriate and authorised persons. Documents should have unambiguous contents and be uniquely identifiable. The effective date should be defined.

iv) Documents containing instructions should be laid out in an orderly fashion and be easy to check. The style and language of documents should fit with their intended use. Standard Operating Procedures (SOPs), work instructions and methods should be written in an imperative mandatory style.

v) Documents within the quality management system should be regularly reviewed and kept up-to-date.

vi) Documents should not be hand-written; although, where documents require the entry of data, sufficient space should be provided for such entries.

3.4. Good documentation practices

i) Handwritten entries should be made in clear, legible, indelible way.

ii) Records should be made or completed at the time each action is taken and in such a way that all significant activities concerning the manufacture of vaccines are traceable.

iii) Any alteration made to the entry on a document should be signed and dated; the alteration should permit the reading of the original information. Where appropriate, the reason for the alteration should be recorded.

3.5. Retention of documents

i) It should be clearly defined which record is related to each manufacturing activity and where this record is located. Secure controls must be in place to ensure the integrity of the record throughout the retention period and validated where appropriate.

ii) Specific requirements apply to batch documentation that must be kept for 1 year after expiry of the batch to which it relates or at least 5 years after certification of the batch by the authorised person, whichever is the longer. For investigational vaccines, the batch documentation must be kept for at least 5 years after the completion or formal discontinuation of the last clinical trial in which the batch was used.

For other types of documentation, the retention period will depend on the business activity that the documentation supports. Critical documentation, including raw data (for example relating to validation or stability), which supports information in the marketing authorisation should be retained whilst the authorisation remains in force. It may be considered acceptable to retire certain documentation (e.g. raw data supporting validation reports or stability reports) where the data has been superseded by a full set of new data.

More information and details can be found on documentation within the PIC/S GMP guide (PIC/S, PE 009-11 [Part I], see References).

4. Rules governing outsourced activities

4.1. Principle

Any activity that is outsourced should be appropriately defined, agreed and controlled in order to avoid misunderstandings that could result in a product or operation of unsatisfactory quality.

4.2. General

i) There should be a written contract covering the outsourced activities, the products or operations to which they are related, and any technical arrangements made in connection with it.

ii) All arrangements for the outsourced activities including any proposed changes in technical or other arrangements should be in accordance with regulations in force, and the marketing authorisation for the product concerned, where applicable.

iii) Where the marketing authorisation holder and the manufacturer are not the same, appropriate arrangements should be in place, taking into account the principles described in this chapter.

4.3. The contract giver

i) The pharmaceutical quality system of the contract giver should include the control and review of any outsourced activities. The contract giver is ultimately responsible to ensure processes are in place to assure the control of outsourced activities. These processes should incorporate quality risk management principles.

ii) Prior to outsourcing activities, the contract giver is responsible for assessing the legality, suitability and the competence of the contract acceptor to carry out successfully the outsourced activities. The contract giver is also responsible for ensuring by means of the contract that the principles and guidelines of GMP are followed.

iii) The contract giver should provide the contract acceptor with all the information and knowledge necessary to carry out the contracted operations correctly in accordance with regulations in force, and the marketing authorisation for the product concerned. The contract giver should ensure that the contract acceptor is fully aware of any problems associated with the product or the work that might pose a hazard to his premises, equipment, personnel, other materials or other products.

iv) The contract giver should audit, monitor and review the performance of the contract acceptor and the identification and implementation of any needed improvement.

v) The contract giver should be responsible for reviewing and assessing the records and the results related to the outsourced activities. He should also ensure, either by himself, or based on the confirmation of the contract acceptor's qualified person, that all products and materials delivered to him by the contract acceptor have been processed in accordance with GMP and the marketing authorisation.

4.4. The contract acceptor

i) The contract acceptor must be able to carry out satisfactorily the work ordered by the contract giver such as having adequate premises, equipment, knowledge, experience, and competent personnel.

ii) The contract acceptor should ensure that all products, materials and knowledge delivered to him are suitable for their intended purpose.

iii) The contract acceptor should not subcontract to a third party any of the work entrusted to him under the contract without the contract giver's prior evaluation and approval of the arrangements. Arrangements made between the contract acceptor and any third party should ensure that information and knowledge, including those from assessments of the suitability of the third party, are made available in the same way as between the original contract giver and contract acceptor.

iv) The contract acceptor should not make unauthorised changes, outside the terms of the contract, that may adversely affect the quality of the outsourced activities for the contract giver.

v) The contract acceptor should understand that outsourced activities, including contract analysis, may be subject to inspection by the competent authorities.

4.5. The contract

i) A contract should be drawn up between the contract giver and the contract acceptor that specifies their respective responsibilities and communication processes relating to the outsourced activities. Technical aspects of the contract should be drawn up by competent persons suitably knowledgeable in related outsourced activities and GMP. All arrangements for outsourced activities must be in accordance with regulations in force and the marketing authorisation for the product concerned and agreed by both parties.

ii) The contract should describe clearly who undertakes each step of the outsourced activity, e.g. knowledge management, technology transfer, supply chain, subcontracting, quality and

purchasing of materials, testing and releasing materials, undertaking production and quality controls (including in-process controls, sampling and analysis).

iii) All records related to the outsourced activities, e.g. manufacturing, analytical and distribution records, and reference samples, should be kept by, or be available to, the contract giver. Any records relevant to assessing the quality of a product in the event of complaints or a suspected defect or to investigating in the case of a suspected falsified product must be accessible and specified in the relevant procedures of the contract giver.

iv) The contract should permit the contract giver to audit outsourced activities, performed by the contract acceptor or his mutually agreed subcontractors

5. Rules governing complaints and products recall

5.1. Principle

All complaints and other information concerning potentially defective products must be reviewed carefully according to written procedures. A system should be designed to evaluate complaints and initiate a recall from the market, if necessary, promptly and effectively for products known or suspected to be defective.

5.2. Complaints

i) A person should be designated responsible for handling the complaints and deciding the measures to be taken together with sufficient supporting staff to assist him.

ii) There should be written procedures describing the risk evaluation action to be taken, including the need to consider a recall, in the case of a complaint concerning a possible product defect.

iii) Any complaint concerning a product defect should be recorded with all the original details and thoroughly investigated. The person responsible for quality control should normally be involved in the study of such problems.

iv) If a product defect is discovered or suspected in a batch, ingredient or equipment used, consideration should be given to checking other batches in order to determine whether they are also affected. In particular, other batches that may contain reworks of the defective batch should be investigated.

v) All the decisions and measures taken as a result of a complaint should be recorded and referenced to the corresponding batch records.

vi) Complaints records should be reviewed regularly for any indication of specific or recurring problems requiring attention and possibly the recall of marketed products.

vii) Special attention should be given to establishing whether a complaint was caused because of counterfeiting.

viii) The competent authorities should be informed as soon as possible if a manufacturer is considering action following possibly faulty manufacture, product deterioration, detection of counterfeiting or any other serious quality problems with a product

5.3. Recalls

i) A person should be designated as responsible for execution and co-ordination of recalls and should be supported by sufficient staff to handle all the aspects of the recalls with the appropriate degree of urgency. This responsible person should normally be independent of the sales and marketing organisation.

ii) There should be established written procedures, regularly checked and updated when necessary, in order to organise any recall activity.

iii) Recall operations should be capable of being initiated promptly, communicated effectively to customers and at any time.

iv) All Competent Authorities of all countries to which products may have been distributed should be informed promptly if products are intended to be recalled because they are, or are suspected of being defective.

v) The distribution records should be readily available to the person(s) responsible for recalls, and should contain sufficient information on wholesalers and directly supplied customers (with addresses, email addresses, phone or fax numbers inside and outside working hours, batches and amounts delivered), including those for exported products and medical samples.

vi) Recalled products should be identified and stored separately in a secure area while awaiting a decision on their fate.

vii) The progress of the recall process should be recorded and a final report issued, including reconciliation between the delivered and recovered quantities of the products.

viii) The effectiveness of the arrangements for recalls should be evaluated regularly.

6. Rules governing self-inspection

6.1. Principle

Self-inspections should be conducted in order to monitor the implementation and compliance with GMP principles laid down in this guide, and to propose necessary corrective measures.

i) Personnel matters, premises, equipment, documentation, production, quality control, distribution of the vaccines, arrangements for dealing with complaints and recalls, and self-inspection, should be examined at intervals following a pre-arranged programme in order to verify their conformity with the principles of quality assurance.

ii) Self-inspections should be conducted in an independent and detailed way by designated competent person(s) from the company. Independent audits by external experts may also be useful.

iii) All self-inspections should be recorded. Reports should contain all the observations made during the inspections and, where applicable, proposals for corrective measures. Statements on the actions subsequently taken should also be recorded.

REFERENCES

INTERNATIONAL ORGANISATION FOR STANDARDIZATION (ISO) (1999). ISO 14644-1:1999. Cleanrooms and associated controlled environments – Part 1: Classification of air cleanliness. International Organization for Standardization (ISO), www.iso.org.

INTERNATIONAL ORGANISATION FOR STANDARDIZATION (ISO) (2000). ISO 14644-2:2000. Cleanrooms and associated controlled environments – Part 2: Specifications for testing and monitoring to prove continued compliance with ISO 14644-1. International Organization for Standardization (ISO), www.iso.org.

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NB: FIRST ADOPTED IN 2016.

CHAPTER 2.3.4.

MINIMUM REQUIREMENTS FOR THE PRODUCTION AND QUALITY CONTROL OF VACCINES

SUMMARY

This chapter provides requirements for the manufacture and quality control of veterinary vaccines in accordance with Chapter 1.1.8 Principles of veterinary vaccine production. *Manufacturers should use the recommendations as a basis for the elaboration of specific rules adapted to their individual needs.*

Production operations should follow clearly defined procedures that comply with the principles elaborated in this chapter, in order to obtain products of the requisite quality in accordance with the marketing authorisations. The manufacture of immunological veterinary medicinal products has special characteristics that should be considered when implementing and assessing the quality assurance system.

Due to the large number of animal species and related pathogenic agents, the variety of products manufactured is very wide and the volume of manufacture is often low. The products should be protected against organic or inorganic contamination and cross-contamination. The environment must also be protected, especially when using pathogenic or exotic biological agents, and personnel must be protected from biological agents pathogenic to humans. The role of the quality assurance system is therefore of paramount importance.

Quality control is concerned with sampling, specifications and testing as well as the organisation, documentation and release procedures to ensure that the necessary and relevant tests are carried out, and that materials are not released for use, nor products released for sale or supply, until their quality has been judged satisfactory. Quality control is not confined to laboratory operations, but must be involved in all decisions that may concern the quality of the product. The independence of quality control from production is considered fundamental to the satisfactory operation of quality control. That is, production or management personnel should not coerce or force quality assurance personnel to depart from approved specifications or procedures.

1. General requirements for vaccine production

1.1. Principle

Production operations must follow clearly defined approved current procedures; they must comply with the following principles in order to obtain products of the requisite quality and be in accordance with the relevant manufacturing and marketing authorisations.

The manufacture of immunological veterinary medicinal products has special characteristics that should be taken into consideration when implementing and assessing the quality assurance system.

Due to the large number of animal species and related pathogenic agents, the variety of products manufactured is very wide and the volume of manufacture is often low. Hence, work on a campaign basis is common, manufacturing a series of batches of the same product in sequence in a given period of time followed by an appropriate cleaning procedure. Moreover, because of the very nature of this manufacture (cultivation steps, lack of terminal sterilisation), the products must be particularly well protected against organic or inorganic contamination and cross-contamination. The environment also

must be protected, especially when the manufacture involves the use of pathogenic or exotic biological agents, and the worker must be particularly well protected when the manufacture involves the use of biological agents pathogenic to humans.

These factors, together with the inherent variability of immunological products and the destructive nature of final product quality control tests so that they can provide only an estimate of quality for the entire batch mean that the role of the quality assurance system is of the utmost importance.

1.2. General

i) Production should be performed and supervised by competent people. Workers must understand the theory behind and practice of their work to the extent that they can predict and prevent problems within the scope of their responsibility.

ii) All handling of materials and products, such as receipt and quarantine, sampling, storage, labelling, dispensing, processing, packaging and distribution should be done in accordance with approved written procedures or instructions and adequately documented.

iii) All incoming materials should be evaluated to assess the quality impact on manufacturing. Appropriate action should be taken when materials are determined to be compromised. Materials should be labelled with the prescribed specifications.

iv) Incoming materials and finished products should be physically or administratively quarantined immediately after receipt or processing, until they have been released for use or distribution.

v) Intermediate and bulk products purchased as such should be handled on receipt as though they were starting materials.

vi) All materials and products should be stored under the appropriate conditions as established by the manufacturer and in an orderly fashion to permit batch segregation and stock rotation according to life expectancy.

vii) Checks on yields, and reconciliation of quantities, should be carried out as necessary to ensure that there are no discrepancies outside acceptable limits.

viii) Operations on different products should not be carried out simultaneously or consecutively in the same room unless there is negligible risk of mix-up or cross-contamination.

ix) At every stage of processing, products and materials should be protected from microbial and other contamination. A method of measuring bioburden within the production facility should be established.

x) When working with dry materials and products, special precautions should be taken to prevent the generation and dissemination of dust particulates. This applies particularly to the handling of highly active or sensitising materials.

xi) At all times during processing, all materials, bulk containers, critical items of equipment and where appropriate rooms used should be labelled or otherwise identified with an indication of the product or material being processed, its strength or concentration (where applicable) and batch number. Where applicable, this indication should also mention the stage of production.

xii) Labels applied to containers, equipment or premises should be clear, unambiguous and in the company's approved format. It is often helpful in addition to the wording on the labels to use colours to indicate status (for example, quarantined, accepted, rejected, clean, etc.).

xiii) Checks should be carried out to ensure that pipelines and other pieces of equipment used for the transportation of products from one area to another are connected in a correct manner.

xiv) Any deviation from instructions or procedures should be avoided as far as possible. If a deviation occurs, it should be approved in writing by a competent authorised person, with the involvement of the quality control department as appropriate to evaluate the effect on product quality and the shelf

life of the product. Deviations from documents approved by regulatory bodies should be reported to them for written approval prior to release of product.

xv) Access to production premises should be restricted to authorised personnel.

xvi) Normally, the production of other products should be avoided in areas destined for the production of vaccines, and should not use the same equipment.

1.3. Prevention of cross-contamination in production

i) Contamination of a starting material or of a product by another material or product should be avoided. This risk of accidental cross-contamination arises from the uncontrolled release of dust, gases, vapours, sprays or organisms from materials and products in process, from residues on equipment, residues from excipients or packaging and from operators' clothing, skin and respiratory tract. The significance of this risk varies with the type of contaminant and of product being contaminated. Amongst the most hazardous contaminants are biological preparations containing living organisms.

ii) Cross-contamination should be avoided by appropriate technical or organisational measures, for example:

a) production in segregated areas or by campaign (separation in time) followed by appropriate cleaning;

b) providing appropriate air-locks and air extraction;

c) minimising the risk of contamination caused by recirculation or re-entry of untreated or insufficiently treated air; routine testing of air;

d) keeping protective clothing inside areas where products with special risk of cross-contamination are processed;

e) using cleaning and decontamination procedures of known effectiveness, as ineffective cleaning of equipment is a common source of cross-contamination;

f) using "closed systems" of production;

g) testing for residues and contamination, and use of cleaning status labels on equipment.

iii) Measures to prevent cross-contamination and their effectiveness should be checked periodically according to set procedures.

1.4. Starting materials

i) The suitability of starting materials should be clearly defined in written specifications. These should include details of the supplier, catalogue or part number, the method of manufacture, the geographical origin and the animal species from which the materials are derived. The controls to be applied to starting materials must be included. Microbiological controls are particularly important.

ii) The results of tests on starting materials must comply with the specifications. Where the tests take a long time (e.g. eggs from SPF flocks) it may be necessary to process starting materials before the results of analytical controls are available. In such cases, the release of a finished product is conditional upon satisfactory results of the tests on starting materials. While vendors' certificates of analysis are useful and may be an acceptance requirement, they should not replace testing by the manufacturer when those test results are critical to acceptance.

iii) Special attention should be paid to knowledge of the supplier's quality assurance system in assessing the suitability of a source and the extent of quality control testing required.

iv) Heat is the preferred method for sterilising starting materials and some equipment. If necessary, other validated methods, such as irradiation, may be used. Steam heat should use live steam at no less than 120°C for not less than 30 minutes. Dry heat should be at no less than 160°C for not less than 1 hour.

1.5. Media

i) The ability of media to support the desired growth and effectiveness should be properly validated in advance.

ii) Media should preferably be sterilised *in situ* or in line. Steam heat under pressure is the preferred method. Gases, media, acids, alkalis, de-foaming agents and other materials introduced into bioreactors should themselves be sterile.

1.6. Seed lot and cell bank system

Where appropriate, the standards for seed lot and cell bank systems in relation to specific-disease chapters in the *Terrestrial Manual* should be consulted.

i) In order to prevent the unwanted drift of properties that ensue from repeated subcultures or multiple generations, the production of immunological veterinary medicinal products obtained by microbial, cell or tissue culture, or propagation in embryos and animals, should be based on a system of limited and controlled passages of seed lots or cell banks with a specified maximum.

ii) The number of generations (doublings, passages) between the seed lot or cell bank and the finished product should be consistent with the relevant dossier regulatory approval.

iii) Seed lots and cell banks should be adequately characterised and tested for contaminants (freedom from extraneous bacteria, fungi, mycoplasma, and viruses) and to ensure identity, purity, safety and efficacy when required by regulators. Cell banks should also be tested for karyology at baseline and maximum passage. Regulatory bodies may require confirmatory testing. Acceptance criteria for new seed lots should be established. Seed lots and cell banks should be established, stored and used in such a way as to minimise the risks of contamination, or any alteration. During the establishment of the seed lot and cell bank, no other living or infectious material (e.g. virus or cell lines) should be handled simultaneously in the same area or by the same person.

iv) Establishment of the seed lot and cell bank should be performed in a suitable environment to protect the seed lot and the cell bank and, if applicable, the personnel handling it and the external environment. The master seed or the cell bank should consist of a single uniform batch or serial that has been mixed and filled into containers as one batch or serial.

v) Only authorised personnel should be allowed to handle the material and this handling should be done under the supervision of a competent person. Different seed lots or cell banks should be stored at the temperature that assures negligible degradation while taking care to avoid confusion or cross-contamination errors. It is desirable to split the seed lots and cell banks and to store the parts at different locations so as to minimise the risk of total loss. Storage equipment should be monitored for proper function and connected to an alarm system for immediate notification of malfunction.

1.6.1. Master seeds and working seeds

i) A master seed (reference culture, parental strain) should be established for each microorganism used in the production of a product to serve as the source of seed for inoculation of all production cultures. Records of the source of the master seed should be maintained. For each seed, the highest and lowest passage levels that may be used for production should be established and specified in the approved production documents for the relevant regulatory procedure.

ii) Working seeds and production seeds may be prepared from the master seed by subculturing. Using a master seed and limiting the number of passages of seed microorganism in this manner assists in maintaining uniformity and consistency in production.

iii) The origin, form and storage conditions of seed material should be described (frozen or desiccated and stored at low temperatures such as –40°C or –70°C, or under other conditions found to be optimal for maintaining viability). Storage containers should be

adequately sealed and clearly labelled. Storage conditions should be properly monitored. An inventory should be kept and each container accounted for. Tamper evident tape may be needed for boxes and containers.

iv) For genetically modified microorganisms, the source of the gene(s) for the immunogenic antigens and the vector microorganism should be identified. Furthermore, the gene sequences introduced into the seed microorganism genome during construction of the modified seed should be provided.

1.6.2. Master cell stocks

i) When cell cultures are used to prepare a product, a master cell stock (MCS) should be established for each type of cell to be used. Records of the source of the master cell stock should be maintained. For each product, the highest and lowest passage levels of cells that may be used for production should be established and specified in approved documents. Each MCS should be characterised to ensure its identity, and its genetic stability should be demonstrated when subcultured from the lowest to the highest passage used for production. The karyotype of the MCS should be shown to be stable with a low level of polyploidy. Freedom from oncogenicity or tumorogenicity should be demonstrated by *in-vivo* studies in appropriate species using the highest cell passage that may be used for production. Purity of MCSs should be established by testing to ensure freedom from extraneous bacteria, fungi, mycoplasma, and viruses.

1.6.3. Primary cells

i) Primary cells are defined as a pool of original cells derived from normal tissue up to and including the tenth subculture used in the production of biologicals.

ii) In the case of products for use in poultry, these cells are usually obtained from SPF embryonating chicken eggs that have originated in an unvaccinated flock subjected to intensive microbiological monitoring.

iii) Other primary cells are derived from normal tissue of healthy animals and are tested for contamination with a wide variety of microorganisms as appropriate, including bacteria, fungi, mycoplasmas, and cytopathic or haemadsorbing-inducing agents or other extraneous viruses. The use of primary cells has an inherently higher risk of introducing extraneous agents compared with the use of cell lines and should be avoided where alternative methods of producing effective vaccines exist. Indeed, some control authorities only allow the use of primary cells in exceptional cases.

1.6.4. Embryonated eggs

i) Embryonated eggs are also commonly used in the production of biologicals. They should be derived from SPF chicken flocks that have been intensively monitored for infectious agents and have not been vaccinated; or, where justified (e.g. for production of some inactivated vaccines) and in line with the marketing authorisation, from healthy chicken flocks. The route of inoculation of the egg and the choice of egg material to be harvested are dependent on the particular organism that is being propagated. Regulatory bodies may have requirements for sources of eggs, and for release testing of products grown on them.

1.7. Operating principles

i) The formation of droplets and the production of foam should be avoided or minimised during manufacturing processes. Centrifugation and blending procedures that can lead to droplet formation should be carried out in appropriate contained or clean or contained areas to prevent transfer of live organisms.

ii) Accidental spillages, especially of live organisms, must be dealt with quickly and safely. Validated decontamination procedures should be available for each organism. Where different strains of single bacterial species or very similar viruses are involved, the process needs to be validated against only one of them, unless there is reason to believe that they may vary significantly in their resistance to the agent(s) involved.

iii) Operations involving the transfer of materials such as sterile media, cultures or products, should be carried out in pre-sterilised closed systems wherever possible. Where this is not possible, transfer operations must be protected by laminar airflow work stations.

iv) Addition of media or cultures to biogenerators (fermenters) and other vessels should be carried out under carefully controlled conditions to ensure that contamination is not introduced. Care must be taken to ensure that vessels are correctly connected when addition of cultures takes place.

v) Where necessary, for instance when two or more fermenters are within a single area, sampling and addition ports, and connectors (after connection, before the flow of product, and again before disconnection) should be sterilised with steam.

vi) Documentation, equipment, glassware, the external surfaces of product containers and other such materials must be disinfected before transfer from a contained area using a validated method. Only the absolute minimum required to allow operations to GMP standards should enter and leave the area. If obviously contaminated, such as by spills or aerosols, or if the organism involved is an exotic, the paperwork must be adequately disinfected through an equipment pass, or the information transferred out by such means as photocopy or fax.

vii) Liquid or solid wastes such as the debris after harvesting eggs, disposable culture bottles, unwanted cultures or biological agents, are best sterilised or disinfected before transfer from a contained area. However, alternatives such as sealed containers or piping may be appropriate in some cases.

viii) Articles and materials, including documentation, entering a production room should be carefully controlled to ensure that only items concerned with production are introduced. There should be a system that ensures that articles and materials entering a room are reconciled with those leaving so that their accumulation within the room does not occur.

ix) Heat-stable articles and materials entering a clean or contained area should do so through a double-ended autoclave or oven. Heat-labile articles and materials should enter through an air-lock with interlocked doors where they are disinfected. Sterilisation of articles and materials elsewhere is acceptable provided that they are double wrapped and enter through an airlock with the appropriate precautions.

x) Precautions must be taken to avoid contamination or confusion during incubation. There should be a cleaning and disinfection procedure for incubators. Containers in incubators should be carefully and clearly labelled.

xi) With the exception of blending and subsequent filling operations (or when totally enclosed systems are used) only one live biological agent may be handled within a production room at any given time. Production rooms must be effectively disinfected between the handling of different live biological agents.

xii) Products should be inactivated by the addition of inactivant accompanied by sufficient agitation, with specified time and conditions. The mixture should then be transferred to a second sterile vessel, unless the container is of such a size and shape as to be easily inverted and shaken so as to wet all internal surfaces with the final culture or inactivant mixture.

xiii) Vessels containing inactivated products should not be opened or sampled in areas containing live biological agents. All subsequent processing of inactivated products should take place in clean areas (as defined in the paragraph related to aseptic preparation) or in enclosed equipment dedicated to inactivated products.

xiv) Methods for sterilisation, disinfection, virus removal and inactivation should be validated.

xv) Filling should be carried out as soon as possible following production. Containers of bulk product prior to filling should be sealed, appropriately labelled and stored under specified conditions of temperature.

xvi) There should be a system to assure the integrity and closure of containers after filling.

xvii) The capping of vials containing live biological agents must be performed in such a way that ensures that contamination of other products or escape of the live agents into other areas or the external environment does not occur.

xviii) There may be a delay between the filling of final containers and their labelling and packaging. Procedures should be specified for the storage of unlabelled containers in order to maintain process control and to ensure satisfactory storage conditions. Special attention should be paid to the storage of heat-labile or photosensitive products. Storage temperatures should be specified and monitored.

xix) For each stage of production, the yield of product should be reconciled with that expected from that process. Any unexpected discrepancies should be investigated.

2. Rules governing quality control

2.1. Principle

Quality control is concerned with sampling, specifications and testing as well as the organisation, documentation and release procedures that ensure that the necessary and relevant tests are carried out, and that materials are not released for use, nor products released for sale or supply, until their quality has been judged satisfactory.

Quality control is not confined to laboratory operations, but must be involved in all decisions that may concern the quality of the product. The independence of quality control from production is considered fundamental to the satisfactory operation of quality control.

2.2. General rules for veterinary medicinal products including vaccines

i) Each holder of a relevant regulatory approval should have a quality control department. This department should be independent from other departments, and under the authority of a person with appropriate qualifications, who has adequate laboratory support. Adequate resources must be available to ensure that all the quality control requirements are effectively and reliably carried out.

ii) The head of the quality control department generally has the following responsibilities:

   a) to approve or reject, as he/she sees fit, starting materials, packaging materials, and intermediate, bulk and finished products;

   b) to evaluate batch records;

   c) to ensure that all necessary testing is carried out;

   d) to approve specifications, sampling instructions, test methods and other quality control procedures;

   e) to approve and monitor any contract analysts;

   f) to check the maintenance of his/her department, premises and equipment;

   g) to ensure that the appropriate validations are done;

   h) to ensure that the required initial and continuing training of department personnel is carried out and adapted according to need.

iii) The quality control department may have other duties, such as to establish, validate and implement all quality control procedures, keep the reference samples of materials and products, provide training and SOPs or Directives to departments to ensure the correct labelling of containers of materials and products, ensure the monitoring of the stability of the products, and participate in the investigation of complaints related to the quality of the product. All these operations should be carried out in accordance with written procedures and recorded.

iv) Finished product assessment should include all relevant factors, including production conditions, results of in-process testing, a review of manufacturing (including packaging) documentation, compliance with finished product specifications and examination of the finished product.

v) In-process controls ensure the quality of product. Those controls should be performed at an appropriate stage of production.

vi) There may be a requirement for the continuous monitoring of data during a production process, for example monitoring of physical parameters during fermentation.

vii) Continuous culture of biological products is a common practice and special consideration needs to be given to the quality control requirements arising from this type of production method.

2.3. Good practice for quality control in laboratories

i) Control laboratory premises and equipment should meet the general and specific requirements for quality control areas given in this chapter.

ii) The personnel, premises, and equipment should be appropriate to the tasks imposed by the nature and the scale of the manufacturing operations. The use of outside laboratories, in conformity with the principles detailed in Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility,* Section 4. *Rules governing outsourced activities,* can be accepted for particular documented reasons.

2.3.1. Documentation

i) Laboratory documentation should follow the principles given in chapter 2.3.3, Section 3. *Rules governing documentation*. The following details should be available to the quality control department:

a) specifications;

b) sampling procedures;

c) testing procedures and records (including analytical worksheets or laboratory notebooks);

d) analytical reports or certificates;

e) data from environmental monitoring, where required;

f) validation records of test methods, where applicable;

g) procedures for and records of the calibration of instruments and maintenance of equipment.

ii) Any quality control documentation relating to a batch record should be retained for one year after the expiry date of the batch or at least 5 years after the certification. Records retention requirements may be specified by the relevant regulatory body or national laws.

iii) For some kinds of data (e.g. analytical tests results, yields, and environmental controls) it is recommended that records are kept in a manner permitting trend evaluation.

iv) In addition to the information that is part of the batch record, other original data such as laboratory notebooks or records should be retained and readily available

2.3.2. Sampling

i) Sampling should be done in accordance with approved written procedures that describe:

a) the method of sampling;

b) the equipment to be used;

c) the amount of the sample to be taken;

d) instructions for any required sub-division of the sample;

e) the type and condition of the sample container to be used;

f) the identification of containers sampled;

g) any special precautions to be observed, especially with regard to the sampling of sterile or noxious materials;

h) the storage conditions;

i) Instructions for the cleaning and storage of sampling equipment.

ii) Quality control personnel should have access to production areas for sampling and investigation.

iii) Samples are retained; firstly to provide a sample for analytical testing and secondly to provide a specimen of the fully finished product. Samples may therefore fall into two categories:

a) *Reference sample*: a sample of a batch of starting material, packaging material or finished product that is stored for the purpose of being analysed should the need arise during the shelf life of the batch concerned.

b) *Retention sample*: a sample of a fully packaged unit from a batch of finished product.

These are stored for identification and retest purposes during or beyond the shelf life of the product. The number of retention samples may be specified by the relevant regulatory authority, otherwise they should be stored at least in duplicate.

iv) Samples should be selected from each batch or serial of product. The selector should pick representative final containers from each batch or serial and store these samples at the storage temperature recommended on the label. The producer should keep these reserve samples at the recommended storage temperature for a minimum of 12 months after the expiry date shown on the label, so that they are available to assist in evaluating the cause of any field problems reported from the use of the vaccine. The samples should be stored in a secure storage area and be tamper-evident.

v) It may be necessary to retain samples of intermediate products in sufficient amount and under appropriate storage conditions to allow repetition or confirmation of a batch control.

vi) Samples should be representative of the batch of materials or products from which they are taken. Other samples may also be taken to monitor the most stressed part of a process (e.g. beginning or end of a process).

vii) Sample containers should bear a label indicating the contents, the batch number, the date of sampling and the containers from which samples have been drawn.

2.3.3. Testing

i) Analytical methods should be validated. All testing operations described in the relevant regulatory approval documents should be carried out according to the approved methods.

ii) The results obtained and associated calculations should be checked and recorded as satisfactory or not. If not, action should be taken according to the manufacturer's procedures.

iii) Records should include at least the following data:

a) name of the material or product and, where applicable, dosage form;

b) batch number and, where appropriate, the manufacturer or supplier;

c) references to the relevant specifications and testing procedures;

d) test results, including observations and calculations, and reference to any certificates of analysis;

e) dates of testing;

f) initials of the persons who performed the testing;

g) initials of the persons who verified the testing and the calculations, where appropriate;

h) a clear statement of release or rejection (or other status decision) and the dated signature of the designated responsible person.

iv) All the in-process controls and procedures should be performed according to methods approved by quality control and the results recorded.

2.4. Batch tests for immunological products

i) It may be necessary to retain samples of intermediate products in sufficient amount and under appropriate storage conditions to allow repetition or confirmation of a batch control.

ii) There may be a requirement for the continuous monitoring of data during a production process, for example monitoring of physical parameters during fermentation.

iii) Continuous culture of biological products is a common practice and special consideration needs to be given to the quality control requirements arising from this type of production method.

2.4.1. Batch or serial release for distribution

i) Prior to release, the manufacturer must test each batch or serial for purity, (safety if required), and potency, as well as perform any other tests described in the firm's Outline of Production or other documentation of the manufacturing process for that product. In countries that have national regulatory programs that include official control authority re-testing (check testing) of final products, samples of each batch or serial should also be submitted for testing in government laboratories by competent authorities. If unsatisfactory results are obtained for tests conducted either by the manufacturer or by competent authorities, the batch or serial should not be released. In such cases, subsequent batches or serials of the product should be given priority for check testing by competent authorities.

2.4.1.1. Batch or serial purity test

i) Purity is determined by testing for a variety of contaminants. Tests to detect contaminants are performed on: master seeds, primary cells, MCSs, ingredients of animal origin if not subjected to sterilisation (e.g. fetal bovine serum, bovine albumin, or trypsin), and each batch or serial of final product prior to release.

ii) Purity test procedures detailed in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

iii) Procedures used to provide evidence that fetal or calf serum and other ingredients of bovine origin are free of pestiviruses are of great importance and should be well documented.

iv) Tests to be used to provide evidence of purity vary with the nature of the product, and should be prescribed in the Outline of Production or other documentation of the manufacturing process.

v) As tests for the detection of TSE agents in ingredients of animal origin have not been developed, vaccine manufacturers should document in their Outlines of Production or SOPs the measures they have implemented to minimise the risk of such contamination in ingredients of animal origin.

This relies on three principles:

a) first, verification that the animal source of all ingredients of animal origin in production facilities are from countries recognised as having the lowest possible risk of bovine spongiform encephalopathy;

b) second, that the tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE agents;

c) third, where relevant, that the processes applied to the material have been validated for inactivation of TSE agents. Methods of production should also

document the measures taken to prevent cross contamination of low risk materials by higher risk materials during processing.

2.4.1.2. Batch or serial safety test

i) Safety tests are not required by many regulatory authorities for the release of each batch or serial where the seed-lot system is used. Other regulatory authorities may allow waiving of target animal batch safety tests in line with VICH GL50 and 55 and waiving of laboratory animal batch safety tests in line with VICH GL59 where alternative methods exist.

ii) Where required, standard procedures are used for safety tests in mice, guinea-pigs, cats, dogs, horses, pigs, and sheep and are generally conducted using fewer animals than are used in the safety tests required for licensing. Batches or serials are considered satisfactory if local and systemic reactions to vaccination with the batch or serial to be released are in line with those described in the relevant regulatory approval dossier and product literature.

2.4.1.3. Batch or serial potency test

i) Batch or serial potency tests, required for each batch or serial prior to release, are designed to correlate with the host animal vaccination–challenge efficacy studies.

ii) For inactivated viral or bacterial products, potency tests may be conducted in laboratory or host animals, or by means of quantitative *in-vitro* methods that have been validated reliably to correlate *in-vitro* quantification of important antigen(s) with *in-vivo* efficacy.

iii) The potency of live vaccines is generally measured by means of bacterial counts or virus titration.

iv) Recombinant DNA or biotechnology-based vaccines should also be tested. Live genetically modified organisms can be quantified like any other live vaccine by titration, and expressed products of recombinant technology are quantified by *in vitro* tests, which can be easier to perform compared with tests on naturally grown antigens because of the in-process purification of the desired product.

v) When testing a live bacterial vaccine for release for marketing, the bacterial count must be sufficiently greater than that shown to be protective in the master seed immunogenicity (efficacy) test to ensure that at any time prior to the expiry date, the count will be at least equal to that used in the immunogenicity test.

vi) When testing a live viral vaccine for release, the virus titre must, as a rule, be sufficiently greater than that shown to be protective in the master seed immunogenicity test in order to ensure that at any time prior to the expiry date, the titre will be at least equal to that used in the immunogenicity test.

vii) Some control authorities specify higher bacterial or viral content than these. It is evident that the appropriate release titre is primarily dependent on the required potency and secondarily dependent on the rate of decay of the bacteria or viruses in the vaccine, as indicated by the stability test.

viii) Standard requirements have been developed and published by competent authorities for potency testing several vaccines. These tests can be found in CFR Title 9 part 113, in the European Pharmacopoeia, and in this *Terrestrial Manual*.

2.4.2. Other tests

i) Depending on the form of vaccine being produced, certain tests may be indicated and should be provided as appropriate in the Outline of Production or other documentation of the manufacturing process.

These tests may concern:

a) The level of moisture contained in desiccated products,

b) The level of residual inactivant in killed products,

c) The complete inactivation of killed products, pH,

d) The level of preservatives and permitted antibiotics,

e) The physical stability of adjuvants,

f) The retention of vacuum in desiccated products,

g) A general physical examination of the final vaccine.

Tests for these purposes may also be found in this *Terrestrial Manual*.

ii) Samples taken for sterility testing should be representative of the whole of the batch, but should in particular include samples taken from parts of the batch considered to be most at risk of contamination, e.g. for products that have been filled aseptically, samples should include containers filled at the beginning and end of the batch and after any significant intervention,

iii) The sterility test applied to the finished product should only be regarded as the last in a series of control measures by which sterility is assured. The test should be validated for the product(s) concerned.

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NB: FIRST ADOPTED IN 2016. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 2.3.5.

MINIMUM REQUIREMENTS FOR ASEPTIC PRODUCTION IN VACCINE MANUFACTURE

SUMMARY

This chapter provides requirements for aseptic production in the manufacture and quality control of veterinary vaccines in accordance with Chapter 1.1.8 Principles of veterinary vaccine production *and Chapter 2.3.4* Minimum requirements for the production and quality control of vaccines. *Manufacturers should use the recommendations as a basis for the elaboration of specific rules adapted to their individual needs.*

The manufacture of vaccines should be carried out in clean areas with controlled entry for personnel, equipment and materials. Clean areas should be maintained to an appropriate cleanliness standard and supplied with air that has passed through filters of an appropriate efficiency. Component preparation, product preparation and filling should be carried out in separate areas within the clean area. Manufacturing operations are conducted aseptically at some or all stages.

Clean areas for the manufacture of vaccines are classified into four grades according to the characteristics of the required environment. Each manufacturing operation requires an appropriate environmental cleanliness level in the operational state in order to minimise the risks of particulate or microbial contamination of the product or materials being handled. In order to meet operational conditions these areas should be designed to reach certain specified air-cleanliness levels in the resting state. The resting state is the condition where the installation is installed and operating, complete with production equipment but with no operating personnel present. The operational state is the condition where the installation is functioning in the defined operating mode with the specified number of personnel working. The operational and resting states should be defined for each clean room or suite of clean rooms.

The chapter sets out specific requirements for air quality, use of isolators, aseptic preparation, personnel, building design, equipment, sanitation, processing of materials, sterilisation and finishing of products.

SPECIFIC REQUIREMENTS FOR ASEPTIC PREPARATION

1. Principle

Most vaccines are injectable products, sterile or aseptically prepared. Their production should meet the following additional requirements for aseptic preparation.

Sole reliance for sterility or other quality aspects must not be placed on finished product test.

2. General

i) The manufacture of vaccines should be carried out in clean areas entry to which should be through airlocks for personnel or for equipment and materials. Clean areas should be maintained to an appropriate cleanliness standard and supplied with air that has passed through filters of an appropriate efficiency.

ii) The various operations of component preparation, product preparation and filling should be carried out in separate areas within the clean area. Manufacturing operations are conducted aseptically at some or all stages.

iii) Clean areas for the manufacture of vaccines are classified according to the required characteristics of the environment. Each manufacturing operation requires an appropriate environmental cleanliness level in the operational state in order to minimise the risks of particulate or microbial contamination of the product or materials being handled.

iv) In order to meet "in operation" conditions these areas should be designed to reach certain specified air-cleanliness levels in the "at rest" occupancy state. The "at-rest" state is the condition where the installation is installed and operating, complete with production equipment but with no operating personnel present. The "in operation" state is the condition where the installation is functioning in the defined operating mode with the specified number of personnel working.

v) The "in operation" and "at rest" states should be defined for each clean room or suite of clean rooms.

vi) For aseptic preparations four grades can be distinguished.

a) *Grade A:* The local zone for high risk operations, e.g. filling zone, stopper bowls, open ampoules and vials, making aseptic connections. Normally such conditions are provided by a laminar air flow work station. Laminar air flow systems should provide a homogeneous air speed in a range of 0.36 to 0.54 m/s (guidance value) at the working position in open clean room applications. The maintenance of laminarity should be demonstrated and validated.

A uni-directional air flow and lower velocities may be used in closed isolators and glove boxes.

b) *Grade B:* For aseptic preparation and filling, this is the background environment for the Grade A zone.

c) *Grade C and D:* Clean areas for carrying out less critical stages in the aseptic preparation.

3. Clean room and clean air device classification

i) Clean rooms and clean air devices should be classified in accordance with EN ISO 14644-1. Classification should be clearly differentiated from operational process environmental monitoring. The maximum permitted airborne particle concentration for each grade is given in the following table.

Maximum permitted number of particles per m^3 equal to or greater than the tabulated size				
	At rest		**In operation**	
Grade	**0.5 μm**	**5.0μm**	**0.5 μm**	**5.0μm**
A	3520	20	3520	20
B	3520	29	352,000	2900
C	352,000	2900	3,520,000	29,000
D	3,520,000	29,000	Not defined	Not defined

ii) For classification purposes in Grade A zones, a minimum sample volume of 1 m^3 should be taken per sample location. For Grade A the airborne particle classification is ISO 4.8 (as defined in ISO 14644-1) dictated by the limit for particles ≥5.0 μm. For Grade B (at rest) the airborne particle classification is ISO 5 for both considered particle sizes. For Grade C (at rest and in operation) the airborne particle classification is ISO 7 and ISO 8 respectively. For Grade D (at rest) the airborne particle classification is ISO 8. For classification purposes EN/ISO 14644-1 methodology defines both the minimum number of sample locations and the sample size based on the class limit of the largest considered particle size and the method of evaluation of the data collected.

iii) Portable particle counters with a short length of sample tubing should be used for classification purposes because of the relatively higher rate of precipitation of particles ≥5.0μm in remote sampling systems with long lengths of tubing. Isokinetic sample heads shall be used in unidirectional airflow systems.

iv) "In operation" classification may be demonstrated during normal operations, simulated operations or during media fills as worst-case simulation is required for this. EN ISO 14644-2 provides information on testing to demonstrate continued compliance with the assigned cleanliness classifications.

v) Clean rooms and clean air devices should be routinely monitored in operation, for the full duration of critical processing, except where justified by contaminants in the process that would damage the particle counter or present a hazard, e.g. live organisms. In such cases, monitoring during routine equipment set up operations should be undertaken prior to exposure to the risk. Monitoring during simulated operations should also be performed.

vi) Airborne particle monitoring systems may consist of independent particle counter; a network of sequentially accessed sampling points connected by manifold to a single particle counter; or a combination of the two.

vii) The sample sizes taken for monitoring purposes using automated systems will usually be a function of the sampling rate of the system used. It is not necessary for the sample volume to be the same as that used for formal classification of clean rooms and clean air devices.

viii) The particle limits given in the table for the "at rest" state should be achieved after a short "clean up" period of 15–20 minutes (guidance value) in an unmanned state after completion of operations.

ix) The monitoring of Grade C and D areas in operation should be performed in accordance with the principle of quality risk management. The requirements and alert or action limits will depend on the nature of the operations carried out, but the recommended "clean up period" should be attained.

x) Other characteristics such as temperature and relative humidity depend on the product and nature of the operations carried out. These parameters should not interfere with the defined cleanliness standard.

xi) Examples of operations to be carried out in the various grades include:

Grade	Examples of operations for aseptic preparations
A	Aseptic preparation and filling
C	Preparation of solutions to be filtered
D	Handling of components after washing

xii) Where aseptic operations are performed monitoring should be frequent using methods such as settle plates, volumetric air and surface sampling (e.g. swabs and contact plates). Sampling methods used in operation should not interfere with zone protection. Results from monitoring should be considered when reviewing batch documentation for finished product release. Surfaces and personnel should be monitored after critical operations. Additional microbiological monitoring is also required outside production operations, e.g. after validation of systems, cleaning and sanitisation.

Recommended limits for microbial monitoring of clean areas during operation:

Recommended limits for microbial contamination[(a)]				
Grade	**Air sample** CFU/m^3	**Settle plates** (diameter 90 mm) CFU/4 hours[(b)]	**Contact plates** (diameter 55 mm) CFU/plate	**Glove print** five fingers CFU/glove
A	< 1	< 1	< 1	< 1
B	10	5	5	5
C	100	50	25	–
D	200	100	50	–

[(a)]These are average values. [(b)]Individual settle plates may be exposed for less than 4 hours.
CFU= colony-forming unit

xiii) Appropriate alert and action limits should be set for the results of particulate and microbiological monitoring. If these limits are exceeded operating procedures should prescribe corrective action.

4. Isolator technology

i) The use of isolator technology to minimise human interventions in processing areas may result in a significant decrease in the risk of microbiological contamination of aseptically manufactured products from the environment. There are many possible designs of isolators and transfer devices. The isolator and the background environment should be designed so that the required air quality for the respective zones can be realised. Isolators are constructed of various materials more or less prone to puncture and leakage. Transfer devices may vary from a single door to double door designs to fully sealed systems incorporating sterilisation mechanisms.

ii) The transfer of materials into and out of the unit is one of the greatest potential sources of contamination. In general the area inside the isolator is the local zone for high risk manipulations, although it is recognised that laminar air flow may not exist in the working zone of all such devices.

iii) The air classification required for the background environment depends on the design of the isolator and its application. It should be controlled and for aseptic processing it should be at least Grade D.

iv) Isolators should be introduced only after appropriate validation. Validation should take into account all critical factors of isolator technology, for example the quality of the air inside and outside (background) the isolator, sanitisation of the isolator, the transfer process and isolator integrity.

v) Monitoring should be carried out routinely and should include frequent leak testing of the isolator and glove or sleeve system.

5. Aseptic preparation

i) Components after washing should be handled in at least a Grade D environment. Handling of sterile starting materials and components, unless subjected to sterilisation or filtration through a microorganism-retaining filter later in the process, should be done in a Grade A environment with Grade B background.

ii) Preparation of solutions that are to be sterile filtered during the process should be done in a Grade C environment; if not filtered, the preparation of materials and products should be done in a Grade A environment with a Grade B background.

iii) Handling and filling of aseptically prepared products should be done in a Grade A environment with a Grade B background.

iv) Prior to the completion of stoppering, transfer of partially closed containers as used in freeze drying should be done either in a Grade A environment with Grade B background or in sealed transfer trays in a Grade B environment.

6. Specific requirements for personnel working in clean areas

i) Only the minimum number of personnel required should be present in clean areas; this is particularly important during aseptic processing.

ii) All personnel (including those concerned with cleaning and maintenance) employed in such areas should receive regular training in disciplines relevant to the correct manufacture of vaccines. This training should include reference to hygiene and to the basic elements of microbiology. When outside staff who have not received such training (e.g. building or maintenance contractors) need to be brought in, particular care should be taken over their instruction and supervision.

iii) Staff who have been engaged in the processing of animal tissue materials or of cultures of micro-organisms other than those used in the current manufacturing process should not enter sterile-product areas unless rigorous and clearly defined entry procedures have been followed.

iv) High standards of personal hygiene and cleanliness are essential. Personnel involved in the manufacture of aseptic preparations should be instructed to report any condition that may cause the shedding of abnormal numbers or types of contaminants; periodic health checks for such conditions are desirable. Actions to be

taken about personnel who could be introducing undue microbiological hazard should be decided by a designated competent person.

v) Wristwatches, make-up and jewellery should not be worn in clean areas.

vi) Changing and washing should follow a written procedure designed to minimise contamination of clean area clothing or carry-through of contaminants to the clean areas.

vii) The clothing and its quality should be appropriate for the process and the grade of the working area. It should be worn in such a way as to protect the product from contamination.

viii) The description of clothing required for each grade is given below:

a) Grade D: Hair and, where relevant, beard should be covered. A general protective suit and appropriate shoes or overshoes should be worn. Appropriate measures should be taken to avoid any contamination coming from outside the clean area.

b) Grade C: Hair and where relevant beard and moustache should be covered. A single or two-piece trouser suit, gathered at the wrists and with high neck and appropriate shoes or overshoes should be worn. They should shed virtually no fibres or particulate matter.

c) Grade A/B: Headgear should totally enclose hair and, where relevant, beard and moustache; it should be tucked into the neck of the suit; a face mask should be worn to prevent the shedding of droplets. Appropriate sterilised, non-powdered rubber or plastic gloves and sterilised or disinfected footwear should be worn. Trouser-legs should be tucked inside the footwear and garment sleeves into the gloves. The protective clothing should shed virtually no fibres or particulate matter and retain particles shed by the body.

ix) Outdoor clothing should not be brought into changing rooms leading to Grade B and C rooms. For every worker in a Grade A/B area, clean sterile (sterilised or adequately sanitised) protective garments should be provided at each work session. Gloves should be regularly disinfected during operations. Masks and gloves should be changed at least for every working session.

x) Clean area clothing should be cleaned and handled in such a way that it does not gather additional contaminants that can later be shed. These operations should follow written procedures. Separate laundry facilities for such clothing are desirable. Inappropriate treatment of clothing will damage fibres and may increase the risk of shedding of particles.

7. Specific requirements for clean areas

i) In clean areas, all exposed surfaces should be smooth, impervious and unbroken in order to minimise the shedding or accumulation of particles or micro-organisms and to permit the repeated application of cleaning agents, and disinfectants where used.

ii) To reduce accumulation of dust and to facilitate cleaning there should be no uncleanable recesses and a minimum of projecting ledges, shelves, cupboards and equipment. Doors should be designed to avoid those uncleanable recesses; sliding doors may be undesirable for this reason.

iii) False ceilings should be sealed to prevent contamination from the space above them.

iv) Pipes and ducts and other utilities should be installed so that they do not create recesses, unsealed openings and surfaces that are difficult to clean.

v) Sinks and drains should be prohibited in Grade A/B areas used for aseptic manufacture. In other areas air breaks should be fitted between the machine or sink and the drains. Floor drains in lower grade clean rooms should be fitted with traps or water seals to prevent back-flow.

vi) Changing rooms should be designed as airlocks and used to provide physical separation of the different stages of changing and so minimise microbial and particulate contamination of protective clothing. They should be flushed effectively with filtered air. The final stage of the changing room should, in the at-rest state, be the same grade as the area into which it leads. The use of separate changing rooms for entering and

leaving clean areas is sometimes desirable. In general hand washing facilities should be provided only in the first stage of the changing rooms.

vii) Both airlock doors should not be opened simultaneously. An interlocking system or a visual or audible warning system should be operated to prevent the opening of more than one door at a time.

viii) A filtered air supply should maintain a positive pressure and an air flow relative to surrounding areas of a lower grade under all operational conditions and should flush the area effectively. Adjacent rooms of different grades should have a pressure differential of 10–15 Pascals (guidance values). Particular attention should be paid to the protection of the zone of greatest risk, that is, the immediate environment to which a product and cleaned components that contact the product are exposed. The various recommendations regarding air supplies and pressure differentials may need to be modified where it becomes necessary to contain some materials, e.g. live viruses or bacteria. Decontamination of facilities and treatment of air leaving a clean area may be necessary for some operations.

ix) It should be demonstrated that air-flow patterns do not present a contamination risk, e.g. care should be taken to ensure that air flows do not distribute particles from a particle-generating person, operation or machine to a zone of higher product risk.

x) A warning system should be provided to indicate failure in the air supply. Indicators of pressure differences should be fitted between areas where these differences are important. These pressure differences should be recorded regularly or otherwise documented.

8. Equipment

i) A conveyor belt should not pass through a partition between a Grade A or B area and a processing area of lower air cleanliness, unless the belt itself is continually sterilised (e.g. in a sterilising tunnel).

ii) As far as practicable equipment, fittings and services should be designed and installed so that operations, maintenance and repairs can be carried out outside the clean area. If sterilisation is required, it should be carried out, wherever possible, after complete reassembly.

iii) When equipment maintenance has been carried out within the clean area, the area should be cleaned, disinfected or sterilised where appropriate, before processing recommences if the required standards of cleanliness or asepsis have not been maintained during the work.

iv) Water treatment plants and distribution systems should be designed, constructed and maintained so as to ensure a reliable source of water of an appropriate quality. They should not be operated beyond their designed capacity.

v) All equipment such as sterilisers, air handling and filtration systems, air vent and gas filters, water treatment, generation, storage and distribution systems should be subject to validation and planned maintenance; their return to use should be approved.

9. Sanitation

i) The sanitation of clean areas is particularly important. They should be cleaned thoroughly in accordance with a written programme. Where disinfectants are used, more than one type should be employed. Monitoring should be undertaken regularly in order to detect the development of resistant strains.

ii) Disinfectants and detergents should be monitored for microbial contamination; dilutions should be kept in previously cleaned containers and should be stored and labelled according to specifications and expiry. Disinfectants and detergents used in Grades A and B areas should be sterile prior to use.

10. Processing

i) Preparations of microbiological origin should not be made or filled in areas used for the processing of other vaccines; however, vaccines of dead organisms or of bacterial extracts may be filled, after inactivation, in the same premises as other sterile medicinal products after appropriate cleaning.

ii) Validation of aseptic processing should include a process simulation test using a nutrient medium (media fill). Selection of the nutrient medium should be made based on dosage form of the product and selectivity, clarity, concentration and suitability for sterilisation of the nutrient medium.

iii) The process simulation test should imitate as closely as possible the routine aseptic manufacturing process and include all the critical subsequent manufacturing steps. It should also take into account various interventions known to occur during normal production as well as worst-case situations.

iv) Process simulation tests should be performed as initial validation with three consecutive satisfactory simulation tests per shift and repeated at defined intervals and after any significant modification to the HVAC-system, equipment, process and number of shifts. Normally process simulation tests should be repeated twice a year per shift and process.

v) The number of containers used for media fills should be sufficient to enable a valid evaluation. For small batches, the number of containers for media fills should at least equal the size of the product batch. The target should be zero growth[1]. For any run size, intermittent incidents of microbial contamination may be indicative of low-level contamination that should be investigated. Investigation of gross failures should include the potential impact on the sterility assurance of batches manufactured since the last successful media fill.

vi) Care should be taken that any validation does not compromise the processes.

vii) Water sources, water treatment equipment and treated water should be monitored regularly for chemical and biological contamination and, as appropriate, for endotoxins. Records should be maintained of the results of the monitoring and of any action taken.

viii) Activities in clean areas and especially when aseptic operations are in progress should be kept to a minimum and movement of personnel should be controlled and methodical, to avoid excessive shedding of particles and organisms due to over-vigorous activity. The ambient temperature and humidity should not be uncomfortably high because of the nature of the garments worn.

ix) Microbiological contamination of starting materials should be within a pre-specified range. Specifications should include requirements for microbiological quality when the need for this has been indicated by monitoring.

x) Containers and materials liable to generate fibres should be minimised in clean areas.

xi) Components, containers and equipment should be handled after the final cleaning process in such a way that they are not re-contaminated.

xii) The interval between the washing and drying and the sterilisation of components, containers and equipment as well as between their sterilisation and use should be minimised and subject to a time-limit appropriate to the storage conditions.

xiii) Components, containers, equipment and any other article required in a clean area where aseptic work takes place should be sterilised and passed into the area through double-ended sterilisers sealed into the wall, or by a procedure that achieves the same objective of not introducing contamination. Non-combustible gases should be passed through micro-organism retentive filters.

xiv) The efficacy of any new procedure should be validated, and the validation verified at scheduled intervals based on performance history or when any significant change is made in the process or equipment.

11. Sterilisation

i) All sterilisation processes should be validated. Particular attention should be given when the adopted sterilisation method is not described in the current editions of Pharmacopoeias, or when it is used for a product that is not a simple aqueous or oily solution. Where possible, heat sterilisation is the method of

1 A rationale to this topic can be found in the PIC/S guideline (PIC/S, 2014).

choice. In any case, the sterilisation process must be in accordance with the marketing and manufacturing authorisations.

ii) Before any sterilisation process is adopted its suitability for the product and its efficacy in achieving the desired sterilising conditions in all parts of each type of load to be processed should be demonstrated by physical measurements and by biological indicators where appropriate. The validity of the process should be verified at scheduled intervals, at least annually, and whenever significant modifications have been made to the equipment. Records should be kept of the results.

iii) For effective sterilisation the whole of the material must be subjected to the required treatment and the process should be designed to ensure that this is achieved.

iv) Validated loading patterns should be established for all sterilisation processes.

v) Biological indicators should be considered as an additional method for monitoring sterilisation. They should be stored and used according to the manufacturer's instructions, and their quality checked by positive controls. If biological indicators are used, strict precautions should be taken to avoid transferring microbial contamination from them.

vi) There should be a clear means of differentiating products that have not been sterilised from those that have. Each basket, tray or other carrier of products or components should be clearly labelled with the material name, its batch number and an indication of whether or not it has been sterilised. Indicators such as autoclave tape may be used, where appropriate, to indicate whether or not a batch (or sub-batch) has passed through a sterilisation process, but they do not give a reliable indication that the lot is, in fact, sterile.

vii) Sterilisation records should be available for each sterilisation run. They should be approved as part of the batch release procedure.

11.1. Sterilisation by heat

i) Each heat-sterilisation cycle should be recorded on a time/temperature chart with a sufficiently large scale or by other appropriate equipment with suitable accuracy and precision. The position of the temperature probes used for controlling or recording should have been determined during the validation, and where applicable also checked against a second independent temperature probe located at the same position.

ii) Chemical or biological indicators may also be used, but should not take the place of physical measurements.

iii) Sufficient time must be allowed for the whole of the load to reach the required temperature before measurement of the sterilising time-period is commenced. This time must be determined for each type of load to be processed.

iv) After the high temperature phase of a heat-sterilisation cycle, precautions should be taken against contamination of a sterilised load during cooling. Any cooling fluid or gas in contact with the product should be sterilised unless it can be shown that any leaking container would not be approved for use.

11.1.1. Moist heat

i) Both temperature and pressure should be used to monitor the process. Control instrumentation should normally be independent of monitoring instrumentation and recording charts. Where automated control and monitoring systems are used for these applications they should be validated to ensure that critical process requirements are met. System and cycle faults should be registered by the system and observed by the operator. The reading of the independent temperature indicator should be routinely checked against the chart recorder during the sterilisation period. For sterilisers fitted with a drain at the bottom of the chamber, it may also be necessary to record the temperature at this position, throughout the sterilisation period. There should be frequent leak tests on the chamber when a vacuum phase is part of the cycle.

ii) The items to be sterilised, other than products in sealed containers, should be wrapped in a material that allows removal of air and penetration of steam but that prevents recontamination after sterilisation. All parts of the load should be in contact with the sterilising agent at the required temperature for the required time.

iii) Care should be taken to ensure that steam used for sterilisation is of suitable quality and does not contain additives at a level that could cause contamination of product or equipment.

11.1.2. Dry heat

i) The process used should include air circulation within the chamber and the maintenance of a positive pressure to prevent the entry of non-sterile air. Any air admitted should be passed through a HEPA filter. Where this process is also intended to remove pyrogens, challenge tests using endotoxins could be used as part of the validation.

11.2. Sterilisation by radiation

i) Radiation sterilisation is used mainly for the sterilisation of heat-sensitive materials and products. Many medicinal products and some packaging materials are radiation-sensitive, so this method is permissible only when the absence of deleterious effects on the product has been confirmed experimentally. Ultraviolet irradiation is not normally an acceptable method of sterilisation.

ii) During the sterilisation procedure the radiation dose should be measured. For this purpose, dosimetry indicators that are independent of dose rate should be used, giving a quantitative measurement of the dose received by the product itself. Dosimeters should be inserted in the load in sufficient number and close enough together to ensure that there is always a dosimeter in the irradiator. Where plastic dosimeters are used they should be used within the time-limit of their calibration. Dosimeter absorbances should be read within a short period after exposure to radiation.

iii) Biological indicators may be used as an additional control

iv) Validation procedures should ensure that the effects of variations in density of the packages are considered.

v) Materials handling procedures should prevent mix-up between irradiated and non-irradiated materials. Radiation sensitive colour disks should also be used on each package to differentiate between packages that have been subjected to irradiation and those that have not.

vi) The total radiation dose should be administered within a predetermined time span.

12. Finishing

i) Partially stoppered freeze drying vials should be maintained under Grade A conditions at all times until the stopper is fully inserted.

ii) Containers should be closed by appropriately validated methods. Containers closed by fusion, e.g. glass or plastic ampoules should be subject to 100% integrity testing. Samples of other containers should be checked for integrity according to appropriate procedures.

iii) The container closure system for aseptically filled vials is not fully integral until the aluminium cap has been crimped into place on the stoppered vial. Crimping of the cap should therefore be performed as soon as possible after stopper insertion.

iv) As the equipment used to crimp vial caps can generate large quantities of non-viable particulates, the equipment should be located at a separate station equipped with adequate air extraction.

v) Vial capping can be undertaken as an aseptic process using sterilised caps or as a clean process outside the aseptic core. Where this latter approach is adopted, vials should be protected by Grade A conditions up to

the point of leaving the aseptic processing area, and thereafter stoppered vials should be protected with a Grade A air supply until the cap has been crimped.

vi) Vials with missing or displaced stoppers should be rejected prior to capping. Where human intervention is required at the capping station, appropriate technology should be used to prevent direct contact with the vials and to minimise microbial contamination.

vii) Restricted access barriers and isolators may be beneficial in assuring the required conditions and minimising direct human interventions into the capping operation.

viii) Containers sealed under vacuum should be tested for maintenance of that vacuum after an appropriate, pre-determined period.

ix) Filled containers of parenteral products should be inspected individually for extraneous contamination or other defects. When inspection is done visually, it should be done under suitable and controlled conditions of illumination and background. Operators doing the inspection should pass regular eye-sight checks, with spectacles if worn, and be allowed frequent breaks from inspection. Where other methods of inspection are used, the process should be validated and the performance of the equipment checked at intervals. Results should be recorded.

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NB: FIRST ADOPTED IN 2016.

PART 3

OIE LISTED DISEASES AND OTHER DISEASES OF IMPOTANCE MULTIPLE SPACIES

SECTION 3.1.

MULTIPLE SPECIES

CHAPTER 3.1.1.

ANTHRAX

SUMMARY

Description and importance of the disease: *Anthrax is primarily a disease of herbivorous animals, although all mammals, including humans, and some avian species can contract it. Mortality can be very high, especially in herbivores. The aetiological agent is the spore-forming, Gram-positive rod-shaped bacterium,* Bacillus anthracis*. The disease has world-wide distribution and is a zoonosis.*

The disease is mediated mainly by exotoxins. Peracute, acute, subacute and, rarely, chronic forms of the disease are reported. Ante-mortem clinical signs may be virtually absent in peracute and acute forms of the disease. Subacute disease may be accompanied by progressive fever, depression, inappetence, weakness, prostration and death. Acute, subacute, and chronic disease may show localised swelling and fever. In chronic disease, the only sign may be enlarged lymph glands.

Detection and identification of the agent: Bacillus anthracis *is readily isolated in relatively high numbers from blood or tissues of a recently dead animal that died of anthrax, and colony morphology of* B. anthracis *is quite characteristic after overnight incubation on blood agar. The colony is relatively large, measuring approximately 0.3–0.5 cm in diameter. It is grey-white to white, non-haemolytic with a rough, ground-glass appearance and has a very tacky, butyrous consistency. The vegetative cells of* B. anthracis *are large, measuring 3–5 μm in length and approximately 1 μm in width. Ellipsoidal central spores, which do not swell the sporangium, are formed at the end of the exponential cell growth phase. The cells stain strongly Gram positive, and long chains are often seen* in vitro*, while paired or short chains are seen* in vivo*. Visualisation of the encapsulated bacilli, usually in large numbers, in a blood smear stained with azure B or polychrome methylene blue* (*M'Fadyean's reaction*) *is fully diagnostic.*

Serological tests: *Antibody detection in serum from infected animals is rarely used for diagnostic purposes and is essentially a research tool. The predominant procedure used is the enzyme-linked immunosorbent assay.*

Requirements for vaccines: *The most widely used livestock anthrax vaccine developed by Max Sterne in 1937 is a live, non-encapsulated, spore former held in suspension. In Russia and Eastern Europe, an equivalent type of vaccine is used* (*strain 55*)*. A list of producers is given in the World Health Organization anthrax guidelines.*

A. INTRODUCTION

Anthrax, an acute bacterial disease primarily of herbivores, is transmissible to humans. The aetiological agent, *Bacillus anthracis*, is a Gram-positive spore-forming rod-shaped bacterium. Anthrax is known by many names around the world including charbon, woolsorters' disease, ragpickers' disease, malignant carbuncle, malignant pustule and Siberian ulcer.

Animals become infected by ingesting spores, or possibly by being bitten by blood feeding insects such as stable flies (*Stomoxys calcitrans*) and mosquitoes (*Aedes aegypti* and *Aedes taeniorhynchus*) that have fed on an infected

animal or carcass. Infected animals are usually found dead as death can occur within 24 hours. To avoid environmental contamination with spores, post-mortem examinations of carcasses of animals suspected to have died of anthrax are discouraged and natural orifices such as mouth, nostril and anus should be closed (for example plugged with cotton wool or other suitable material soaked in an approved disinfectant) to prevent the formation of spores. There are regulations in most countries that prohibit post-mortem examination when anthrax is suspected. Recently dead animals may show any number of lesions, none of which is pathognomonic or entirely consistent.

Lesions most commonly seen are those of a generalised septicaemia often accompanied by an enlarged spleen with a dark semi-fluid pulp ('blackberry jam' consistency) and poorly clotted blood. Haemorrhage from the nose, mouth, vagina and/or anus at death may be found.

Gram-positive rod-shaped *B. anthracis* is an obligate pathogen. Most of the other species of *Bacillus* are common ubiquitous environmental saprophytes, although a number, notably *B. cereus, B. licheniformis* and *B. subtilis*, are occasionally associated with food poisoning in humans and with other clinical manifestations in both humans and animals.

1. Zoonotic risk and biosafety requirements

More than 95% of human anthrax cases take the cutaneous form and result from handling infected carcasses or hides, hair, meat or bones from such carcasses. *Bacillus anthracis* is not invasive and requires a lesion to infect. Protection for veterinarians and other animal handlers involves wearing gloves and other protective clothing (including full personal protective equipment [PPE] depending on the situation) when handling specimens from suspected anthrax carcasses and never rubbing the face or eyes. The risk of gastrointestinal anthrax may arise if individuals eat meat from animals infected with anthrax.

The risk of inhaling infectious doses becomes significant in occupations involving the processing of animal by-products for manufacturing goods (industrial anthrax). These include the tanning, woollen, animal hair, carpet, bone processing, and other such industries, where the potential for aerosolisation of substantial numbers of spores increases the risk of exposure to infectious doses. It is important that industrial workers use appropriate personal protective clothing and equipment and follow standard operating procedures that minimise the risk of transmission. Efficient air extraction equipment should be positioned over picking, combing, carding and spinning machines. Air blowing machinery should never be used for cleaning equipment due to the risk of spore dispersal.

Clinical specimens and cultures of *B. anthracis* should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Vaccination of laboratory personnel is recommended.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of anthrax and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Demonstration of capsule	–	–	–	++	–	–
Demonstration of lack of motility	–	–	–	++	–	–
Gamma phage lysis	–	–	–	++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Penicillin susceptibility	–	–	–	++	–	–
PCR	–	–	–	++	+++/++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

Demonstration of encapsulated *B. anthracis* in smears of blood or tissues from fresh anthrax-infected carcasses and growth of the organism on blood agar plates is relatively uncomplicated and within the capability of most bacteriology laboratories. Difficulty may be encountered in the case of pigs and carnivores in which the terminal bacteraemia is frequently not marked, or in animals that received antibiotics before death.

Recovery of *B. anthracis* from old decomposed carcasses, processed specimens (bone meal, hides), or environmental samples (contaminated soil) is often difficult, requiring demanding and labour-intensive procedures. However live spores may be recovered from the turbinate bones of dead livestock and wildlife for an extended period after death (M. Hugh-Jones, personal communication).

1.1. Culture and identification of *Bacillus anthracis*

1.1.1. Fresh specimens

Bacillus anthracis grows readily on most types of nutrient agar, however, 5–7% horse or sheep blood agar is the diagnostic medium of choice. Blood is the primary clinical material to examine. Swabs of blood, other body fluids or swabs taken from incisions in tissues or organs can be spread over blood agar plates. After overnight incubation at 37°C, *B. anthracis* colonies are grey-white to white, 0.3–0.5 cm in diameter, non-haemolytic, with a ground-glass surface, and very tacky when teased with an inoculating loop. Tailing and prominent wisps of growth trailing back toward the parent colony, all in the same direction, are sometimes seen. This characteristic has been described as a 'medusa head' or 'curled hair' appearance. Confirmation of *B. anthracis* should be accomplished by the demonstration of a capsulated, spore-forming, Gram-positive rod in blood culture. Absence of motility is an additional test that can be done.

Anthrax-specific phages were first isolated in the 1950s, and the specifically named gamma phage was first reported in 1955 (Brown & Cherry, 1955) and quickly became the standard diagnostic phage for anthrax. Gamma phage belongs to a family of closely related anthrax phages (World Health Organization [WHO], 2008).

Two tests for confirming the identity of *B. anthracis* are gamma phage lysis and penicillin susceptibility. The typical procedure for these tests is to plate a lawn of suspect *B. anthracis* on a blood or nutrient agar plate and place a 10–15 µl drop of the phage suspension on one side of the lawn and a 10-unit penicillin disk to the other side. Allow the drop of phage suspension to soak into the agar before incubating the plate at 37°C. A control culture, e.g. the Sterne vaccine or the NCTC strain 10340, should be tested at the same time as the suspect culture to demonstrate the expected reaction for gamma phage lysis and penicillin susceptibility. If the suspect culture is *B. anthracis*, the area under the phage will be devoid of bacterial growth, because of lysis, and a clear zone will be seen around the penicillin disk indicating antibiotic susceptibility. Note that some field isolates of *B. anthracis* may be phage resistant or penicillin resistant. As the performance of the gamma phage lysis assay may be affected by the density of bacterial inoculum, Abshire *et al.* (2005) recommend streaking the suspect culture on the agar plate over several quadrants instead of using a lawn format and inoculating a drop of gamma phage on the

first and second quadrants on the plate. If antibiotic or phage resistant *B. anthracis* is suspected then polymerase chain reaction (PCR) diagnostic methods may be applied.

Phage suspensions may be obtained from central veterinary laboratories or central public health laboratories.

The phage can be propagated and concentrated by the following protocol. Store phage at 2–4°C and do not freeze phage as it will quickly become non-viable.

1.1.1.1. Stage one

i) Spread a blood agar (BA) plate of the Sterne vaccine strain of *B. anthracis*. Incubate overnight at 37°C.

ii) Inoculate approximately 10 ml of nutrient broth (NB) with growth from the BA plate and incubate at 37°C for approximately 4 hours or until just cloudy, then refrigerate.

iii) Spread 100 µl of the culture from step ii on three pre-dried BA plates and incubate at 37°C for 30–60 minutes.

iv) Spread 100 µl of the phage suspension to be amplified over the same plates. Incubate at 37°C overnight.

v) Harvest the phage-lysed growth on the BA plate in 5 ml of NB followed by a second 'wash' of 5 ml NB. Incubate at 37°C overnight.

vi) Filter (0.45 µm) and count by dropping 20 µl drops (three drops per dilution) of tenfold dilutions of the filtrate in saline onto lawns of the *B. anthracis* culture prepared as in step iii.

1.1.1.2. Stage two

This is essentially the same procedure as Stage one, only uses the filtrate from step vi to harvest the phage from the plates.

vii) Prepare three Sterne strain lawns on BA, as in step iii. Incubate at 37°C for 30–60 minutes.

viii) Spread 100 µl phage from step vi. Incubate at 37°C overnight.

ix) To 9 ml of filtrate from step vi, add 1 ml of 10× concentrated NB.

x) Harvest the phage from step viii with 5 ml of the solution from step ix, followed by a second 5 ml wash with the rest of the solution from step vi.

xi) Add 10 ml of 1× NB.

xii) Incubate at 37°C overnight, filter and count.

1.1.1.3. Stage three

xiii) Inoculate 100 ml of brain–heart infusion broth with approximately 2.5 ml of the culture from step ii. Incubate on a rotary shaker at 37°C until just turbid.

xiv) Add the 20 ml of filtrate from step xii and continue incubation overnight.

xv) The resultant filtrate is checked for sterility and titrated in tenfold dilutions on lawns of the vaccine strain as in step vi to determine the concentration of the phage. This should be of the order of 10^8–10^9 plaque forming units per ml.

1.1.2. Capsule visualisation

Virulent encapsulated *B. anthracis* is present in tissues and blood and other body fluids from animals that have died from anthrax. Thin smears may be prepared from blood from ear veins or other peripheral veins, exudate from orifices and, for horses and pigs, from oedematous fluid or superficial lymph nodes in the neck region. However if the animal has been dead more than 24 hours, the capsule may be difficult to detect. The bacteria should be looked for in smears of these specimens that have been dried, fixed and then stained with azure B (M'Fadyean's reaction). The capsule stains pink, whereas the bacillus cells stain dark blue. The cells are found in pairs or short chains and are often square-ended (the chains are sometimes likened to a set of railway

carriages – so-called 'box-car' or 'jointed bamboo-rod' appearance). Gram stain does not reveal the capsule. The capsule is not present on *B. anthracis* grown aerobically on nutrient agar or in nutrient broths but can be seen when the virulent bacterium is cultured for a few hours in a few millilitres of blood (defibrinated horse or sheep blood seems to work best). Alternatively, the capsule is produced when the virulent *B. anthracis* is cultured on nutrient agar containing 0.7% sodium bicarbonate and incubated in the presence of CO_2 (20% is optimal, but a candle jar works well). The agar is prepared by weighing nutrient agar base powder required for a final volume of 100 ml but reconstituting the measured agar in only 90 ml of water. Autoclave and cool to 50°C in a water bath. Add 10 ml of a filter-sterilised (0.22–0.45 µm filter) 7% solution of sodium bicarbonate. Mix and pour into Petri dishes. The encapsulated *B. anthracis* will form mucoid colonies and the capsule can be visualised by making thin smears on microscope slides, fixing, and staining with azure B or polychrome methylene blue (M'Fadyean's stain).

Polychrome methylene blue can be prepared as follows: 0.3 g of methylene blue is dissolved in 30 ml of 95% ethanol; 100 ml of 0.01% potassium hydroxide (KOH) is mixed with the methylene blue solution. Ideally, this should be allowed to stand exposed to the air, with occasional shaking, for at least 1 year to oxidise and mature. Addition of K_2CO_3 (to a final concentration of 1%) hastens the 'ripening' of the stain, but before it is regarded as diagnostically reliable, its efficacy should be established by testing it in parallel with an earlier, functional batch of stain on *bona fide* samples. It has been found that stains that give positive reactions with cultures of *B. anthracis* cultured artificially in horse blood sometimes do not give positive results in the field.

The method for capsule visualisation has been revised according to the description by Owen *et al.* (2013). The established simple polychrome methylene blue (PMB) staining method for blood or tissue smears from dead animals (M'Fadyean's reaction) is therefore replaced by azure B staining. It must be noted that the rarity of anthrax disease worldwide due to improvements in the control of the disease has led to quality controlled commercially produced PMB being difficult to obtain. In addition, reports of inaccurate results using alternative methylene blue-based stains has also become a concern. Hence, for laboratories requiring a reliable M'Fadyean stain for rapid detection, the recommended approach is to use commercially pure azure B (Owen *et al.,* 2013).

This microscopy method was validated by Aminu *et al.* (2020), for the detection of *B. anthracis* in field-collected blood smears. Four capsule-staining techniques were evaluated in an investigation of livestock mortalities suspected to be caused by anthrax. Field-prepared blood samples were tested by microscopy and the results indicated that the median sensitivity and specificity of microscopy using azure B were found to be comparable with those of the recommended standard, polychrome methylene blue (Aminu *et al.* 2020).

Azure B stain is prepared by constituting into a solution of 0.03 g azure B in 3 ml of 95% ethanol or methanol to which is then added 10 ml of 0.01% KOH (0.23% final azure B concentration). This can then be used immediately and throughout the tests. If stored in the dark at room temperature, the shelf life is at least 12 months. Smears to be stained should be fixed with ethanol or methanol (95–100%), not by heat, and the stain left for 5 minutes before washing off for optimum effect (Owen *et al.,* 2013).

In making smears for staining, only small drops of blood or tissue fluid are needed, and a thin, small smear is best. After fixing by dipping the smear in 95–100% alcohol for about 5 minutes and drying, a small (approximately 20 µl) drop of stain is placed on the smear and spread over it with an inoculating loop. After 5 minutes, the stain is washed with water, blotted, air-dried, and observed initially using the ×10 objective lens under which the short chains appear like short hairs; once found, these can be observed under oil immersion (×1000) for the presence of the pink capsule surrounding the blue/black-staining bacilli. To avoid laboratory contamination, the slide and blotting paper should be autoclaved or left for some hours in a 10% sodium hypochlorite solution.

1.1.3. Other specimens

Identification of *B. anthracis* from old, decomposed specimens, processed materials, and environmental samples, including soil, is possible but these samples often have saprophytic contaminants that outgrow and obscure *B. anthracis* on non-selective agars. The following procedure is suggested:

i) The sample is blended in two volumes of sterile distilled or deionised water and placed in a water bath at 62.5 ± 0.5°C for 15–30 minutes. Turnbull *et al.* (2007) have demonstrated that heat activation of spores can be conducted at a temperature range of 60–70°C with holding times not exceeding 15–30 minutes for best recovery.

ii) Tenfold dilutions to 10^{-2} or 10^{-3} are then prepared. From each dilution, 10–100 µl are plated on to blood agar and optionally 250–300 µl on to PLET agar (polymyxin, lysozyme, EDTA [ethylene diamine tetra-acetic acid], thallous acetate) (Knisely, 1966; WHO, 2008). All plates are incubated at 37°C. For combined selection and differentiation, blood agar-based media can provide advantages. In addition, PLET agar contains the toxic thallium acetate. An alternative for this is the so-called TSPB agar, a blood agar that has high selectivity against Gram-negative bacteria due to the addition of trimethoprim, sulfamethoxazole and polymyxin B (Hudson *et al.,* 2007; Rosenblatt & Stewart, 1974). TSPB agar is prepared by dissolving 40 g/litre nutrient agar base (e.g. SIFIN). The mixture is autoclaved and uniformly cooled to 45°C before adding 50 ml/litre sterile sheep blood (5%), trimethoprim (13.1 mg/litre), sulfamethoxazole (20 mg/litre) and polymyxin B (30,000 IU/litre). After mixing thoroughly, the agar is dispensed into Petri dishes.

iii) Blood agar plates are examined for typical colonies as previously described after overnight incubation, and the PLET plates are examined after 40–48 hours. Confirmation of the identity of suspect colonies as *B. anthracis* is done as described above.

PLET medium (Knisely, 1966; WHO, 2008) is prepared by using heart-infusion agar base (DIFCO) made up to the manufacturer's instructions with the addition of 0.25–0.3 g/litre EDTA and 0.04 g/litre thallous acetate. The mixture is autoclaved and uniformly cooled to 50°C before adding the polymyxin at o30,000 units/litre and lysozyme at 300,000 units/litre. After mixing thoroughly, the agar is dispensed into Petri dishes.

Reports of procedures for direct detection of *B. anthracis* in soils and other environmental specimens using the PCR are emerging. None of these has become routinely applicable at the present time.

Animal inoculation may be considered for recovery of *B. anthracis* if all other methods fail. Examples of when this might occur are specimens from animals that received antibiotic therapy before death or environmental samples containing sporostatic chemicals. Due to the increasing concern to eliminate the use of animals for biological testing, this approach should be used as a last resort and only if justified. Adult mice or guinea-pigs are the animals of choice. If the samples involved are soils, the animals should be pretreated, the day before testing, with both tetanus and gas gangrene antiserum. The samples are prepared as described for culturing, including heat-shocking at 62.5°C for 15 minutes. Mice are injected subcutaneously with 0.05–0.1 ml; guinea-pigs are inoculated intramuscularly with up to 0.4 ml (0.2 ml in each thigh muscle). Any *B. anthracis* present will result in death in 48–72 hours and the organism can be cultured from the blood as described above.

1.2. Immunological detection and diagnosis

It needs to be borne in mind that *B. anthracis* is antigenically very closely related to *B. cereus,* which is considered a ubiquitous component of the environmental microflora. The only unshared antigens that lend themselves to differentiating these two species by immunological approaches are the anthrax toxin antigens, produced during the exponential phase of growth, and the capsule of *B. anthracis*. This places considerable constraints on the extent to which immunological methods can be used in routine detection methodology.

1.2.1. Ascoli test

Ascoli (1911) published a procedure for the detection of thermostable anthrax antigen in animal tissue being used for by-products. This uses antiserum raised in rabbits to produce a precipitin reaction. The test lacks high specificity, in that the thermostable antigens of *B. anthracis* are shared by other *Bacillus* spp., and is dependent on the probability that only *B. anthracis* would proliferate throughout the animal and deposit sufficient antigen to give a positive reaction. This test appears to be used only in Eastern Europe.

To perform the Ascoli test, put approximately 2 g of sample in 5 ml of saline containing 1/100 final concentration of acetic acid and boil for 5 minutes. The resultant solution is cooled and filtered through filter paper. A few drops of rabbit antiserum (see preparation below) are placed in a small test tube. The filtrate from the previous step is gently layered over the top of the antiserum. A positive test is the formation of a visible precipitin band in under 15 minutes. Positive and negative control specimen suspensions should be included.

Antiserum is prepared in rabbits by the subcutaneous inoculation of Sterne anthrax vaccine on days 1 and 14. On days 28 and 35, the rabbits receive 0.5 ml of a mixture of several strains of virulent *B. anthracis* not exceeding 10^5 colony-forming units (CFU)/ml suspended in saline. Alternatively, the live virulent bacteria can be inactivated by prolonged suspension in 0.2% formalised saline, but the antigen mass needs to be increased to 10^8–10^9 CFU/ml. The suspension should be checked for inactivation of the *B. anthracis* before animal inoculation by culture of 0.1 ml into 100 ml of nutrient broth containing 0.1% histidine and, after incubation at 37°C for 7 days, subculture on to blood or nutrient agar. The dose regimen for the formalised suspension after initial vaccination on days 1 and 14 is increasing doses of 0.1, 0.5, 1, and 2 ml given intravenously at intervals of 4–5 days. Following either procedure, a test bleed at 10 days after the last injection should determine whether additional 2 ml doses should be administered to boost the precipitin titre.

1.2.2. Immunofluorescence

While some success has been achieved with immunofluorescence for capsule observation in the research situation (Ezzell & Abshire, 1996), it does not lend itself to routine diagnosis.

1.3. Confirmation of virulence with the polymerase chain reaction

Confirmation of virulence can be carried out using the PCR. The following instructions are taken from the WHO (2008). Template DNA for PCR can be prepared from a fresh colony of *B. anthracis* on nutrient agar by suspension of a loop of growth in 25 µl sterile deionised (or distilled) water and heating to 95°C for 20 minutes. Following cooling to approximately 4°C, and brief centrifugation, the supernatant can be used for the PCR reaction.

Examples of suitable primers (Beyer *et al.,* 1996; Hutson *et al.,* 1993) for confirming the presence of the pXO1 and pXO2 plasmids are given in the table below.

Target	Primer ID	Sequence (5' → 3')	Product size	Concentration
Protective antigen (PA)	PA 5 3048–3029	TCC-TAA-CAC-TAA-CGA-AGT-CG	596 bp	1 mM
	PA 8 2452–2471	GAG-GTA-GAA-GGA-TAT-ACG-GT		
Capsule	1234 1411–1430	CTG-AGC-CAT-TAA-TCG-ATA-TG	846 bp	0.2 mM
	1301 2257–2238	TCC-CAC-TTA-CGT-AAT-CTG-AG		

PCR can be carried out in 50 µl volumes using the above primers, 200 µM each of dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$ and 2.5 units of DNA polymerase, all in NH_4 buffer, followed by the addition of 5 µl of template DNA. A 2% agarose gel has been found to work best with these small fragments.

Alternatively, premixed, predispensed, dried beads available commercially can be used. These are stable at room temperature, containing all the necessary reagents, except primer and template, for performing 25 µl PCR reactions. The template can be added in a 2.5 µl volume.

The following PCR cycle can be used: 1 × 95°C for 5 minutes; 30 × 95°C for 0.5 minute followed by 55°C for 0.5 minute followed by 72°C for 0.5 minute; 1 × 72°C for 5 minutes; cool to 4°C.

It should be noted that the primers given in the table above have proved successful for confirming the presence or absence of pXO1 and/or pXO2 in pure cultures of isolates from animal (including human) specimens or environmental samples. They may be unsuitable, however, for direct detection of *B. anthracis* in such specimens or samples. A choice of alternatives can be found in Jackson *et al.* (1998) and Ramisse *et al.* (1996). For the rare possibility that an isolate may lack both pXO1 and pXO2, a chromosomal marker should also be run; primers for these are also described in Jackson *et al.* (1998) and Ramisse *et al.* (1996). Ågren *et al*. (2013) published a very comprehensive study on the *in-silico* and *in-vitro* evaluation of 35 PCR-based methods for 20 chromosomal markers of *B. anthracis*. The PL3 (target: part of pro-phage type 3) assay (Wielinga *et al*., 2011) was identified as one of the best performing assays in this study and could be used for routine diagnostics.

Real-time PCR assays have been developed for enhanced speed, sensitivity and specificity of detection of pXO1, pXO2 and chromosomal genes of *Bacillus anthracis* and other closely related *Bacillus* spp. (e.g. Hadjinicolaou *et al*., 2009; Hoffmaster *et al*., 2002; Irenge *et al*., 2010; Qi *et al*., 2001; Rao *et al*., 2010). Selection of a particular assay will be dependent on the fitness for purpose and source of starting material (e.g. isolates, clinical specimen, environmental sample), requirement to differentiate from other *Bacillus* spp. or vaccine strains, demonstration of genetic diversity or confirmation of isolate identity. It is important that the laboratory conducting real-time PCR evaluate the performance of the test for their purpose and complete a validation analysis to ensure that it has been optimised and standardised for its intended use (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

Molecular typing techniques for *B. anthracis* like canonical single-nucleotide polymorphisms typing (e.g. Van Ert *et al*., 2007) and variable number tandem repeat analysis (e.g. Keim *et al*., 2000) are appropriate for use in specialised laboratories. Molecular typing based on whole genome sequencing e.g. core genome multilocus sequence typing may be useful to elucidate the diversity of *B. anthracis* genotypes circulating, to determine connections between outbreak events and supports infection chain tracing (Abdel-Glil *et al*., 2021).

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

The most widely used vaccine for prevention of anthrax in animals was developed by Sterne (1937). He derived a rough variant of virulent *B. anthracis* from culture on serum agar in an elevated CO_2 atmosphere. This variant, named 34F2, was incapable of forming a capsule and was subsequently found to have lost the pXO2 plasmid, which codes for capsule formation. It has become the most widely used strain world-wide for animal anthrax vaccine production. In Central and Eastern Europe, an equivalent $pXO2^-$ derivative, Strain 55, is the active ingredient of the current livestock vaccine. A list of manufacturers of anthrax vaccine for use in animals is given in Annex 5 of WHO (2008).

The following information concerning preparation of the anthrax vaccine for use in animals is based on Misra (1991) and the WHO (1967). Generalised procedures are given; national regulatory authorities should be consulted in relation to Standard Operating Procedures that may pertain locally.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Anthrax vaccine production is based on the seed-lot system. A seed lot is a quantity of spores of uniform composition processed at one time and maintained for the purpose of vaccine preparation. Each seed lot is no more than three passages from the parent culture and must produce a vaccine that is efficacious and safe for use in animals. It is recommended that a large

seed lot be prepared from the parent strain and preserved by lyophilisation for future production lots. The parent culture can be purchased[1].

2.1.2. Quality criteria

The seed lot is acceptable for anthrax vaccine if a vaccine prepared from the seed lot or a suspension harvested from a culture derived from a seed lot meets the requirements for control of final bulk with respect to freedom from bacterial contamination, safety and efficacy (immunogenicity).

2.2. Method of manufacture

2.2.1. Procedure

i) Preparation of the master seed

Seed lots are cultured on solid media formulated to promote sporulation of the organism. The solid medium formula for casein digest agar (sporulation agar) given in Misra (1991) is: 50 g tryptic digest of casein; 10 g yeast extract; 0.1 g $CaCl_2.6H_2O$; 0.01 g $FeSO_4.7H_2O$; 0.05 g $MgSO_4.7H_2O$; 0.03 g $MnSO_4.4H_2O$; 5.0 g K_2HPO_4; 1.0 g KH_2PO_4; 22 g agar; 1000 ml deionised or distilled water. The ingredients are dissolved in the water with the appropriate amount of heating; the solution is adjusted to pH 7.4, distributed into Roux bottles (120 ml per bottle) or other appropriate container, sterilised by autoclaving and cooled in the horizontal position. After the agar has solidified, excess liquid should be removed aseptically and the bottles left in an incubator (37°C) for at least 2 days to dry and to check the sterility.

Volumes of 2 ml of vaccine seed should be spread across the agar in Roux bottles, which should be incubated at 37°C until at least 80% sporulation is apparent by microscopic examination of aseptically extracted loopfuls (at least 72 hours). The growth is harvested with 10 ml per bottle of sterile deionised or distilled water and checked for purity. After washing three times in sterile deionised or distilled water with final suspension, also in sterile deionised or distilled water, sterilised lyophilisation stabiliser is added and the suspension is dispensed into lyophilisation vials and freeze-dried.

Attenuated vaccine strains can gradually lose their antigenicity over repeated subculturing conditions. Therefore, it is recommended that master seed lots be made in bulk and kept within three passages from the original seed culture. A large number of master seed stocks should be prepared.

ii) Preparation and testing of the working seed

Reconstitute a vial of seed stock and inoculate several slants (approximately 10 ml) of sporulation (casein digest) agar. Incubate at 37°C for 72 hours and store in a refrigerator. Test the slants for purity by culture on to nutrient agar plates and in nutrient broth (0.1 ml in 100 ml of nutrient broth). The latter should be subcultured on to nutrient agar after incubation at 37°C for 7 days and should be a pure culture of *B. anthracis*. A sample of the broth culture should also be checked for lack of motility.

Volumes of seed needed for a production run should be calculated on the basis of harvesting the spores from each slant with 10 ml of sterile deionised or distilled water and using this to inoculate five Roux bottles.

iii) Preparation of vaccine concentrate

Roux bottles with casein digest agar are prepared as for the master seed in Section C.2.2.1.i above. One Roux bottle can be expected to yield about 2000 doses of vaccine. Each Roux bottle is inoculated with 2 ml of working seed suspension and incubated at 37°C with porous plugs for several days until small loopfuls of culture from randomly selected bottles show at least 90% of the organisms to be in sporulated forms when examined in wet mounts by phase contrast (phase bright spores) or following staining for spores. The growth from each

1 UK Health Security Agency, Microbiology Services, Porton Down, Salisbury SP4 0JG, UK (www.culturecollections.org.uk/)

bottle is then harvested with 20 ml of physiological saline. Tests for contaminants should be carried out by subculture to nutrient agar plates and inoculation of 100 ml nutrient broth with 0.1 ml of harvested spores followed by subculture to nutrient agar after 7 days at 37°C and by tests for motility. Acceptable harvests (i.e. those showing no evidence of contaminants) are pooled.

iv) Glycerination

Twice the volume of sterile, pure, neutral glycerol should be added to the bulk pool of vaccine concentrate. Saponin (0.1% final concentration) may also be added at this point if it is to be included as an adjuvant. Mix thoroughly (the inclusion of sterilised glass beads may be helpful). Carry out a purity test and hold for 3 weeks at ambient temperature to allow lysis of any vegetative bacteria, determine the viable spore count and store under refrigeration thereafter.

v) Determining titre and dilution for use

The number of culturable spores in the product is then calculated by spreading tenfold dilutions on nutrient agar plates. The suspension is diluted so that the final bulk contains the number of culturable spores desired. The diluent should contain the same proportions of saline, glycerol and (if being included) saponin as present in the vaccine concentrate. The vaccine should contain a minimum of $2–10 \times 10^6$ culturable spores per dose for cattle, buffaloes and horses, and not less than $1–5 \times 10^6$ culturable spores per dose for sheep, goats and pigs.

vi) Filling the containers

Distribution of aliquots of vaccine into single and multidose containers is performed as outlined in WHO (1965). Basically, the final bulk is distributed to containers in an aseptic manner in an area not used for production, and any contamination or alteration of the product must be avoided. The vaccine may be lyophilised after distribution into appropriate dosage containers. Containers are sealed as soon as possible with a material that is not detrimental to the product and that is capable of maintaining a hermetic seal for the life of the vaccine.

2.3. Requirements for substrates and media

Please refer to Misra (1991) for detailed information on substrates and media used for anthrax vaccine production.

2.3.1. In-process controls

i) Purity of the seed lot

Purity tests consist of microscopic examination of stained smears with culture and motility tests as described in Section C.2.2.

ii) Safety of the seed lot

Not less than 5×10^9 culturable spores should be injected subcutaneously into each of three healthy, 1–2-year-old, unvaccinated sheep, which must survive an observation period of at least 10 days.

iii) Immunogenicity of the seed lot

At least 10 healthy guinea-pigs, 300–500 g in weight should be inoculated with 5×10^6 viable spores and observed for 21 days. At least 80% of the animals should survive. The immunised animals, together with three unimmunised controls, should then be challenged with 10 median lethal doses (LD_{50}) of the strain 17 JB of *B. anthracis*. During a 10-day observation period, none of the immunised animals should succumb to the challenge while all the controls should die from anthrax. The test should be repeated if one of the immunised animals dies.

2.3.2. Final product batch tests

i) Sterility and purity

The vaccine is a live culture of *B. anthracis* spores; sterility does not apply, but the batches must be tested for freedom from contamination (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

ii) Safety

Safety testing is performed on two healthy sheep or goats and consists of inoculating subcutaneously twice the recommended vaccination dose. The animals are observed for 10 days. The final bulk passes the test if no systemic reactions develop and if not more than a transient oedema is observed at the injection site. If the test is carried out in sheep only, a progressive oedema indicates that the vaccine may be unsuitable for goats.

iii) Batch potency

Efficacy or immunogenicity is tested on the final bulk as follows: at least ten healthy 300–500 g guinea-pigs are inoculated with a sheep dose of the vaccine. The guinea-pigs are observed for 21 days, and at least 80% of the animals must survive the observation period. Surviving immunised guinea-pigs and three non-vaccinated controls are challenged with an appropriate dose of virulent *B. anthracis*. A recommended challenge is 200 LD_{50} of the Pasteur II strain (17JB). If, by 10 days after challenge, all vaccinated guinea-pigs survive and control animals die, the final bulk is deemed to be satisfactory. If any vaccinated animals die during the post-challenge observation period from a cause other than anthrax, and death is not associated with the vaccine, the test may be repeated.

2.4. Requirements for authorisation

2.4.1. Safety requirements

i) Target and non-target animal safety

The vaccine has been shown to cause disease in some goats and llamas; this may be related to the saponin adjuvant. The vaccine is not recommended for use in pregnant animals, nor in animals destined for slaughter within 2–3 weeks of vaccination. Local regulations may specify other time periods in some countries or regions, but there is no scientific reason for regarding meat from clinically healthy animals as unfit for human handling or consumption after a holding period of 2 weeks following vaccination. Concurrent administration of antibiotics to vaccinated animals is contraindicated as the antibiotic will interfere with the vaccine. Antibiotics should not be given for several days before and after vaccination.

Accidental human inoculation is treated by expressing as much of the inoculum as possible from the injection site and washing the wound thoroughly with soap and water. Medical attention should be sought if infection develops.

ii) Reversion–to-virulence for attenuated/live vaccines

The 34F2 strain of *B. anthracis* is known to be stable and cannot produce capsule *in vitro*.

iii) Environmental consideration

Leftover vaccine, empty vials, and equipment used for vaccinating are contaminated with the live spores and should be autoclaved, disinfected, or incinerated.

2.4.2. Efficacy requirements

i) For animal production

Not applicable.

ii) For control and eradication

The recommended dose for cattle and horses is a minimum of 2–10 × 10^6 culturable spores; for sheep, goats and pigs, it is 1–5 × 10^6 culturable spores. The vaccine should contain these

spores in an appropriate volume, e.g. 2×10^6/ml. Immunity should be good for at least 1 year and it is recommended that an annual booster be given. Horses may be slow to develop immunity following initial vaccination; some manufacturers therefore recommend a two-dose initial vaccination, administered 1 month apart, followed by a single annual booster.

Bacillus anthracis spores are stable in unlyophilised or lyophilised vaccine and preservatives are not required. Storage under refrigeration is recommended (4°C).

As there is no generally acceptable test for stability of anthrax vaccines, it is recommended that, in each filling lot, the number of culturable spores be determined before and after holding at an appropriate temperature for an appropriate period. There should be no evidence of a fall in the number of culturable spores.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantage

There are no vaccines based on biotechnology available for anthrax.

3.2. Special requirements for biotechnological vaccines, if any

Not applicable.

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NB: There are WOAH Reference Laboratories for anthrax (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on anthrax diagnostic tests, reagents and vaccines

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.1.2.

AUJESZKY'S DISEASE (INFECTION WITH AUJESZKY'S DISEASE VIRUS)

SUMMARY

Description and importance of the disease: *Aujeszky's disease, also known as pseudorabies, is caused by an alphaherpesvirus that infects the central nervous system and other organs, such as the respiratory tract, in a variety of mammals except humans and the tailless apes. It is associated primarily with suidae (pigs or wild boars), the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks of age, which die from encephalitis). The disease is controlled by containment of infected herds and by the use of vaccines and/or removal of latently infected animals.*

A diagnosis of Aujeszky's disease is established by detecting the agent (by virus isolation or polymerase chain reaction [PCR]), as well as by detecting a serological response in the live animal.

Identification of the agent: *Isolation of Aujeszky's disease virus can be made by inoculating a tissue homogenate, for example of brain and tonsil or material collected from the nose/throat, into a susceptible cell line such as porcine kidney (PK-15 or SK6), or primary or secondary kidney cells. The specificity of the cytopathic effect is verified by immunofluorescence, immunoperoxidase or neutralisation with specific antiserum. The viral DNA can also be identified using PCR; this can be accomplished using real-time PCR techniques.*

Serological tests: *Aujeszky's disease antibodies are demonstrated by virus neutralisation, latex agglutination or enzyme-linked immunosorbent assay (ELISA). A number of ELISA kits are commercially available world-wide. A WOAH International Standard Reference Serum defines the lower limit of sensitivity for routine testing by laboratories that undertake the serological diagnosis of Aujeszky's disease.*

It is possible to distinguish between antibodies resulting from natural infection and those from vaccination with gene-deleted vaccines.

Requirements for vaccines: *Vaccines should prevent or at least limit the excretion of virus from the infected pigs. Recombinant DNA-derived gene-deleted or naturally deleted live Aujeszky's disease virus vaccines, lack a specific glycoprotein (gG, gE, or gC), which enables the use of companion diagnostic tests to differentiate vaccinal antibodies from those resulting from natural infection.*

A. INTRODUCTION

Aujeszky's disease, also known as pseudorabies, is caused by *Suid herpesvirus 1* (SHV-1), a member of the subfamily *Alphaherpesvirinae* and the family *Herpesviridae.* The virus should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). The virus infects the central nervous system and other organs, such as the respiratory tract, of a variety of mammals (such as dogs, cats, cattle, sheep, rabbits, foxes, minks, etc.) except humans and the tailless apes. It is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks of age, which die from encephalitis). In consequence, the pig is the only species able to survive a productive infection and therefore, serves as the reservoir host. In pigs, the severity of clinical signs depends on the age of the pig, the route of infection, the virulence of the infecting strain and the immunological status of the animal. Young piglets are highly susceptible with mortality rates reaching 100% during the first 2 weeks of life.

These animals show signs of hyperthermia and severe neurological disorders: trembling, incoordination, ataxia, nystagmus to opisthotonos and severe epileptiform-like seizures. When pigs are older than 2 months (grower-finisher pigs), the respiratory forms become predominant with hyperthermia, anorexia, and mild to severe respiratory signs: rhinitis with sneezing and nasal discharge that may progress to pneumonia. The frequency of secondary bacterial infections is high, depending on the health status of the infected herd. In this group of pigs, the morbidity can reach 100%, but in cases of the absence of complicated secondary infections, mortality ranges from 1% to 2% (Pejsak & Truszczynski, 2006). Sows and boars primarily develop respiratory signs, but in pregnant sows, the virus can cross the placenta, infect and kill the fetuses, inducing abortion, return to oestrus, or stillborn fetuses. Virus may be found in the semen of infected boars (van Rijn *et al.,* 2004). In the other susceptible species, the disease is fatal, the predominant sign being intense pruritus causing the animal to gnaw or scratch part of the body, usually head or hind quarters, until great tissue destruction is caused. For that reason, the disease was named "mad-itch" in the past.

Focal necrotic and encephalomyelitis lesions occur in the cerebrum, cerebellum, adrenals and other viscera such as lungs, liver or spleen. In fetuses or very young piglets, white spots on liver are highly suggestive of their infection by the virus. Intranuclear lesions are frequently found in several tissues.

Aujeszky's disease is endemic in many parts of the world, but several countries have successfully completed eradication programmes, e.g. the United States of America, Canada, New Zealand and many Member States of the European Union.

The disease is controlled by containment of infected herds and by the use of vaccines or removal of latently infected animals (Pejsak & Truszczynski, 2006). Stamping out has been or is used in several countries usually when the infected farms are small or when the threat to neighbouring farms is very high in free countries.

Whereas isolation of the Aujeszky's disease virus or detection of the viral genome by the polymerase chain reaction (PCR) are used for diagnosis in the case of lethal forms of Aujeszky's disease or clinical disease in pigs, serological tests are required for diagnosis of latent infections and after the disappearance of the clinical signs. Affected animals except suids, do not live long enough to produce any marked serological response. Serological tests are the tests to be used to detect subclinically or latently infected pigs, especially in the case of qualification of the health status of the animals for international trade or other purposes.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of Aujeszky's disease and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	–	–	–	+++	–	–
Real-time PCR	–	+	+	+++	+	–
Detection of immune response						
Latex agglutination	+++	+++	+++	+	+++	+++
ELISA	+++	+++	+++	+	+++	+++
VN	+	+	+	+	+	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Virus isolation

The diagnosis of Aujeszky's disease can be confirmed by isolating the virus from the oro-pharyngeal fluid, nasal fluid (swabs) or tonsil swabs from living pigs, or from samples from dead pigs or following the presentation of clinical signs such as encephalitis in herbivores or carnivores. For post-mortem isolation of SHV-1, samples of brain, tonsil, and lung are the preferred specimens. In cattle, infection is usually characterised by a pruritus, in which case a sample of the corresponding section of the spinal cord may be required in order to isolate the virus. In latently infected pigs, the trigeminal ganglia is the most consistent site for virus isolation, although latent virus is usually non-infective unless reactivated, making it difficult to recover in culture.

Tissue samples are homogenised in normal saline or cell culture medium with antibiotics. The method used should be suitable for the subsequent diagnostic test. The amount of tissue homogenised should take into account a possible non-homogeneous distribution of the virus. A tissue homogenate of approximately 10% is recommended. The resulting suspension is clarified by low speed centrifugation, e.g. at 900 ***g*** for 10 minutes. The supernatant fluid is used to inoculate any sensitive cell culture system. Numerous types of cell line or primary cell cultures are sensitive to SHV-1, but a porcine kidney cell line (PK-15 or SK6) is generally employed. The overlay medium for the cultures should contain antibiotics (such as: 200 IU/ml penicillin; 100 μg/ml streptomycin; 100 μg/ml polymyxin; and 3 μg/ml fungizone).

SHV-1 induces a cytopathic effect (CPE) that usually appears within 24–72 hours, but cell cultures may be incubated for 5–7 days. The monolayer develops accumulations of birefringent cells, followed by complete detachment of the cell sheet. Syncytia also develop, the appearance and size of which are variable. In the absence of any obvious CPE, it is advisable to make one blind passage into further cultures. Additional evidence may be obtained by staining infected cover-slip cultures with haematoxylin and eosin to demonstrate the characteristic herpesviral acidophilic intranuclear inclusions with margination of the chromatin. Virus identity should be confirmed by immunofluorescence, immunoperoxidase, neutralisation using specific antiserum following the method described in Section B.2.1. or by PCR.

The isolation of SHV-1 makes it possible to confirm Aujeszky's disease, but failure to isolate does not guarantee freedom from infection.

1.2. Identification of virus by the polymerase chain reaction

The PCR can be used to identify SHV-1 genomes in secretions or organ samples. Many individual laboratories have established effective protocols, but there is as yet no internationally agreed standardised approach.

The PCR is based on the selective amplification of a specific part of the genome using two primers located at each end of the selected sequence. In a first step, the complete DNA may be isolated using standard procedures (e.g. proteinase K digestion and phenol–chloroform extraction) or commercially available DNA extraction kits. Using cycles of DNA denaturation to give single-stranded DNA templates, hybridisation of the primers, and synthesis of complementary sequences using a thermostable DNA polymerase, the target sequence can be amplified up to 10^6-fold. The primers must be designed to amplify a sequence conserved among SHV-1 strains, for example parts of the gB or gD genes that code for essential glycoproteins have been used (Mengeling *et al*., 1992; Van Rijn *et al*., 2004; Yoon *et al*., 2006). Real-time PCRs have been developed that can differentiate gE-deleted vaccine viruses from wild-type virus based on the specific detection of gB and gE genes (Ma *et al*., 2008; Wernike *et al*., 2014).

The amplified product may be identified from its molecular weight as determined by migration in agarose gel, with further confirmation where possible by sequencing the amplified product. More recent techniques include the use of fluorescent probes linked to an exonuclease action and real-time monitoring of the evolution of product, enabling simultaneous amplification and confirmation of the template DNA thus increasing the rapidity and specificity of the PCR assays.

In all cases, the main advantage of PCR, when compared with conventional virus isolation techniques, is its rapidity; with the most modern equipment, the entire process of identification and confirmation can be completed within one day. However, because of the nature of the test, many precautions need to be taken to avoid contamination of samples with extraneous DNA from previous tests or from general environmental contamination in the laboratory (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*). This may limit the value of the test for many laboratories unless care is taken to avoid DNA carry-over contamination. The use of an internal control is recommended so as to avoid false-negative results by ensuring adequate efficiency of DNA extraction and confirming the absence of PCR inhibitors in each sample. In practice, different systems can be used for detection of endogenous or exogenous genes (Hoffman *et al.,* 2009). Kits for the test are commercially available (Pol *et al.,* 2013).

2. Serological tests

Virus neutralisation (VN) has been recognised as the reference method for serology (Moennig *et al*., 1982), but for general diagnostic purposes it has been widely replaced by the enzyme-linked immunosorbent assay (ELISA) because of its suitability for large-scale testing (Moennig *et al*., 1982). The tests can be performed on a variety of matrices (e.g. serum, whole blood, milk, muscular exudates, and filter paper), but the preferred matrix is serum.

A latex agglutination test has also been developed, and can be used for screening for antibodies. Kits for the test are commercially available (Schoenbaum *et al*., 1990).

Serological tests are carried out only for suids, as other animals (herbivores and carnivores) die too quickly to produce antibodies. In free areas where pigs are not vaccinated, an active epidemiological survey can be carried out using ELISA gB or gE or latex agglutination kits. As antibodies can be detected between 7 and 10 days post-infection, these serological tools can also be used to confirm infection in pigs in the case of a suspected outbreak. In area where pigs are vaccinated with gE deleted vaccines, the ELISA gE kits permit the differentiation between infected and vaccinated pigs (DIVA), but to assess the level of immunity induced by vaccination, gB ELISA, latex agglutination kits or viral neutralisation should be used.

Any serological technique used should be sufficiently sensitive to give a positive result with the WOAH International Standard Reference Serum or a calibrated secondary serum. Reference serum can be obtained from the WOAH Reference Laboratory for Aujeszky's Disease in France (see Table given in Part 4 of this *Terrestrial Manual*). For international trade purposes, the test should be sensitive enough to detect the standard serum diluted 1/2. To authorise pig movement from an area where deleted gE vaccines are used to a free area, serological assays should be able to detect at least the dilution of 1/8 for ELISA gE of the WOAH International Standard Reference Serum as prescribed by the European Commission (2008).

2.1. Virus neutralisation

VN in cell culture can be performed in several ways, which vary according to the length of incubation of the virus/serum mixtures (e.g. 1 hour at 37°C or 24 hours at 4°C) and the presence or absence of complement. Most laboratories use a reaction period of 1 hour at 37°C in the absence of complement, because this is easy and rapid. However, the sensitivity can be improved by increasing the incubation period to 24 hours at 4°C, which facilitates the detection of antibody levels 10–15 times lower than in the 1-hour method. For international trade purposes, the test method should be validated as being sensitive enough to detect the WOAH Standard Reference Serum diluted 1/2.

VN cannot be used to differentiate antibodies of vaccinal origin from those caused by natural infection. It is one of the two tests available that complies with the requirement in the WOAH *Terrestrial Animal Health Code* chapter when it refers to "a diagnostic test to the whole virus".

i) Cells

Cells susceptible to infection with SHV-1 are used; they may be cell lines (e.g. PK-15, SK6, MDBK), or primary or secondary cell cultures (e.g. porcine kidney).

ii) Cell culture medium

The medium depends on the type of cells. For example, the medium for PK-15 cells is Eagle's minimal essential medium (MEM) + 10% fetal bovine serum and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin or, alternatively, 50 μg/ml gentamycin).

iii) Maintenance of the cells

The cells are cultured in cell culture vessels of, for example, 75 cm^2. They are trypsinised once or twice per week. For weekly trypsinisation, the cells are usually cultured in 50 ml of medium, with a multiplication rate of 5. For two trypsinisations a week, the cells are cultured in 30 ml of medium, with a multiplication rate of 3.

For trypsinisation, the growth medium is removed once the cell sheet is complete. The cell sheet is washed with about 5 ml of recently thawed trypsin/ethylene diamine tetra-acetic acid (EDTA) (0.25%) in an isotonic buffer. The washing fluid is discarded and the preparation is washed again, retaining only a few drops of trypsin. The container is placed in an incubator at 37°C for 5–10 minutes until the cells have become detached. Once the sheet is detached and the cells are well separated, for twice weekly passage they are suspended in 90 ml of growth medium, and this suspension is distributed into three 75 cm^2 cell culture vessels. For weekly trypsinisation the cells are suspended in 150 ml of growth medium and the suspension distributed into five 75 cm^2 cell culture bottles.

iv) Virus

A suitable strain of SHV-1, such as the Kojnok strain or the NIA-3 strain, is stored at a temperature of –65°C or below, or in freeze-dried form at 4°C.

v) Preparation of stock virus suspension

The culture fluid is removed from a cell culture vessel containing a complete cell sheet. About 1 ml of stock virus suspension of known titre (about 10^7 $TCID_{50}$/ml [50% tissue culture infective dose]) is added, and the vessel is incubated at 37°C±2°C for 1 hour. 30 ml of culture medium is added and the vessel is again incubated at 37°C±2°C. The vessel is examined frequently until there is about 75% cell destruction (after about 36–48 hours). It is then frozen at a temperature of –65°C or lower to disrupt the cells.

The vessel is then thawed and shaken vigorously. Medium is collected and centrifuged at 1500 ***g*** for 15 minutes. The supernatant fluid is divided into portions (of about 0.5 ml) in small tubes that are labelled (date and virus reference) before being stored at a temperature of –65°C or lower until required.

vi) Titration of the stock virus suspension

Titration of the stock suspension is performed by the method of Reed & Muench or that of Kärber, and the titre is expressed per 50 μl and per ml.

The VN test requires an internal quality control serum with a known titre of neutralising antibody to SHV-1 (it can be calibrated against an international standard serum or a secondary standard prepared from that serum), and a negative control serum (from a specific antibody free pig, e.g. from an official Aujeszky's disease free herd). The test sera themselves should be of good quality, clearly labelled, of known provenance with clinical history, stored in refrigeration at all times, free from fungal or bacterial contamination, non-haemolysed and of sufficient quantity. Serum should be separated from the coagulum without delay, thereby preventing toxicity.

There are qualitative and quantitative procedures for VN, both of which are described below.

2.1.1. Qualitative virus neutralisation technique

i) Complement in the serum samples is destroyed by heating in a water bath at 56–59°C for 30 minutes.

ii) Each undiluted serum sample is placed in two to three wells, at 50 μl per well, of a 96-well cell-culture grade microtitre plate. Each serum can also be diluted 1/2 in the MEM, before being placed in two other wells.

iii) 50 µl of virus suspension containing 100 $TCID_{50}$ (or 2×10^3 $TCID_{50}$/ml), obtained by diluting stock virus suspension of known titre with MEM, is added to each well.

iv) The plate is gently shaken and placed in an incubator for 1 hour at 37°C (±2°C) (5% CO_2 optional).

v) 150 µl of cell suspension containing about 150,000 cells/ml is added to each well.

vi) The plate is covered (for incubation in CO_2), or a plastic sheet is sealed carefully around the edges of the plate (for incubation in air). The plate is shaken lightly to obtain an even distribution of cells at the bottom of the wells, and placed in the incubator at 37°C (±2°C) (CO_2 optional) for 3–5 days.

vii) *Controls:* Each set of plates must include the following controls:

a) Virus control

This is to verify the amount of virus actually used for the test. The virus dose used for VN (target titre 100 $TCID_{50}$/50 µl) is diluted with MEM at 1/10, 1/100 and 1/1000. Of each dilution, 50 µl is placed in at least four wells, to which 50 µl of medium is added before the wells are incubated for 1 hour at 37°C (±2°C). The cell suspension is added in the same way as for the sera under test.

b) Cell control

150 µl cell suspension and 100 µl MEM are placed in each of at least four wells.

c) Positive serum control

A serum of known SHV-1 neutralising antibody titre is used. Five dilutions are prepared in the same way as for the sera under test: a dilution corresponding to the serum titre, two-fold and four-fold dilutions, and 1/2 and 1/4 dilutions (equivalent to T, T/2, T/4, 2T and 4T, where T is the serum titre, i.e. undiluted serum for the qualitative test). Add 50 µl of virus suspension containing 100 $TCID_{50}$/50 µl to 50 µl of positive control sample dilutions. The cells are incubated and the cell suspension is added in the same way as for the sera under test.

d) Serum control

This is to verify the absence of a toxic effect of the sera on the cells. Wells containing 50 µl of each serum are incubated for 1 hour at 37°C in the presence of 50 µl of medium. Then, 150 µl of cell suspension is added in the same way as for the sera under test.

e) Negative serum control

This is done in the same way as for sera under test.

viii) *Reading the results:* An inverted-image microscope (×100) is used to examine the wells for toxic effects and CPEs after 3 to 5 days. The controls must give the following results if the tests are to be considered valid:

a) Virus control

The titre of the viral suspension should be between 30 and 300 $TCID_{50}$/50 µl.

b) Cell control

The cell sheet must be intact.

c) Positive serum control

The titre obtained must be equal to the predicted titre, within one dilution.

d) Serum control

Examination for a CPE should take into account a possible toxic effect on cells.

e) Negative serum control

A CPE should be present.

ix) For the sera under test if distributed in three wells, the following results may be seen:

a) presence of a CPE in three wells = negative result;

b) absence of a CPE in three wells on day 3 = positive result;

c) presence of a CPE in one well but not in the others = inconclusive result, test must be repeated;

d) small plaques indicating a CPE on day 3 = inconclusive result, test must be repeated;

e) toxicity in serum control and test wells = unreadable result, test must be repeated. (NB replacement of medium with fresh medium after 16 hours' incubation will reduce the toxicity without affecting the titre of specific antibody.) Plates can be read until day 5 of incubation.

f) If the serum was initially diluted 1/2 and distributed in two wells, it is considered positive if CPE is absent in one of the two wells, and it is highly recommended to retest using the quantitative technique. Diluting the serum to 1/2 can prevent the toxicity effect of the tested sera.

x) *Interpretation of the results:* This test is capable of detecting the presence or absence of neutralising antibody to SHV-1. It is incapable of distinguishing vaccinated animals from infected animals.

The technique described (VN for 1 hour at 37°C) can give false-negative and false-positive results. The sensitivity can be increased (leading to fewer false negatives) by adopting a method based on neutralisation involving 24 hours of contact between virus and serum at 4°C, before the addition of cells.

A qualitative technique such as this one, which employs undiluted serum samples (1/2 final dilution), can give a false-positive result in certain cases due to nonspecific neutralisation of the virus. This problem can be addressed by carrying out a confirmatory test using the quantitative technique (see Section B.2.1.2 below).

Samples giving inconclusive results may be tested by an alternative technique with better sensitivity such as an ELISA or the animal should be re-bled to confirm status.

2.1.2. Quantitative virus neutralisation technique

The quantitative VN technique is similar to the qualitative procedure, but each serum is used both undiluted and in a series of dilutions. Depending on the desired precision, the purpose of testing and the expected titre, two wells are used for each dilution of serum, and a range of dilutions appropriate for the purpose. The procedure below describes the test for an initial maximum dilution of 1/16. It is possible to reach higher titres using more wells (e.g. A1 to A12 for 1/256 dilution).

i) Complement in the serum samples is destroyed by heating in a water bath at 56–59°C for 30 minutes.

ii) 75 µl of MEM is added to well A2 and 50 µl of MEM is added to wells A1, and A3 to A6 of a 96-well cell-culture grade microtitre plate and continued for comparable wells in rows B, C, etc., for additional serum samples.

iii) 75 µl of undiluted serum sample is added to well A2, and continued for wells in rows B, C, etc., with other serum samples.

iv) Using a multichannel pipette, the contents of wells in column 2 are mixed, then 50 µl is transferred to column 1 and 3, and so on to column 6 or further to a predetermined row, using the same nozzles. The 50 µl portions remaining after the last row are discarded.

v) 50 µl of virus suspension containing 100 $TCID_{50}$ (or 2×10^3 $TCID_{50}$/ml), obtained by diluting stock virus suspension of known titre with MEM, is added to each well in columns 2 to 6. No virus is added to wells in column 1, this is a control column of serum samples.

vi) The plate is shaken and placed in an incubator for 1 hour at 37°C (±2°C) (5% CO_2 optional).

vii) 150 µl of cell suspension containing about 150,000 cells/ml is added to each well.

viii) The plate is covered (for incubation in CO_2), or a plastic sheet is sealed carefully around the edges of the plate (for incubation in air). The plate is shaken lightly to obtain an even distribution of cells at the bottom of the wells, and placed in the incubator at 37°C (±2°C) (CO_2 optional) for 3–5 days.

ix) Controls are set up as described for the qualitative technique.

x) *Reading the results:* The neutralising titre of a serum is expressed by the denominator of the highest initial dilution that brings about complete neutralisation of the CPE of the virus in 50% of the wells. Neutralisation at any dilution (even undiluted, equivalent to a final dilution of 1/2) is considered to be positive. If the serum shows neutralisation only when undiluted (with growth of virus and CPE at the 1/2 and subsequent dilutions), it would be advisable to apply alternative tests (ELISA or latex agglutination) to provide confirmation of the result, or to request another sampling of the animal, at least 8 days after the first.

2.2. Enzyme-linked immunosorbent assay

The sensitivity of the ELISA is generally superior to that of the VN test using 1-hour neutralisation without complement. Some weak positive sera are more readily detected by VN tests using 24-hour neutralisation, while others are more readily detectable by ELISA.

ELISA kits, which are available commercially, use indirect or competitive techniques for detecting antibodies. They differ in their mode of preparation of antigen, conjugate, or substrate, in the period of incubation and in the interpretation of the results. Their general advantage is that they enable the rapid processing of large numbers of samples. This can also be automated and the results analysed by computer. Some of these kits make it possible to differentiate between vaccinated and naturally infected animals when used with a 'matching' vaccine (Eloit et al., 1989; Van Oirschot et al., 1986). Alternatively, non-commercial ELISA protocols may be adopted (Toma & Eloit, 1986) provided they are shown to detect the WOAH International Standard Reference Serum as positive at a dilution of 1/2 (the minimum sensitivity for international trade purposes). It is recommended to use a kit or in-house assay that has been validated to this standard or a secondary standard prepared against the International Reference Standard by external quality control tests by an independent laboratory. A suitable test protocol for whole virus antibodies is presented below (Toma & Eloit, 1986).

2.2.1. Preparation of antigen

i) A cell line sensitive to SHV-1, such as PK-15 or fetal pig testis, is used. It must be free from extraneous viruses, such as bovine viral diarrhoea virus. The cells should be split and seeded into fresh 75 cm^2 flasks the day before inoculation. A suitable medium such as MEM, without serum, is used to overlay the cultures.

ii) Virus inoculated, and control uninoculated flasks are processed in parallel throughout. A suitable well characterised strain of SHV-1 is used, e.g. Kojnock strain. When a confluent cell monolayer has developed (approximately 24 hours after seeding), it is inoculated with 10^8 $TCID_{50}$ SHV-1 in 5 ml medium; and 5 ml medium (without virus) is placed in control flasks. The cultures are left for adsorption for 30 minutes at 37°C, and then overlaid with 20 ml medium.

iii) When CPE is just beginning, the supernatant medium is discarded and 4 ml KCl (4 mM solution) and glass beads are added. The flasks are shaken gently to detach cells.

iv) Cells are washed by centrifuging three times at 770 ***g*** in 4 mM KCl. The pellet is resuspended in 4 mM KCl with 0.2% Triton X-100 (1 ml per flask) by applying 60 strokes with a glass homogeniser

v) The cell homogenate is layered on to 0.25 mM sucrose in 4 mM KCl and centrifuged for 10 minutes at 770 ***g***.

vi) The pellet is resuspended in antigen-diluting buffer, pH 9.6 (0.1 M Tris, 2 mM EDTA, 0.15 mM NaCl) at 1/50 the volume of the original culture medium. It may then be stored in small aliquots at –70°C. Antigen is stable in this form for 2 years.

2.2.2. Coating microtitre plates

i) Virus antigen and control (no virus) antigen are diluted in diluting buffer, pH 9.6 (see above) to a dilution predetermined in chequerboard titrations.

ii) 200 µl of antigen is dispensed into each well of 96-well ELISA-grade plates, coating alternate rows with SHV-1 positive and control antigen. Incubation is for 18 hours at 4°C.

iii) The plates are washed three times with washing solution (Tween 20, 0.5 ml/litre).

iv) Coated plates are stored at –20°C or –70°C. They are stable for several months.

2.2.3. Test procedure

i) Test serum samples are diluted 1/30 in PBS/Tween buffer, pH 7.2 (137 mM NaCl, 9.5 mM phosphate buffer, 0.5 ml/litre Tween 20).

ii) Diluted samples are added to virus and control antigen coated wells, and incubated at 37°C for 30 minutes.

iii) The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

iv) Protein A/peroxidase conjugate is added to all wells at a predetermined dilution in PBS/Tween buffer, pH 7.2 (see above), with added bovine serum albumen fraction V (10 g/litre), and the plates are incubated at 37°C for 30 minutes.

v) The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

vi) A suitable chromogen/substrate mixture, such as tetra methyl benzidine (TMB)/hydrogen peroxide, is added to each plate.

vii) The reaction is stopped with 2 M sulphuric acid. The absorbance is read at 492 nm.

The test must be fully validated using known positive and negative sera, and calibrated against the WOAH International Standard Reference Serum. It is highly recommended to carry out a batch control for each batch of the test, to determine sensitivity and specificity in relation to the original validation criteria (criteria to accept or refuse the batch have to be set). For routine analysis, all tests must include positive and negative internal controls, including at least one weak positive sample that, when diluted at the appropriate dilution for the test, has equivalent activity to a 1/2 dilution of the WOAH International Standard Reference Serum. Internal controls are also used to monitor the sensitivity, specificity and reproducibility of the test over time. For further details see Toma & Eloit, 1986 and Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*. Commercial ELISA kits also have to be validated in the setting in which they are going to be used.

As well as testing sera, the ELISA can be adapted to test pools of sera, filter paper disks that have been moistened with a small quantity of blood obtained by puncturing a superficial vein (Banks, 1985; Toma *et al.,* 1986), or muscle exudates (Le Potier *et al.,* 1998). These techniques make it convenient to collect blood samples from large numbers of pigs (Vannier *et al.,* 2007). The disks are air-dried before shipment to the laboratory. The (analytical) sensitivity may be lower than for a standard ELISA due to the type of sample or unavoidable dilution of the sample. Use of an adapted ELISA is therefore more appropriate for testing at the population level rather than for individual testing (e.g. prior to animal movement), unless a validation study has shown a comparable (analytical) sensitivity to the standard ELISA.

Requirements for the detection of gE antibodies by ELISA in pigs destined for slaughter that are to be introduced into zones free from Aujeszky's disease have been defined by several control authorities. For example, in the European Union, ELISA gE kits must be able to detect activity at least equivalent to a 1/8 dilution of the WOAH International Standard Reference Serum (European Commission, 2008). The WOAH *Terrestrial Animal Health Code* specifies circumstances in which gE-specific tests may be used. The gE ELISAs can also be adapted to test blood on filter paper disks depending on its sensitivity.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Aujeszky's disease may be controlled by the use of vaccines containing either modified live or inactivated virus antigens. In addition, these conventional vaccines have been supplemented by recombinant DNA-derived gene-deleted or naturally deleted live SHV-1 vaccines. These vaccines, referred to as marker or DIVA-vaccines, are made with a virus that lacks a specific glycoprotein (most commonly gE-, although gG- or gC-deleted vaccines have also been described, as have vaccines with multiple deletions[1]). These gene-deleted DIVA-vaccines have the advantage over conventional whole virus vaccines that it is possible to distinguish infected animals from non-infected vaccinated animals. This is done by testing for the antibodies directed against the protein coded for by the deleted gene, which will be absent in non-infected DIVA-vaccinated pigs but present in field-infected pigs. Therefore, in countries with infected pigs, where the eradication of Aujeszky's disease is planned, these DIVA-vaccines are the vaccines of choice (Pensaert *et al*., 2004). Standards applicable to the manufacture of live and inactivated virus vaccines are described. For DIVA-vaccines, the tests should include demonstrable absence of a serological response in vaccinated pigs to the protein coded for by the deleted gene, and in addition a demonstrable response to the same protein in vaccinated pigs that become infected by field virus.

Other vaccines are inactivated and constituted of adjuvanted, viral subunit of purified and concentrated immunogenic glycoproteins (except the gE) allowing differentiation of vaccinated from infected pigs.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Vaccines are made using a seed-lot system in which a master seed virus (MSV) is prepared from a suitable strain of Aujeszky's disease virus. A number of strains are used for vaccine manufacture. The antigen in an inactivated vaccine can be one of a number of wild-type strains, or the naturally deleted Bucharest virus. Modified live conventional vaccines use numerous strains, such as Bartha or are derived from Aujeszky's original isolate or from other field isolates, such as the NIA-3 strain (Marchioli *et al*., 1987; McFerran & Dow, 1975; Van Oirschot *et al*., 1990; Visser & Lutticken, 1988).

It is recommended that for differentiating between infected and vaccinated animals, deleted strains should be used.

A virus identity test (using either a fluorescent antibody test, neutralisation test, [constant serum/decreasing virus method], or any other suitable identity test) must be conducted on the MSV.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Most of the cell lines used to propagate SHV-1 are continuous lines, such as the PK-15 line. A master cell stock (MCS) is established at a specified passage level. The MCS and the highest passage level (MCS × n) intended for use in the preparation of a biological product is specified in

1 The nomenclature for the genes changed several years ago, but the old designation is still in the literature. The old and the new nomenclature is: gII = gB; gIII = gC; gp50 = gD; gI = gE; gX = gG; gp63 = gI. Note that some commercial serological kits may still be named by the old nomenclature.

an Outline of Production. Both MCS and MCS × n are monitored by a variety of procedures to characterise the cell line and to ensure freedom from adventitious agents. The extraneous agents to be detected are generally defined in monographs or guidelines (e.g. European Pharmacopoeia, US Code of Federal Regulations, EU guidelines, etc.). In general, the type of agents to be looked for is founded on a risk analysis depending on the history of the viral strain and cells on which the vaccinal strain was isolated and on which it is cultivated. The MCS must be monitored for species of origin. A minimum of 50 mitotic cells should be examined at both the MCS and MCS × n passage levels. The modal number in the MCS × n must not exceed 15% of the modal number of the MCS. Any marker chromosomes in the MCS must also be present in the highest cell passage.

If there is evidence that the cell line may induce malignancies in the species for which the product is intended, the cell line is tested for tumorgenicity and oncogenicity.

Both the MSV and the MCS must be shown to be free from mycoplasma, bacteria, fungi, cytopathogenic or haemadsorbing viruses, porcine parvovirus, cytopathic and noncytopathic ovine and bovine pestiviruses and other extraneous agents, such as circovirus, as determined by culturing and by fluorescent antibody procedures or others, such as PCR.

2.2. Method of manufacture

2.2.1. Procedure

Only MSV that has been established as pure, safe and immunogenic may be used as seed for a vaccine product. Cells from the MCS are propagated in a variety of growth media. All batches of vaccine must be from the first to the twentieth passage of MCS.

2.2.2. In-process controls

It is necessary to carry out tests at each critical step of the manufacturing process. The control tests are also carried out on intermediate products with a view to verifying the consistency of the production process and the final product.

2.2.3. Final product batch tests

It is essential to differentiate the tests that are carried out on a routine basis to release batches of final product from those that are performed to define the biological properties of a vaccine. The trials carried out for batch release are not the same as the ones carried out once only to determine the safety and efficacy of a vaccine. The batch release controls are always short-term trials, as inexpensive as possible, and not always carried out in pigs. Their purpose is mainly to attest the reproducibility of the quality of the finished product, which has to be in compliance with the quality initially defined in the application for marketing authorisation.

i) Sterility and purity

Tests must be carried out for sterility and freedom from contamination (see chapter 1.1.9 and Section C.2.1.2 of this chapter).

Each batch of SHV-1 vaccines must be tested for freedom from extraneous viruses. Using a minimum amount of a monospecific antiserum, the live vaccinal strain is neutralised and inoculated into cell cultures known to be sensitive to viruses pathogenic for pigs. No CPE and no haemadsorbing agents should be detected. The vaccines have to be free from pestiviruses.

ii) Inactivation

For inactivated vaccines, inactivation must be checked using two passages in the same type of cell culture as used in the production of the vaccine. Tests can be carried out by vaccinating susceptible animals such as rabbits.

iii) Identity

Where necessary, a specific test for virus identification should be carried out.

iv) Safety

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures should be statistically relevant and target the smallest number of animals required for the relevant regulatory approval.

v) Batch potency

The potency of the vaccine must be demonstrated using a suitable method, the results of which have to be correlated with the efficacy tests described previously.

In this kind of test, the most difficult point is to determine an acceptability threshold for using or rejecting the batch according to the results that are obtained.

Virus content tests should be carried out using each of at least three containers. The virus titre of the vaccine must be determined and must normally not be higher than 1/10 of the dose at which the vaccine has been shown to be safe, and not lower than the minimum release titre.

vi) Preservatives

If no preservative is included in the final product, the manufacturer must demonstrate that the product remains acceptable for its recommended period of use after opening the vial.

The efficacy of preservatives in multidose containers must be demonstrated. The concentration of the preservative in the final filled vaccine and its persistence throughout shelf life must be checked.

vii) Precautions (hazards)

All information about possible adverse reactions induced by the vaccine must be indicated. Any putative risk for human health if the user is accidentally given a small quantity of the product has to be indicated. The manufacturer should indicate all the conditions of use of the vaccine: mixing, reconstitution, storage, asepsis, length of needle, route of administration and health status of the vaccinated animals.

2.2.4. Stability tests

Tests have to be carried out to verify the shelf life proposed by the manufacturer. These tests must always be real-time studies; they must be carried out on a sufficient number of batches (at least three) produced according to the described production process and on products stored in the final container, and normally include biological and physicochemical stability tests. The manufacturer has to provide the results of analyses that support the proposed shelf life under all proposed storage conditions. Usually, the proposed shelf life corresponds to the period for which the product is considered to be stable minus 3 months.

2.3. Requirements for authorisation

2.3.1. Safety requirements

Local and general reactions must be examined. When a live vaccine is used, it is necessary to differentiate the exact safety properties of the vaccinal strain from those of the finished product if this includes an adjuvant.

Objective and quantifiable criteria to detect and measure adverse reactions should be used; these would include temperature changes, weight gain, litter size, reproductive performance, etc., of vaccinated and control groups. The tests must be performed by administering the vaccine to the pigs in the recommended dose and by each recommended route of administration.

In general, safety is tested initially under experimental conditions, following the requirements of the WOAH *Terrestrial Animal Health Code,* Chapter 7.8 *Use of animals in research and education*.

When the results of these preliminary tests are known, it is necessary to increase the number of animals vaccinated in order to evaluate the safety of the vaccine under practical conditions.

i) Laboratory testing

All tests must be carried out on pigs that do not have antibodies against Aujeszky's disease virus or against a subunit of the virus.

a) General effects

1. Live vaccines

Intranasal tests and vaccination of 3- to 5-day-old piglets are very useful for ascertaining the degree of safety of a strain. At least five piglets should be used.

It is also essential to assess the properties of a vaccine, especially live ones, in the target animals under normal conditions of use and at the youngest age intended for vaccination, e.g. fattening pigs, which are generally vaccinated when they are between 9 and 12 weeks old, and pregnant sows when this use of the vaccine is claimed by the manufacturer and is authorised. No clinical signs, including significant thermal reactions (data have to be recorded before vaccination and on a schedule such as 6 hours, 24 hours and 48 hours later, then on a daily basis during the observation period), should be observed after vaccination. These assays have to be performed on at least ten vaccinated pigs, with five unvaccinated pigs as controls.

Reversion to virulence following serial passage must be examined. Primary vaccination is done by the intranasal route. Series of at least four passages in piglets are made. No fewer than two fully susceptible animals must be used for each passage.

The object of these assays is to test the genetic stability of live vaccine strains. The tests appear to be less necessary when a genetically modified live strain is concerned, especially if it is produced by gene deletion.

It is recommended to test for possible excretion of the vaccine strain. For this purpose, no fewer than 14 piglets, 3–4 weeks old each receive one dose of vaccine by the recommended route and at the recommended site (except for vaccines administered by the intranasal route). Four unvaccinated piglets are kept as controls. Suitably sensitive tests for the virus are carried out individually on the nasal and/or oral secretions of vaccinated and in-contact pigs as follows: nasal and oral swabs are collected daily from 1 day before vaccination to 10 days after vaccination. Vaccine strains that are isolated from the nasal/oral secretion collected from pigs in which the vaccine was administered by the parenteral route are not recommended for eradication purposes.

The ability of the Aujeszky's disease vaccine strain to spread from vaccinated pigs to unvaccinated ones (lateral spread) must be tested by using the recommended route of administration that presents the greatest risk of spread (except for vaccines administered by the intranasal route). A repetition of the assays (four times) is necessary as this phenomenon is difficult to detect. Four piglets should be used each time for vaccination and placed in contact, 1 day later, with two unvaccinated piglets. It may also be necessary to examine the spread of the strain to nontarget species that may be susceptible to the vaccine strain.

Live attenuated vaccine strains are tested with regard to their general effects by administering to 5- to 10-day-old piglets ten times the field dose. This administration of an overdose makes it possible to detect reactions not produced under normal conditions of use. Such reactions may be produced inadvertently when large numbers of animals are vaccinated. If vaccines are administered by the intranasal route, the manufacturer has to indicate clearly that the vaccine will spread from vaccinated pigs to unvaccinated ones.

2. Inactivated vaccines

It is essential to test inactivated vaccines in the target animals under normal conditions of use for fattening pigs and for sows when this use is claimed by the manufacturer and authorised (European Pharmacopoeia, 2008; Vannier *et al*., 2007). As described previously, it is fundamental to use objective and quantifiable criteria to detect and to measure adverse reactions, such as temperature changes, weight performance, litter size, reproductive performance, etc., on vaccinated and control groups. The tests must be performed by administering the vaccine in the recommended dose and by each recommended route of administration to the pigs for which it is intended.

Pigs or sows are usually observed until there is no further evidence of vaccine reaction. The period of observation must not be fewer than 14 days from the day of administration. This period has to be extended when, for example, the vaccine is used in pregnant sows and it is necessary to assess the possible effects of the vaccine on reproductive performance. In this case, the period of observation lasts the full duration of the pregnancy.

Control authorities generally request vaccination with a double dose so that adverse reactions, which may be at the limit of detection when a single dose is administered, are more likely to be detected.

b) Local reactions

Local reactions are often associated with the use of inactivated vaccines, as these side-effects can be induced by adjuvants, particularly oil adjuvants. However, some Aujeszky's disease live vaccines are mixed with different adjuvants, which modify what has been observed in the past.

Local reactions are mainly inflammatory and can be more or less complicated (necrotic or suppurative), depending on the nature of the adjuvants used and the aseptic conditions of the vaccination. Oil adjuvants can induce a variety of effects including muscular degeneration, granuloma, fibrosis and abscessation. In addition to the nature of the oil used (the intensity of the reaction is reduced when metabolisable oils are used in the vaccine), the type of emulsion used (water/oil, oil/water, water/oil/water) induces these reactions to a greater or lesser extent. In consequence, it is necessary to observe the site of injection not only from the outside, but also by dissection after slaughter, especially for growing and finishing pigs.

ii) Field testing

Field trials are necessary to assess the safety of an Aujeszky's disease vaccine in a large number of pigs or sows. In Europe (European Pharmacopoeia, 2008), tests must be carried out in each category of animals for which the vaccine is intended (sows, fattening pigs). At least three groups of no fewer than 20 animals each are used with corresponding groups of no fewer than 10 controls. The rectal temperature of each animal is measured at the time of vaccination, 6, 24 and 48 hours later. At slaughter, the injection site must be examined for local reactions. If the vaccine is intended to be used in sows, reproductive performances have to be recorded. Field trials are supplemented by laboratory studies of efficacy correlated to vaccine potency.

2.3.2. Efficacy requirements

i) Laboratory trials

All tests must be carried out on pigs that do not have antibodies against Aujeszky's disease virus or against a subunit of the virus, except that some tests may be done using maternally immune animals.

a) Assessment of passive immunity

To test the efficacy of vaccines, it is important to mimic the natural infection conditions (European Commission, 2008). SHV-1 infection gives rise to important losses of young piglets from nonimmune sows. Thus, when vaccinating sows, the main goal is to protect the young piglets through passive immunity conferred by the colostrum ingested immediately after birth, with the secondary objective of preventing abortion.

To measure this passive immunity and the protection induced by vaccinating the sows, experimental models have been established. The sows are vaccinated according to the vaccinal protocol during pregnancy. When the piglets are, for example, 6–10 days old they are given an intranasal challenge exposure with a virulent SHV-1 strain. It is preferable to use a strain titrated in median lethal doses (LD_{50}). Pigs should be inoculated by the nasal route, 10^2 LD_{50} per pig in 1 ml. The efficacy of the vaccine is assessed by comparing clinical signs, but also and more importantly, mortality, or humane euthanasia, in piglets from unvaccinated dams with that observed in piglets from vaccinated sows.

Piglets from vaccinated sows can be found to have 80% protection against mortality compared with those from the control sows. In order for the results to be significant, it is recommended that eight vaccinated sows and four control sows be used (subject to satisfactory numbers of piglets from each sow).

b) Assessment of active immunity

1. Clinical protection

   Several criteria can be considered when measuring active immunity induced by vaccinating pigs. Generally, pigs are vaccinated at the beginning of the growing period, i.e. when they are between 9 and 12 weeks old. Laboratory trials are performed by challenging pigs at the end of the finishing period, when they weigh between 80 and 90 kg.

   In general, at least three criteria, such as rectal temperature, weight loss and clinical signs, along with mortality, are used to measure the clinical protection of pigs after vaccination and challenge (De Leeuw & Van Oirschot, 1985). The antibody titres have little predictive value for the efficacy of the vaccines. Weight loss compared between the vaccinated and control groups is the most reproducible and reliable parameter when the challenge conditions are well standardised. The measure of the difference in weight gain or loss between the two groups of pigs and, in the interval of time between challenge (day 0 and day 7), has a very good predictive value for the efficacy of the vaccines (Stellmann *et al*., 1989). Significant results can be obtained when weight performances are compared between one group of at least eight vaccinated pigs and another group of eight unvaccinated control pigs

   For challenge, it is usually preferable to use a high titre of a virulent strain, as this makes it possible to obtain a more marked difference between vaccinated and control pigs. On the basis of previous work, a challenge dose with at least 10^6 $TCID_{50}$/ml virulent strain having undergone not more than three passages on primary cells can be sufficient, but a higher titre ($10^{7.5}$ $TCID_{50}$/ml) is recommended. The oro-nasal route should be used to challenge the pigs by introducing the virulent strain in an appropriately high volume (≥4 ml).

   This method of evaluating the efficacy of SHV-1 vaccines is now well tested and has made it possible to establish an objective index for determining the efficacy of a vaccine. This index, which compares the relative weight losses between vaccinated and control pigs, can also be used for potency testing batches before release and for batch efficacy testing. However, the value of the cut-off index will be different as the conditions of the assay will not be identical. The influence of passively acquired, maternally derived antibodies on the efficacy of a vaccine must be evaluated adequately.

2. Virulent virus excretion

Additionally, it is desirable that vaccines should prevent or at least limit viral excretion from infected pigs (Vannier *et al*., 1991). When a control programme against Aujeszky's disease is based on large-scale vaccination, it is essential to choose the vaccines or the vaccinal scheme that best limits the replication of virulent virus in infected pigs. Several assays have been performed to compare vaccines on that basis.

Generally, the pigs are vaccinated and challenged at different periods. It is better, but more time-consuming, to infect pigs at the end of the finishing period. To measure the virus excretion, nasal swabs (taken at 10 cm depth in the nostrils) are taken daily from each pig from the day before challenge to at least 12 days after challenge. The swabs can be weighed before the sampling and immediately after to calculate the exact weight of collected mucus. Medium is then added to each tube containing a swab. The virus is titrated from the frozen and thawed medium.

Different indexes can be used to express the quantity of virulent virus excreted by pigs, taking into consideration the duration and the level of viral excretion, and the number of pigs excreting virulent virus.

3. Duration of immunity

It is recommended that any claims regarding the onset and duration of immunity should be supported by data from trials. Assessment of duration of immunity can be based on challenge trials or, as far as it is possible, on immunological and serological tests.

ii) Field trials

In general terms, it is extremely difficult to assess vaccine efficacy in animal populations. In order to do this, it would be necessary to vaccinate the animals in the absence of the pathogen that the vaccine protects against, then to await the moment of infection and to compare the effects of infection in vaccinated animals (or the offspring of vaccinated dams) with the effects in the unvaccinated animals of the same age, in the same building and in the same batch as the vaccinated animals (or those protected passively). As all these conditions are difficult to achieve in the field, field trials are certainly more appropriate to safety testing than to efficacy testing, except for the development of DIVA-vaccines that offer the opportunity to evaluate the effectiveness of vaccines under field conditions (Bouma, 2005).

2.3.3. Stability

Tests have to be carried out to verify the shelf life proposed by the manufacturer. These tests must always be real-time studies; they must be carried out on a sufficient number of batches (at least three) produced according to the described production process and on products stored in the final container, and normally include biological and physicochemical stability tests. The manufacturer has to provide the results of analyses that support the proposed shelf life under all proposed storage conditions. Usually, the proposed shelf life corresponds to the period for which the product is considered to be stable minus 3 months.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Biotechnology combined with a better knowledge of the functions and characteristics of the SHV-1 glycoproteins helped to develop new vaccines. For example, Quint *et al.* (1987) deleted glycoprotein E-coding sequence from the NIA3 strain. This resulted in an efficient DIVA-vaccine against Aujeszky's disease, allowing differentiation of vaccinated from infected animals (DIVA vaccines). Most of the vaccines used at the moment are obtained from recombinant DNA-derived gene-deleted virus. The deletion of the genes coding for the glycoprotein E is the most commonly used, allowing an attenuated live virus vaccine to be obtained but still protecting against the clinical signs and reducing significantly

the level of the viral excretion by the pigs vaccinated and infected. Because of the ability of some glycoproteins of SHV-1 to induce strong immune responses, efficiencies of DNA vaccines, consisting of plasmids encoding these glycoproteins, were tested. Indeed, DNA vaccination has a number of advantages: ease of construction and standardised production of plasmids, no handling of infectious particles, induction of humoral and cellular immune responses, bypass of the maternal derived immunity. The pioneering study on DNA vaccination against Aujeszky's disease infection was published in 1997 (Gerdts *et al.,* 1997). The use of a novel generation of plasmid amplifying the level of gene transcription of the proteins of interest (Dory *et al.,* 2005) have been shown to be efficient strategies. These vaccines are not yet commercialised.

3.2. Special requirements for biotechnological vaccines, if any

Criteria to assess quality, safety and efficacy of the vaccines derived from the biotechnology are the same as the ones defined for conventional vaccines (see section C.2). Nevertheless special attention has to be paid to the stability of the recombinant DNA construction.

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NB: There are WOAH Reference Laboratories for Aujeszky's disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Aujeszky's disease

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.1.3.

BLUETONGUE (INFECTION WITH BLUETONGUE VIRUS)

SUMMARY

Bluetongue (BT) is an infectious, primarily vector-borne viral disease that affects wild and domestic ruminants such as sheep, goats, cattle, buffaloes, deer and most species of African antelope, and camelids as vertebrate hosts. The virus is primarily transmitted between susceptible vertebrate hosts by competent species of Culicoides *midges, and the global distribution of the disease is largely determined by the distribution of competent vectors. Infection with bluetongue virus (BTV) is inapparent in the vast majority of animals but can cause fatal disease in a proportion of infected sheep, deer and wild ruminants. Infection of cattle is usually subclinical, with the exception of BTV-8 infection. Cattle are particularly significant in the epidemiology of the disease due to the prolonged viraemia that occurs following infection. When apparent, clinical signs of BT are mainly attributable to an increase in vascular permeability and include fever, hyperaemia and congestion, facial oedema and haemorrhages, erosion of the mucous membranes, coronitis and laminitis, and pleural and pericardial haemorrhages.*

Detection of the agent: *BTV is the type member of the* Orbivirus *genus of the family* Reoviridae*. There are 27 recognised BTV serotypes and several recently isolated but as yet unclassified unique 'atypical' strains. Virus serotype identification traditionally requires isolation and amplification of the virus in embryonated chicken eggs,* Culicoides *cells or other tissue culture and the subsequent application of serogroup- and serotype-specific tests including virus neutralisation assays. The reverse-transcription polymerase chain reaction (RT-PCR)* assay *has permitted rapid amplification of BTV cDNA in clinical samples and well validated real-time RT-PCR-based systems for virus detection are now routine, allowing for more rapid and sensitive diagnostic testing. The combination of RT-PCR amplification and capillary sequencing or whole genome sequencing now offer relatively rapid unequivocal agent identification at the genome level. Collectively, these procedures augment the classical virological techniques to provide information on virus serogroup, serotype and topotype.*

Serological tests: *Serological responses appear 7–14 days after BTV infection. Infected animals produce both neutralising and non-neutralising anti-BTV antibodies that are generally long-lasting. Enzyme-linked immunosorbent assay (ELISA) and virus neutralisation (VN) are the most frequently used serological tests. A monoclonal antibody-based competitive ELISA to specifically detect anti-BTV (serogroup-specific) antibodies is the test recommended to certify animals as free from infection prior to movement; the test is highly sensitive and specific (compared with the agar gel immunodiffusion), quick, inexpensive and reliable. However, the ELISA may have reduced sensitivity for some serotypes. Procedures to identify and quantify BTV serotype-specific antibodies are more complex, being typically based on cell culture- and live virus-dependent neutralisation tests.*

Requirements for vaccines: *Vaccination is the preferred method of BT control in endemic regions. Vaccination has been used successfully to limit direct losses, minimise the circulation of BTV, eradicate BTV from country/regions and allow safe movement of animals. However, use of live vaccines can be associated with adverse outcomes and live attenuated vaccine strains can be spread by vectors, with eventual reversion to virulence or reassortment of vaccine virus genes with those of wild-type virus strains. Inactivated vaccines are safer, but require multiple doses to become efficacious and incur higher costs. Such vaccines have been very effective in combatting the spread of BTV-8, BTV-1 and BTV-4 in Europe.*

A. INTRODUCTION

1. Description of the disease

Bluetongue (BT) is an infectious, primarily vector-borne viral disease that affects wild and domestic ruminants. Midges of an increasing number and geographical range of species (Carpenter *et al*., 2015) in the genus *Culicoides* (the insect host) typically transmit bluetongue virus (BTV) among susceptible ruminants, having become infected by feeding on viraemic animals (the vertebrate host). Other routes of transmission have been documented including direct vertical, oral and possibly venereal transmission and indirect transmission through reused needles; however the epidemiological significance of these routes remains uncertain (Belbis *et al*., 2017; Darpel *et al*., 2016; Kirkland *et al*., 2004). The more recently recognised serotypes, such as BTV-25, BTV-26 and BTV-27 appear to be transmitted exclusively by these vector-independent routes, and may result in persistent infection in goats (Belbis *et al*., 2017; MacLachlan *et al*., 2015; Vogtlin *et al.,* 2013).

The global distribution of BTV is determined by epidemiological systems (episystems) that are in turn primarily delimited by specific vector species, the presence of susceptible host species and their collective natural history. Observations in Europe, India and the USA indicate that strains of BTV can move between episystems through movement of animals, wind dispersion of infected vectors and by adaptations of both vector species and virus (Carpenter *et al*., 2015; Jacquot *et al*., 2019; Maan *et al*., 2015). The expanding global range of BTV infection has been notable, particularly at the traditional northern- and southern-most limits of BTV distribution with incursions of multiple serotypes into Europe, Australia, both North and South America, and Asia, in countries that had not previously reported BTV infections (MacLachlan *et al*., 2015; MacLachlan & Mayo, 2013). In addition to detecting and confirming an increasing number of serotypes, developing sequencing technologies, phenotyping software and molecular assays have revealed that two major ancestral lineages, a Western (Africa, Europe, the Americas) and an Eastern (Australia, and Asia) exist globally (Maan *et al*., 2015; Mertens *et al*., 2007).

In temperate parts of the world, infection has a seasonal occurrence (Verwoerd & Erasmus, 2004), whereas in tropical regions BTV infection can occur year-round (MacLachlan *et al*., 2015). Survival of BTV in the environment is associated with insect factors such as over-wintering (Mayo *et al*., 2016). Additionally, long-term persistence of BTV in a region only occurs when multiple virus serotypes circulate (MacLachlan *et al*., 2015). The ability of the more recently detected BTV serotypes 25, 26 and 27 to be transmitted in the absence of vector insects reduces the likelihood of seasonality in their occurrence or dependence on the geographical distribution of vector species for transmission.

The vertebrate hosts for BTV include both domestic and wild ruminants, such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and other *Artiodactyla* such as camels. Although detection of antibodies to BTV antigen or viral nucleic acid or live virus has been demonstrated in some carnivores, black and white rhinoceroses and elephants, the role of non-ruminant species in BTV epidemiology is considered minimal. The outcome of infection ranges from subclinical in the vast majority of infected animals, especially wild African ruminants, cattle and goats, to serious or fatal in a proportion of infected sheep, goats, deer and some wild ruminants (Verwoerd & Erasmus, 2004). Infection in cattle and goats is typically subclinical and these species are considered to be amplifying reservoir hosts in endemic regions, making control measures for BTV in those animals important. However, a higher incidence and severity of clinical disease has been observed in naïve cattle infected with BTV-8. As breeds of sheep have varying levels of susceptibility to disease, BTV infections of livestock can occur unobserved and be detected only by active surveillance (Daniels *et al*., 2004).

Clinical signs of disease in sheep vary markedly in severity, influenced by the type or strain of the infecting virus, husbandry factors and animal breed (Verwoerd & Erasmus, 2004). In severe cases there is an acute febrile response characterised by hyperaemia and congestion, leading to oedema of the face, eyelids and ears, and haemorrhages and erosions of the mucous membranes. The tongue may show intense hyperaemia and become oedematous, protrude from the mouth and in severe cases become cyanotic. Hyperaemia may extend to other parts of the body particularly the coronary band of the hoof, the groin, axilla and perineum (MacLachlan & Mayo, 2013). Sheep that develop chronic disease often have severe muscle degeneration, and breaks in the wool may occur associated with pathology in the follicles. A reluctance to move is common and torticollis may occur in severe cases (Maclachlan *et al.,* 2009). In fatal cases, the lungs may show interalveolar hyperaemia, severe alveolar oedema and the bronchial tree may be filled with froth. The thoracic cavity and pericardial sac may contain varying quantities of plasma-like fluid. Most cases show a distinctive haemorrhage near the base of the pulmonary artery (Verwoerd & Erasmus, 2004). In cases of vertical transmission of BTV-8, the central nervous system may be severely affected resulting in a failure of the cerebral hemispheres to develop (dummy calf syndrome). With virulent strains, infection in naïve sheep can result in mortality rates as high as 70%.

Control of BTV in animals is covered in Chapter 8.3 of the WOAH *Terrestrial Animal Health Code*. Because of the high percentage of subclinical infections, persistent viraemia, and challenges with vector control, traditional methods of control and eradication of BTV, such as movement controls, stamping out and vector control have not always been successful in controlling the disease. Thus, a safe and efficacious vaccine that meets the objectives of control, eradication and prevention requirements is an important component of disease control in many settings (MacLachlan & Mayo, 2013).

2. Nature and classification of the pathogen

Taxonomically, BTV is classified as the type species in the *Orbivirus* genus in the family *Reoviridae*, one of 22 recognised species in the genus that also includes epizootic haemorrhagic disease virus (EHDV), equine encephalosis virus and African horse sickness virus. There is significant immunological cross-reactivity among members of the BTV serogroup. Within species, individual BTV are differentiated on the basis of genotype and neutralisation tests; currently 27 serotypes of BTV are recognised including Toggenburg Orbivirus (BTV-25), BTV-26 from Kuwait and BTV-27 from Corsica. These most recently detected serotypes and several other as yet serotypically unclassified unique BTV strains (Belbis *et al*., 2017) may exhibit quite distinct modes of transmission associated with their respective abilities to infect *Culicoides* spp. and specific ruminant hosts (Breard *et al*., 2018, Chaignat *et al*., 2009, Maan *et al*., 2015; Savini, 2015). Both genetic shift and genetic drift account for the diversity and heterogenicity of the BTV strains in the field (Pritchard *et al*., 2004).

BTV particles are composed of three protein layers. The outer capsid layer contains two proteins, VP2 and VP5. VP2 is the major neutralising antigen and determinant of serotype specificity. Removal of the outer VP2/VP5 layer leaves a bi-layered icosahedral core particle that comprises an outer layer composed entirely of capsomeres of VP7 and a complete inner capsid shell (the subcore layer) comprising VP3, which surrounds 10 dsRNA genome segments and minor structural proteins (VP1, VP4 and VP6). VP7 is a major determinant of serogroup specificity and the site of epitopes used in competitive enzyme-linked immunosorbent assays (C-ELISAs) to detect anti-BTV antibodies (Mertens *et al*., 2005). VP7 can also mediate attachment of BTV to insect cells.

Genetic sequencing of specific BTV genome segments and movement towards full genome analyses allows for additional differentiation and analysis of strains apart from serotyping, with potential to identify multiple different clades for each genome segment[1] (Gould, 1987; Jacquot *et al*., 2019). Even for strains within one serotype it is therefore possible to identify the likely geographical origin (topotype) for each genome segment (Gould, 1987). Identification of apparent associations between some genotypes of virus and some vector species has led to further development of the concept of viral-vector episystems (Daniels *et al*., 2004). Movements of several BTV serotypes between vector species and into new geographical regions, leading to multiple reassortment events and the emergence of novel strains containing new combinations of genome segments from different origins (Jacquot *et al.,* 2019; Nomikou *et al.,* 2015), indicate that a more complete identification of the genetic makeup of each strain would contribute to a better understanding of BTV epidemiology.

3. Zoonotic potential and biosafety and biosecurity requirements

There is no known risk of human infection with BTV. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

4. Differential diagnosis

Depending on the clinical presentation, affected species and epidemiological data, differential diagnoses for suspected cases of BTV infection may include other infectious diseases including but not limited to epizootic haemorrhagic disease, foot and mouth disease, peste des petits ruminants, sheep and goat pox, vesicular stomatitis and contagious ecthyma. In cases of dummy calf syndrome, bovine viral diarrhoea virus should be taken into consideration. Non-infectious causes, including photosensitisation, should also be considered.

1 See BTV-Glue database http://btv-glue.cvr.gla.ac.uk

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of bluetongue and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Real-time RT-PCR	–	+++	–	+++	++	–
RT-PCR	–	+++	–	+++	++	–
Classical virus isolation	–	+++	–	+++	–	–
Detection of immune response						
C-ELISA (serogroup specific)	+++	+++	++	–	+++	++
VN (serotype specific)	+	+	++	+	++	++
AGID	+	+	+	–	+	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; C-ELISA = competitive enzyme-linked immunosorbent assay;
VN = virus neutralisation; AGID = agar gel immunodiffusion
[a]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the BTV agent

1.1. *In-vitro* and *in-vivo* cultures

Specimens for virus isolation can include unclotted blood (heparin or EDTA [ethylene diamine tetra-acetic acid]-treated whole blood) from suspected viraemic animals, blood clots after separation of serum, spleen or lymph nodes collected at necropsy of clinical cases, or midges. Several virus isolation systems for BTV are in common use, including inoculation of embryonated chicken eggs (ECE) and primary inoculation of cell cultures such as the KC cell line (a cell line derived from *C. sonorensis* midges). The KC cell line has been proven to be very sensitive (McHolland & Mecham, 2003) and from an animal ethics perspective reduces the use of embryonated eggs. Attempts to isolate virus *in vitro* in mammalian cell culture systems may be considered more convenient, however the success rate is frequently much lower than that achieved with embryonated chicken egg and KC cell systems. The same diagnostic procedures are used for domestic and wild ruminants. Inoculation of sheep continues to be used for *in-vivo* testing and amplification of virus but should be avoided wherever possible in accordance with chapter 7.8 of the *Terrestrial Animal Health Code*.

1.1.1. Isolation in embryonated chicken eggs

i) Blood is collected from suspected viraemic animals into an anticoagulant such as EDTA, heparin or sodium citrate, and the blood cells are washed three times with sterile phosphate-buffered saline (PBS). Washed cells are re-suspended in PBS or isotonic sodium chloride

and either stored at 4°C or used immediately for attempted virus isolation. Tissue and midge suspension can be also prepared and stored as described above or immediately used.

ii) For long-term storage at 4°C or where refrigeration is not possible for short periods of time, blood samples are collected in oxalate–phenol–glycerine. Washed red blood cells and tissue homogenates can be stored directly at –70°C. The virus is not stable at –20°C. BTV has remained viable for several months or even years in whole blood in anticoagulant stored at 4°C.

iii) In fatal cases, spleen and lymph nodes are the preferred organs for virus isolation attempts. Organs and tissues should be kept and transported at 4°C to a laboratory where they are homogenised in PBS or isotonic saline (1/10), centrifuged at 1500 rpm for 10 minutes, and filtered (0.2–0.4 μm). The tissue suspensions can be used as described below for blood cells.

iv) Washed blood cells are re-suspended 1/1 in distilled water (to disrupt whole red blood cells) then diluted to 1/10 in PBS to ensure isotonic balance. 0.1 ml amounts are inoculated intravascularly into 5–12 ECE that are 9–12 days old. This procedure requires skill and practice. Details are provided by Clavijo *et al*. (2000).

v) The eggs are incubated in a humid chamber at 32–33.5°C and candled daily. Any embryo deaths within the first 24 hours post-inoculation are regarded as nonspecific.

vi) Embryos that die between days 2 and 7 and embryos remaining alive at 7 days are retained at 4°C overnight before harvesting. Infected embryos may have a haemorrhagic appearance. Dead embryos and those that live to 7 days are homogenised as two separate pools. Whole embryos, after removal of their heads, or pooled organs such as the liver, heart, spleen, lungs and kidney, are homogenised and the debris removed by centrifugation at 1500 ***g*** for 10 minutes.

vii) Virus in the supernatant may be identified either directly as described in Section B.1.2 below or after further amplification in cell culture, as described in Section B.1.1.2.

1.1.2. Isolation in cell culture

Virus isolation is best achieved by primary isolation (or amplification) of virus in ECE, followed by a passage in the *Aedes albopictus* (AA) clone C6/36 insect cell culture or primary isolation in cells derived from *Culicoides sonorensis* (free of BT viruses) and designated as KC or CuVa cells (McHolland & Mecham, 2003). These two amplification steps are then followed by a passage in mammalian cell lines such as baby hamster kidney (BHK 21) or African green monkey kidney (Vero). Passages in BHK 21 and Vero will enable further replication of virus and visual confirmation of virus isolation by cytopathic effect (CPE).

There have been occasions where CPE is not seen in a mammalian cell line, but BTV antigen has been visualised when staining by direct immunofluorescence. Cell monolayers are incubated at 37°C in 5% CO_2 with humidity and monitored for the appearance of CPE after 5–7 days. If no CPE appears, a second passage is made in the mammalian cell culture.

Positive CPE or immunofluorescence detection or negative cell culture results for BTV must be confirmed after each ECE, KC or cell culture passage by antigen detection ELISA or polymerase chain reaction (PCR) techniques.

1.2. Virus detection and characterisation

The success of virus isolation techniques is assessed by testing for the presence of BTV in the cell culture supernatants or embryo tissues using a variety of detection systems. Virus detection systems include antigen-capture ELISA, direct immunofluorescence, reverse-transcription PCR (RT-PCR) or real-time RT-PCR, as described in Section B.1.3 below. Currently, testing of the isolation media by real-time RT-PCR is the preferred screening method.

Detection and characterisation are typically step-wise processes, with serogroup-specific tests used initially to detect the presence of a BTV. Subsequent genotype and serotype identification of BTV isolates provides valuable epidemiological information and is critical for the implementation of vaccines or for vaccine development. RT-PCR assays employing serotype-specific primers will provide the most rapid

and specific information regarding isolate serotype (Mertens *et al.,* 2007). Genotyping for molecular epidemiology can be based on RT-PCR tests and sequencing of the amplicon. Different laboratories have standardised several different gene sequences for this purpose. Where available, full genome sequencing may also be performed to provide serotype, as well as other unique sequence information of isolates.

Neutralisation procedures using individual serotype antisera may also be employed for serotyping, although some serotypes are cross-reactive and interpretation can be difficult. For laboratories without serotyping capabilities, BTV isolates may be submitted to any WOAH BT Reference Laboratory for serotyping of isolates.

1.2.2. Immunological serogrouping of viruses

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP7, that are conserved within each serogroup. The cross-reactivity between members of the BT and EHD serogroups raises the possibility that an isolate of EHDV could be mistaken for BTV on the basis of a weak immunofluorescence reaction with a polyclonal anti-BTV antiserum. Polyclonal and monoclonal antibodies (MAbs) used for serogrouping BTV isolates must be characterised as appropriate for the purpose. There exists significant VP7 variation within BTV, as well as antigenic relatedness between other closely related orbiviruses, such as EHDV, that will influence antibodies binding in different assay formats (IFA, ELISA, AGID). For this reason, a BT serogroup-specific MAb can be used. A number of laboratories have generated such serogroup-specific reagents. Commonly used methods for the identification of viruses to serogroup level are as follows.

i) *Immunofluorescence/immunoperoxidase staining*

Monolayers of BHK or Vero cells in various tissue culture substrates (including chamber slides, glass cover-slips, 96 or 24 well plates or other suitable formats) are infected with either tissue culture-adapted virus or virus in ECE lysates. After 24–48 hours at 37°C, or after the appearance of a mild CPE, infected cells are fixed with agents such as paraformaldehyde, acetone or methanol, dried and viral antigen detected using anti-BTV antiserum or BTV-specific MAbs and standard immunofluorescent procedures. Standard immunoperoxidase staining procedures can also be used when fluorescent microscopes are not available for reading immunofluorescence, as stained antigen can be read by eye or using light microscope at 100× magnification.

ii) *Antigen capture enzyme-linked immunosorbent assay*

Viral antigen in ECE, culture medium harvests and infected insects may be detected directly using an antigen-capture ELISA (Ag ELISA). In this technique, virus derived proteins are captured by antibody adsorbed to an ELISA plate and bound materials detected using a second antibody. The capture antibody may be polyclonal or a serogroup-specific MAb. Serogroup-specific MAbs and polyclonal antibody raised to VP7 proteins or whole virus have been used successfully to detect captured virus. Antigen detection from whole blood is not always successful using the Ag ELISA.

1.2.2. Serotyping of isolates by virus neutralisation

Neutralisation tests are type specific for the currently recognised BTV serotypes that have been isolated in culture. Reference antibodies can be used to serotype a virus. In the case of an untyped isolate, the characteristic regional localisation of BTV serotypes can generally obviate the need to attempt neutralisation by antisera to all isolated serotypes, particularly when endemic serotypes are well known.

There is a variety of tissue culture-based methods available to aid in typing isolates. Cell lines commonly used are BHK, Vero and L929. Three methods to serotype BTV isolates are outlined briefly below. For antibody typing virus neutralisation methods, see Section B.2. below. There is also a fluorescence inhibition test, not described. BTV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included.

i) *Plaque reduction*

The virus to be serotyped is diluted to contain approximately 100 plaque-forming units (PFU) and incubated with either no antiserum (virus control) or with serial dilutions of individual standard antisera to a panel of BTV serotypes. Virus/antiserum mixtures are added to monolayers of cells. After adsorption and removal of inoculum, monolayers are overlaid with agarose or carboxy-methyl cellulose (medium viscosity) for easy removal of semi-solid agar before staining. The neutralising antibody titres are determined as the reciprocal of the serum dilution that causes a fixed reduction (e.g. 80%) in the number of PFU. The unidentified virus is considered serologically identical to a standard serotype if the latter is run in parallel with the untyped virus in the test and is similarly neutralised.

ii) *Plaque inhibition*

Tests are performed in 90 mm diameter Petri dishes containing confluent cell monolayers that are infected with approximately 5×10^4 PFU standard or untyped virus. After adsorption and removal of inoculum, monolayers are overlaid with agarose. Standard anti-BTV antisera are added to individual filter paper discs and placed on the agarose surface. Dishes are incubated for at least 4 days. A zone of virus neutralisation, with concomitant survival of the cell monolayer, will surround the disc containing the homologous antiserum.

iii) *Microtitre neutralisation*

Approximately 100 $TCID_{50}$ (50% tissue culture infective dose) of the standard or untyped virus is added in 50 µl volumes to test wells of a flat-bottomed microtitre plate and mixed with an equal volume of standard antiserum serially diluted in tissue culture medium. Approximately 10^4 cells are added per well in a volume of 100 µl, and plates incubated for up to 7 days, depending on the level of CPE observed in negative serum wells of the test. When CPE has developed to 4+ (100%) in the negative control serum wells of each serotype tested, the test is read using an inverted microscope. Wells are scored for the degree of CPE observed. Those wells that contain cells only or cells and antiserum, should show no CPE. In contrast, wells containing cells and virus should show 75–100% CPE. The unidentified virus is considered serologically identical to a standard BTV serotype if both are neutralised in the test to a similar extent.

1.3. Molecular methods

1.3.1. Detection of nucleic acid

RT-PCR techniques provide rapid identification of BT viral nucleic acid in blood and other tissues of infected animals. Importantly, RT-PCR-based diagnostics should be interpreted with caution because the RT-PCR procedure will detect virus-specific nucleic acid after the virus is no longer viable and capable of establishing a new infection in either insects or mammalian hosts. Hence a positive RT-PCR result, does not necessarily indicate the presence of infectious virus. In addition, RNA originating from vaccine strains can be detected.

Multiple RT-PCR formats are available that can be used to detect BTV specifically to 'serogroup' Orbiviruses and to 'serotype' BTV. These molecular approaches are much more rapid than traditional virological and immunological approaches, which may require up to 4 weeks to generate information on serogroup and serotype. Developments in molecular assays, new sequencing technologies and phenotyping software have identified increasing numbers of new serotypes, methods of transmission, and host-specific susceptibility patterns.

The nucleic acid sequence of cognate BTV genes may differ depending on the geographical area of virus isolation (Gould, 1987; Maan *et al.,* 2015). This has provided a unique opportunity to complement studies of BTV epidemiology by providing information on the potential geographical origin of virus isolates, a process termed genotyping or topotyping. While sequencing of BTV isolates from different parts of the world may allow detection of various clades for each genome segment, which may permit finer discrimination of geographical origin, the relationship between sequence and geographical origin is not absolute in all cases. Thus, BTV sequencing information is vitally important and all data regarding BTV segment sequences should be made widely available by submitting appropriately validated data to reputable sequence databases such as

GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The BTV-Glue website (http://btv-glue.cvr.gla.ac.uk) can provide phylogenetic tree analyses of BTV isolates based on the sequence of RNA segments. These compiled data will provide a resource for epidemiological studies, the identification of new isolates, as well as supporting *in-silico* analyses for maintenance and validation of existing RT-PCR assays and the design of new primers probes for development of additional assays, e.g. for novel/variant BTV types/strains.

The capacity of RT-PCR assays to detect very small numbers of nucleic acid molecules means that such tests are exquisitely sensitive to contamination by extraneous nucleic acids leading to false positive results. False negatives, due for example to poor nucleic acid preparation or inappropriate primers, may also be encountered. This is covered in detail in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*.

There are many RT-PCR assays currently in use that use different extraction methods, reverse transcriptases, amplification enzymes, primers and conditions. Technology is changing rapidly, and the genetic diversity of the BTV genes makes the choice and validation of RT-PCR assays conditional on its application in a regional setting. Therefore, the procedures listed below are examples only.

Two BTV RT-PCR assays are presented here: a real-time assay (Hofmann *et al*., 2008), targeting the NS3 gene segment and a conventional nested assay targeting the NS1 gene segment, using primers designed by Katz *et al*. (1993). The nested assay has been successfully used for over 20 years and can detect serotypes 1–24 and 26 (there are no reports of testing of other serotypes from multiple species. The nested assay may be beneficial for laboratories without the capacity to perform real-time RT-PCR. The real-time RT-PCR assay presented below has been tested at several laboratories world-wide and has been found capable of detecting all 27 serotypes of BTV (as well as other recently detected novel BTV strains), and equals or surpasses the sensitivity of the nested assay while providing rapid, quantitative detection of BTV without the contamination risks associated with nested RT-PCR assays.

1.3.1.1. Real-time reverse-transcription polymerase chain reaction

Real-time RT-PCR methods provide sensitive and rapid detection of BTV in a one-step procedure. Advantages of real-time methods over traditional PCR methods include rapidity of testing, quantitation of the virus present, and the reduced opportunity for contamination to occur as no post-amplification handling such as gel electrophoresis is needed. The real-time RT-PCR assays are the tests of choice for diagnosis.

The method presented here is an adaption from Hofmann *et al.* (2008) and is capable of detecting all known BTV serotypes and strains currently circulating. The assay targets BTV segment 10 (NS3). The procedure given may require modification to accommodate individual laboratory or different RT-PCR kit requirements.

i) RNA extraction from blood, tissue samples, and midges

Commercial kits are widely available; the RNA extraction step can be performed according to the procedures specified in each kit.

ii) Real-time reverse-transcription polymerase chain reaction

Kits for the one-step real-time PCR are available commercially. Below are some basic steps as described by Hofmann *et al*. (2008), which can be modified depending upon local/case-specific requirements, kits used and equipment available.

Primer and probe sequences for the detection of BTV species viruses:

BTV_IVI_F 5'-TGG-AYA-AAG-CRA-TGT-CAA-A-3'

BTV_IVI_R 5'-ACR-TCA-TCA-CGA-AAC-GCT-TC-3'

BTV_IVI_P 5'FAM-ARG-CTG-CAT-TCG-CAT-CGT-ACG- C-3' BHQ1

a) Primer stock solutions are diluted to a working concentration of 20 pmol/µl, whereas probe is diluted to a working concentration of 5 pmol/µl.

b) A test plate layout should be designed and loaded into the real-time PCR machine software. Using the layout as a guide, 0.5 µl of each primer working stock (20 pmol/µl) is added to each well that will go on to contain RNA samples, positive or negative controls. The plate is held on ice.

Note: PCR plates can be replaced with tubes or strips as appropriate.

c) 2 µl of RNA samples, including test and positive and negative controls, are added to appropriate wells of the plate following the layout (note: these wells already contain primers from step b).

d) Heat denaturation: 95°C for 5 minutes, hold on ice for further 3 minutes.

e) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared, following the manufacturer's instructions. Probe should be included in the master mix to give a final concentration of 0.2 pmol/µl per sample.

f) 22 µl of master mix is distributed in each well on the PCR plate containing the denatured primers and RNA.

g) The plate is placed in a real-time thermal cycler programmed for reverse transcription and cDNA amplification/fluorescence detection as suggested by the manufacturers. The following thermal profile is an example:

	48°C	30 minutes
	95°C	2 minutes
50 cycles:	95°C	15 seconds
	56°C	30 seconds
	72°C	×30 seconds

1.3.1.2. Reverse-transcription polymerase chain reaction – nested PCR

Select RT-PCR (reverse-transcription and first stage amplification) and PCR (nested amplification) kits are available to perform the nested assay. The assay presented below for illustration uses the parameters associated with a specific kit. The PCR parameters should be adjusted according to the manufacturer's recommendations for the specific kits to be used.

The nested assay employs the use of the following primers:

First stage amplification (outer) (dilute to 25 pmol/µl; final concentration in PCR is 0.6 µM each):

FW: GTT-CTC-TAG-TTG-GCA-ACC-ACC
RV: AGG-CCA-GAC-TGT-TTC-CCG-AT

Nested amplification (dilute to 25 pmol/µl; final concentration in PCR is 0.5 µM each):

nFW: GCA-GCA-TTT-TGA-GAG-AGC-GA
nRV: CCC-GAT-CAT-ACA-TTG-CTT-CCT

i) Prepare the first stage amplification mixture (one-step RT-PCR kit) of the following reagents (per sample):

Nuclease-free water	11.8 µl
5× one-step RT-PCR buffer	5.0 µl
dNTP mix	1.0 µl
Enzyme	1.0 µl
FW primer (25 pmol/µl	0.6 µl
RV primer (25 pmol/µl)	0.6 µl

ii) Dispense 20 µl of the mixture into each PCR tube included in the assay. Add 5 µl of sample or control denatured RNA (described above) to the appropriate tube. Place tubes in a thermal cycler and run the following programme:

Reverse transcription	50°C	30 minutes
Taq activation	95°C	15 minutes
Followed by 35 cycles of:		
Denature:	94°C	45 seconds
Anneal:	58°C	45 seconds
Extension	72°C	60 seconds (final extension 10 minutes)

iii) Prepare a nested PCR mixture (HotStarTaq DNA Polymerase kit) of the following reagents (per sample):

Nuclease-free water	40.75 µl
10×HotStar buffer	5.0 µl
dNTP mix	1.0 µl
HotStarTaq	0.25 µl
nFW primer (25 pmol/µl)	1.0 µl
nRV primer (25 pmol/µl)	1.0 µl

iv) Dispense 49 µl of the mixture into each PCR tube. Transfer 1.0 µl of the amplified DNA from the first stage reaction (step 2) to the appropriate nested tube. Change gloves between samples and use caution when transferring the DNA to avoid cross contamination of samples. Place tubes in a thermal cycler and run the following programme:

Taq activation	95°C	15 minutes
Followed by 28 cycles of:		
Denature:	94°C	45 seconds
Anneal:	62°C	45 seconds
Extension:	72°C	1 minute (final extension 10 minutes)

Perform gel electrophoresis followed by modern gel visualisation methods on the nested PCR product. The positive control(s) and any positive samples will have a 101 base pair band. Negative controls and negative samples should not have a visible band. Positive samples may be sequenced for verification.

1.3.2. Nucleic acid sequencing

Although PCR-based methods can provide a rapid prospective determination of the serotype and genotype of a BTV isolate, the nucleic sequence of specific BTV genome segments is required for ultimate unequivocal identification. Through the exploitation of information provided by real-time RT-PCR or serology, and the now extensive availability of BTV sequence databases that better inform primer design, the combination of RT-PCR amplification and high throughput capillary sequencing can offer a relatively rapid, confirmative diagnostic approach. The now more economically viable option of whole genome sequencing (WGS) is also being increasingly routinely applied to BTV diagnosis (Belbis *et al*., 2017). However, the concentration of BTV in field isolations can provide limitations to the effectiveness of these sequencing approaches and prior establishment of growth in cell culture may be required for some isolates. Care should be taken concerning interpretation of results obtained with tissue culture grown virus samples, as the process of adaptation to cell culture inevitably involves selection of those viruses that can infect and replicate in the culture system used. The sequences generated may not therefore fully represent the virus population present in the original diagnostic sample.

2. Serological tests

Serological responses appear 7–14 days after BTV infection. Infected animals produce both neutralising and non-neutralising anti-BTV antibodies that are generally long-lasting. Anti-BTV antibodies generated in infected animals can be detected in a variety of ways that vary in sensitivity and specificity. Both serogroup-specific and serotype-specific antibodies are elicited and if the animal was not previously exposed to BTV, the neutralising antibodies generated are specific for the serotype of the infecting virus. Multiple infections with different BTV serotypes lead to the production of antibodies capable of neutralising serotypes to which the animal has not been exposed.

2.1. Competitive enzyme-linked immunosorbent assay

The BT competitive or blocking ELISA (C-ELISA) was developed to measure BTV-specific antibody without detecting cross-reacting antibody to other Orbiviruses (Afshar *et al*., 1989; Lunt *et al*., 1988). The specificity is the result of using BT serogroup-reactive MAbs. These antibodies were derived in separate laboratories, and although possessing different properties or epitope specificities, all appear to bind to the amino-terminal region of the major core protein VP7. In the C-ELISA, antibodies in test sera compete with the MAbs for binding to antigen. The following procedure for the C-ELISA has been standardised after comparative studies in a number of international laboratories. Importantly, monoclonal antibody-based competitive ELISA formats have been known to not detect all serotypes of BTV (particularly some BTV-15). Use of such ELISAs therefore requires an understanding of the test's fitness for purpose, and the assay must be validated accordingly to avoid missing detection of some BTV serotypes, including the novel serotypes recently identified.

2.1.1. Test procedure

There are several test procedures described; this is an example of one BT ELISA procedure.

i) First, 96-well microtitre plates are coated at 4°C overnight or at 37°C for 1 hour with 50–100 µl of either tissue culture-derived sonicated cell culture antigen or the major core antigen VP7 expressed in either *Baculovirus* (Oldfield *et al*., 1990) or yeast (Martyn *et al*., 1990) and diluted in 0.05 M carbonate buffer, pH 9.6.

ii) The plates are washed five times with PBST (0.01 M PBS containing 0.05% or 0.1% Tween 20, pH 7.2).

iii) Next, 50 µl of test sera is added in duplicate at a single dilution, either 1/5 (Afshar *et al.,* 1989) or 1/10 (Lunt *et al*., 1988) in PBST containing 3% bovine serum albumin (BSA).

iv) Immediately, 50 µl of a predetermined dilution of MAb diluted in PBST containing 3% BSA is added to each well. MAb control wells contain diluent buffer in place of test serum.

v) Plates are incubated for 1 hour at 37°C or 3 hours at 25°C, with continuous shaking.

vi) After washing as described above, wells are filled with 100 µl of an appropriate dilution of horseradish peroxidase-labelled rabbit anti-mouse IgG (H+L) in PBST containing 2% normal bovine serum.

vii) Following incubation for 1 hour at 37°C, the conjugate solution is discarded and plates are washed five times using PBS or PBST. Wells are filled with 100 µl substrate solution containing 1.0 mM ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]), 4 mM H_2O_2 in 50 mM sodium citrate, pH 4.0, and the plates are shaken at 25°C for 30 minutes. (Other substrates may be used and the reaction continued with shaking for an appropriate length of time to permit colour development.)

viii) The reaction is stopped by addition of a stopping reagent, such as sodium azide.

ix) After blanking the ELISA reader on wells containing substrate and stopping reagent, the absorbance values are measured at 414 nm. Results are expressed as per cent inhibition and are derived from the mean absorbance values for each sample by the following formula:

% inhibition = 100 – [(mean absorbance test sample)/(mean absorbance MAb control) × 100].

NB: Some laboratories prefer to use a negative control serum that has previously been shown to have a percentage inhibition of zero as an alternative to the MAb control.

x) Percentage inhibition values >50% are deemed positive. Inhibition between 40% and 50% is considered to be suspicious. The results of the test sera duplicates can vary as long as the results do not lie either side of the positive cut -off.

xi) Strong and weak positive sera and a negative serum should be included on each plate. The weak positive should give 60–80% inhibition and the negative should give less than 40% inhibition.

A number of commercially produced C-ELISAs based on recombinant VP7 and anti-VP7 MAb are now available. These commercial assays are routinely used in many laboratories across the world and have been proved to be fit for purpose in ring-trials (Batten *et al*., 2008). Formal acceptance for trade purposes should depend on adoption of individual kits to the WOAH Register.

Genetic divergence of certain BTV strains (e.g. different regional groups or topotypes) may affect the nature of serogroup-reactive antibodies. It is therefore possible that diagnostic characteristics for antibody detection are not uniform for all viruses encompassed by the serogroup. Diagnostic reagents and kits produced in one region may not have the same performance characteristics when used in another region. This should be addressed in considerations of fitness for purpose.

2.2. Indirect ELISA

An indirect ELISA for bulk milk samples has been shown to be reliable and useful for surveillance purposes (Kramps *et al*., 2008). It should be validated for relevant serotypes before use. While an indirect ELISA may have the disadvantage of cross reactivity with related viruses such as EHDV, it does have high analytical sensitivity and could be a useful screening assay in some situations.

2.3. Virus neutralisation serology

VN serology can identify serotype-specific neutralising antibodies as well as determine their titre. It is an important additional test in endemic areas where multiple serotypes are likely to be present. Its capability to identify the serotype involved in an outbreak is essential for putting in place appropriate control measures such as vaccination or animal movement restrictions. It is also useful for epidemiological surveillance, transmission studies and for determining useful antibody response to vaccination. Cross-neutralising antibodies can develop in animals that have experienced BTV infection. Importantly, infection with a second or third serotype can broaden the neutralising antibody response to include antibodies to serotypes to which the animal has not been exposed. The application of VN serology is frequently most useful in conjunction with other virological investigations that, in combination, can provide a more definitive basis for resolving serotype distribution. The use of the plaque reduction test (modified from Section B.1.2.2) can improve resolution of serotype involvement, where this is unclear on a standard VN test due to its quantitative nature.

2.3.1. Test procedure

Several methods to determine titre and serotype of BTV have been described; here the procedure that has been standardised after comparative studies in various international laboratories is briefly outlined. Indicator cell lines commonly used are BHK and Vero. It is important that well characterised, reference positive and negative control antiserum be included in each test.

i) 50 µl of serial sera dilutions, from 1/10 to 1/1280, are added to each test well of flat-bottomed microtitre plates and each well is mixed with an equal volume of medium containing approximately 100 $TCID_{50}$ of suitable, well characterised, BTV reference viruses. Note the selection of reference strains used will be dependent on the circulating (or possibly circulating) serotypes in the testing population.

ii) The plates are incubated in a humid chamber at 37°C in 5% CO_2.

iii) After 1 hour of incubation, approximately 10^4 Vero or BHK-21 cells are added per well in a volume of 100 µl of suitable media (containing) able to sustain growth of the chosen cells.

iv) Incubate at 37°C for up to 7 days, depending on the level of CPE obtained in normal serum wells of the test. When CPE has developed to 100% in the normal serum wells of each serotype tested, the test is read using a light microscope at 100× magnification to determine presence of CPE.

v) Wells are scored for the degree of CPE observed. A sample is considered positive when it nears full neutralisation, allowing for the acceptance of trace CPE at the lowest dilution (1/10). The presence of trailing trace CPE may indicate evidence of partial neutralisation. In these situations, the reference virus used may not be representative of contemporary circulating viruses and further investigation may be required (for example, use of the plaque reduction

neutralisation test, which is superior in resolving cross reactivity between related serotypes). The serum titre represents the highest serum dilution capable of near complete neutralisation of 50% of replicate test wells. A fourfold difference in endpoint titre obtained from different serotypes tested can indicate the serotype involved in an infection. Cross reactions are known to occur between serotypes in the VN test even when only one serotype is involved.

2.4. Agar gel immunodiffusion

It must be recognised that a major disadvantage of the AGID used for BT is its lack of specificity in that it does not exclusively differentiate between antibodies to the BT and EHD serogroups. Hence, it cannot be used definitively to detect antibodies to BTV as a positive reaction may have been the result of an infection to another *Orbivirus* species. Notably, the AGID test is simple to perform and the antigen used in the assay relatively easy to produce. Since 1982, the test has been one of the standard testing procedures for international movement of ruminants, however, it is no longer considered sufficiently accurate for use in the support of international trade. The assay does have a role in the investigation of samples that give inconsistent results in other assays. AGID positive sera should be retested using a BT serogroup-specific assay if BTV specificity is required. The preferred test, a C-ELISA, is described in Section B.2.1.

C. REQUIREMENTS FOR VACCINES

1. Background

Vaccination with effective vaccines is the preferred method of BTV control in endemic countries. Vaccination has been used successfully to limit direct losses, minimise the circulation of BTV, eradicate BTV from a region, and allow safe movement of animals. Both live attenuated and inactivated BTV vaccines are currently available for use in sheep and sheep and cattle, respectively. Recombinant BT vaccines based on various approaches are under development (van Rijn, 2019), but none has been licensed and these vaccines will not be addressed here.

Live attenuated vaccines are inexpensive to produce in large quantities; they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease in the areas where they are used. In South Africa, live attenuated vaccines have been used for over 50 years and are known to induce an effective and lasting immunity. Live attenuated vaccines are produced by adapting BTV field isolates to growth *in vitro* through serial passages in tissue culture or in ECEs. Stimulation of a strong antibody response by these vaccines is directly correlated to their ability to replicate in the vaccinated host. However, live attenuated BTV vaccines suffer from a variety of documented or potential adverse outcomes, including depressed milk production, abortion/embryonic death, teratogenesis and congenital defects, and have been documented to be spread by vectors with considerable potential for reversion to virulence and reassortment of vaccine virus genes with those of wild-type virus strains (Ferrari *et al*., 2005; Ranjin *et al*., 2019; Savini *et al.,* 2014). Under-attenuation, the impact of which may vary with different breeds of sheep (Veronesi *et al*., 2010), and reassortment with other vaccine and field strains (Nomikou *et al*., 2015) may also occur. The frequency and significance of these events remain poorly defined but transmission of vaccine strains by vector midges has already been documented in the USA, South Africa and Europe (Ferrari *et al*., 2005).

Inactivated vaccines containing tissue-culture grown and chemically inactivated vaccine strains are far safer but require multiple doses to become efficacious and incur higher costs. Such vaccines have been very effective in combatting the spread of BTV-8 in Europe. Whilst inactivated vaccines (as with live vaccines) are not compatible with serological assays for detection of infection in vaccinated animals (DIVA strategies), surveillance can be maintained after their use by RNA based (RT-PCR) assays.

Additionally, non-BTV vaccines for both target and non-target species have been found to be contaminated with BTV leading to abortion, heart failure, respiratory distress and death in non-ruminant species. These drawbacks have resulted in a great deal of research and development of BTV vaccines that are safe, efficacious against multiple serotypes, inexpensive, are DIVA capable, only require a single dose with rapid onset of immunity, and block viraemia. Recent efforts with reverse genetics and molecular technology has led to next generation vaccines that balance these often competing requirements while meeting specific field situation needs.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

See chapter 1.1.8 for general requirements for master seeds and allowable passages for vaccine production.

2.1.1. Biological characteristics of the master seed

For live attenuated vaccines, the master or primary virus seed is prepared from a single plaque of serially passaged, attenuated BTV. Vaccine viruses have been attenuated by either passage in ECE, tissue culture cells or a combination of both. Each primary seed virus lot should also be tested for transmissibility and reversion to virulence prior to vaccine manufacture. Samples of vaccine prepared from secondary seed virus at the maximum permitted passage level should be tested in sheep for avirulence, safety and immunogenicity.

For inactivated vaccines, the issues of attenuation do not apply, and the approach adopted has been to use field strains of low passage level with the intent of achieving high antigenicity.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Primary seed virus must be free of contaminating bacteria, viruses, prions, fungi and mycoplasmas, particularly pestivirus contamination. For the latter, attention should be paid to the fetal bovine serum used in cell cultures, as it may be contaminated. Seed viruses must be shown to have the desired serotype specificity. BTV seed lot viruses should be sequenced and the data made available to relevant databases. Secondary seed lots, which are used as inocula for vaccine production, are usually not more than three passages beyond the primary seed lot.

2.1.3. Validation as a vaccine strain

Live attenuated BT vaccines must be safe and efficacious, and a brief description of appropriate tests for these parameters is given below. In addition, attenuated viruses should not revert to virulence during replication in vaccinated animals or be able to be transmitted from such animals by insect vectors. The latter criterion is very important because insect-mediated transmission of attenuated virus from vaccinated to non-immune animals, with the subsequent replicative steps in each host species, increases the possibility of reversion to virulence. Although tests for reversion to virulence and transmissibility are rarely, if ever performed, a brief description of what may be necessary is outlined.

There is a variation in BT susceptibility between breeds of sheep; it is important that sheep that have been proven to be susceptible to infection with BTV be used for vaccine validation.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic

In a number of different circumstances (see Chapter 1.1.10 *Vaccine banks*) and in the event that a new or different serotype or variant of BTV results in an emergency epizootic situation that cannot be controlled by currently available vaccines, and where there is not enough time to fully test a new MSV for all extraneous agents, provisional acceptance of the new strain could be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account the characteristics of the process, including the nature and concentration of the inactivant for inactivated vaccines, before allowing or not the early release of the new product.

2.2. Method of manufacture

2.2.1. Procedure

Attenuation of field isolates of BTV was first achieved by serial passage in ECE. Because of the concern about transmission of the egg propagated attenuated virus, it has been recommended that animals receiving vaccines produced in ECE should not be moved internationally. More recently, it is accepted that passage in cultured cells will also result in attenuation of virulence. No studies have been done to precisely relate passage number and extent of attenuation for individual virus isolates or serotypes. To prepare attenuated virus, field isolates are adapted to cell culture and passaged *in vitro* up to 40 times or more. Ideally, a number of plaque-purified viruses are picked at this stage and each is examined to determine the level of viraemia they generate and their ability to elicit a protective immune response in vaccinated sheep. The most suitable virus is one that replicates to low titre but generates a protective immune response, and this may represent the source of vaccine primary seed stock virus.

BTV for inactivated vaccines is produced in large-scale suspension cell systems under aseptic and controlled conditions. Cell lines adapted for large scale industrial cultures are used and these are proven to be free from contaminating microorganisms. When the viral suspension virus reaches its maximum titre, followed by cell disruption, the culture is clarified and filtered. Subsequently inactivation is performed according to processes adopted by the manufacturer, such as by addition of binary ethyleneiminee (BEI) or other inactivating agents. The process must comply with legislation relevant for the intended market, be validated to ensure complete inactivation and supported by appropriate documentation. The inactivation process should not significantly alter the immunogenic properties of the viral antigens. Purification is carried out by chromatography. The inactivated virus is then concentrated by ultrafiltration and stored. The inactivated, chromatography-purified and concentrated BTV antigens are made into vaccine by dilution in a buffer solution and addition of adjuvants.

2.2.2. Requirements for ingredients

All ingredients of animal origin, including serum and cells must be checked for the presence of viable bacteria, viruses, fungi or mycoplasma. For further details, see chapter 1.1.8 for general guidance on ingredients of animal origin. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies.

2.2.3. In-process controls

Virus concentration of live attenuated vaccines is assessed by infectivity and ELISAs.

For inactivated vaccines, during inactivation of the virus, timed samples are taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of BHK-21 or other appropriate cell cultures. At the end of the inactivation process, the vaccine is checked to ensure that there is no live virus.

2.2.4. Final product batch tests

i) Sterility

Every batch of vaccine should be tested for the presence of contaminant viruses, viable bacterial, fungi or mycoplasma. For example, in South Africa a pool of ten randomly selected ampoules are inoculated into soya broth and thioglycolate broth, and incubated at room temperature and 37°C for 14 days, respectively. If contaminated, the batch is disqualified.

ii) Safety

Every batch of attenuated vaccine is safety tested in newborn and adult mice or guinea-pigs. If any adverse reactions or significant signs are noted, the test is repeated. Any increase in the body temperature of the test animal that is above the level expected for the strain of attenuated virus under test should be regarded as symptomatic. If the results are unsatisfactory after a second attempt, the batch is disqualified.

Safety testing of inactivated vaccines is conducted to ensure side effects are not observed.

iii) Potency

Each batch is tested by inoculation of susceptible sheep. Pre-vaccination, 21- and 28-day post-vaccination sera are tested by VN assay to determine neutralising antibody levels. To be passed, the antibody titre must be equal to or higher than a set standard based on international vaccine standards.

iv) Duration of immunity

Studies with live attenuated BTV vaccine have shown that antibodies in sheep may appear before day 10 post-vaccination, reach a maximum approximately 4 weeks later and persist for well over a year. There is a temporal relationship between the increase in neutralising antibody titre and clearance of virus from the peripheral circulation. Live attenuated BTV vaccines have been in use for over 50 years and are known to induce an effective and lasting immunity in sheep (Verwoerd & Erasmus, 2004). Many serotypes of BTV may be present in endemic areas of South Africa, and polyvalent vaccines are used. The inclusion of 15 serotypes in three polyvalent vaccines that are administered sequentially sometimes means that an effective immune response is not generated to all serotypes, presumably because of the antigenic mass of individual serotype-specific antigens is small. In an attempt to broaden the response, vaccination is repeated annually.

Initial studies with inactivated vaccines show that antibody against BTV can be detected by day 7 post-vaccination and increase in titre to days 14–21. A second dose of vaccine boosts the titre. Data to demonstrate the expected duration of immunity are being acquired.

v) Stability

Procedures have been developed for attenuated vaccines. Stability should be tested over a period of 2 years. Vaccines in liquid and lyophilised forms are deemed to have shelf lives of 1 and 2 years, respectively. Each batch of vaccine is subjected to an accelerated shelf-life test by storing it at 37°C for 7 days. It is then titrated and evaluated according to a set standard, as determined in the initial testing of the vaccine. Shelf life of stock aliquots stored at +4°C should be tested periodically.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

All vaccines must be safety tested. Safety tests for attenuated vaccines do not address the issue of their teratogenicity. Attenuated virus vaccines are teratogenic and should not be administered to pregnant sheep during the first half of pregnancy as this may cause fetal abnormalities and embryo death.

i) Target and non-target animal safety

Demonstration of avirulence is necessary for live, attenuated vaccines. A number of sheep seronegative by an appropriate, sensitive serological test (that will reliably detect antibodies even in vaccinated animals), are inoculated with the primary seed stock. Temperatures are noted twice daily. The animals are monitored at regular intervals over a period of 28 days for clinical signs and any local or systemic reactions to ensure avirulence and innocuity. Blood samples removed at regular intervals can be used to measure level of viraemia and antibody responses. The test shall be valid if all of the vaccinated sheep show evidence of virus replication and do not display signs of disease other than mild transient illness. In South Africa, a clinical reaction index is calculated for each animal between days 4 and 14 and must be below a specific standard value.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Transmissibility is an issue with live attenuated vaccines but not with killed vaccines. Procedures to determine if attenuated virus can be transmitted by insects that feed on vaccinated, viraemic sheep are difficult to perform and analyse statistically, and consequently, this criterion of vaccine validation is rarely sought. Laboratory data indicate that laboratory-adapted viruses can be transmitted by insect vectors (Ferrari *et al.,* 2005). A suitable procedure to determine attenuated virus transmissibility requires that sheep be vaccinated and, during viraemia, that they be exposed to competent, uninfected *Culicoides*, which are then permitted to feed on uninfected animals that are monitored for the presence of BTV and anti-BTV antibody. As the titre of attenuated virus in the blood of vaccinated sheep is usually low, very large numbers of *Culicoides* may be needed and only a small proportion of these would become infected and live long enough to feed on and potentially transmit the virus to other uninfected sheep. It is difficult to design a laboratory experiment that takes account of the large numbers of vaccinated sheep and insects that would be present in field situations. Although virus titres in blood less than 10^3 $TCID_{50}$/ml have traditionally been considered a "safe" threshold, authentic instances of insects acquiring BTV from animals with viraemic titres much less than 10^3 $TCID_{50}$/ml have been reported. Given the complex interaction of BTV, *Culicoides* vectors and animal hosts in the life cycle of infection, virus titres induced by live attenuated vaccine should be kept to an absolute minimum especially if field transmission of vaccine strains is a concern.

Current data indicate that during viraemia and in contrast to wild-type virus, laboratory-adapted strains of BTV may be found in the semen of bulls and rams (Kirkland *et al.,* 2004). The implications of these observations for virus transmissibility are unclear. A recent study of semen from rams vaccinated with BTV-2 live attenuated vaccine showed that even if BTV was not detected in the semen, the vaccine caused a decrease in the quality of the semen (Breard *et al.,* 2007).

Validation studies confirm that attenuated viruses do not revert to virulence in vaccinated sheep. However, if attenuated viruses can be transmitted from vaccinated animals, reversion to virulence following several sheep-insect replication cycles becomes a distinct prospect. The only appropriate way to monitor for reversion to virulence under these circumstances is to compare the virulence of the vaccine virus with that which had been subject to several sheep–insect replication cycles as described above. As indicated, this is difficult to achieve. Consequently, the effect of sheep/insect passages on the virulence of attenuated viruses has not been determined. In South Africa, it is accepted that if blood from vaccinated animals during the viraemic stages is serially passaged three times in sheep without reversion to virulence, the chances of reversion in the field will be infinitely small. In Europe, five passages are required.

iii) Precautions (hazards)

Attenuated vaccines should be used in the cooler months when adult *Culicoides* vector populations are at a minimum. They should not be used in ewes during the first half of pregnancy and in rams 2 months before the breeding season

2.3.3. Efficacy requirements

Vaccinated and unvaccinated sheep known to be susceptible to BT disease should be challenged with virulent homologous serotype. It is recommended that the challenge model preferably use virus passaged only in ruminant animals and with no or limited ECE or cell culture passages. Passage in such an isolation system results in viral cultures that might induce clinical BT disease that is milder than the natural disease. Animals are monitored for clinical signs of BT disease, rectal temperatures are taken twice daily and blood samples removed at regular intervals to measure viraemia and antibody responses. Unvaccinated control sheep should show clinical signs of BT disease and viraemia. However, it is difficult to be certain of the appearance of clinical disease following inoculation of sheep with certain BTV serotypes and isolates, and consequently, evidence of infection of unvaccinated control sheep may rest on the appearance of a temperature rise above 40°C and a viraemia. As a further evidence of infection pre- and post-vaccination sera are checked for the presence of neutralising antibody.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

The live attenuated and inactivated products now commercially available do not allow any DIVA strategy via serological testing.

RT-PCR may be used as DIVA assays, allowing surveillance to be maintained during inactivated vaccination campaigns. DIVA strategies may be possible using PCR-based test systems in populations in which inactivated vaccines have been applied. The inactivated vaccines may generate a very weak RT-PCR signal although this disappears a few days post-vaccination. In contrast, infected animals usually maintain a high RT-PCR signal that can last for several weeks to months, even in the presence of neutralising antibodies, due to the haemagglutinin activity associated with outer capsid protein VP2. A similar approach is not possible with currently available live attenuated vaccines. The generation of weak signal due to very early infection should also be considered if using this strategy.

2.3.5. Duration of immunity

Studies to determine a minimum duration of immunity should be conducted before the vaccine receives final approval. Duration of immunity should be demonstrated in a manner similar to the original efficacy study, challenging animals at the end of the claimed period of protection. At a minimum, the duration should be for the length of the mosquito season in areas with seasonal infections. It may be desirable to demonstrate longer immunity for animals at higher risk and in infected areas with year-round mosquito activity.

2.3.6. Stability

Live and inactivated vaccines are typically assigned an initial dating of 18 or 24 months, respectively, before expiry. Real-time stability studies should be conducted to confirm the appropriateness of all expiration dating. Product labelling should specify proper storage conditions.

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NB: There are WOAH Reference Laboratories for bluetongue
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for bluetongue

NB: First adopted in 1991. Most recent updates adopted in 2021.

CHAPTER 3.1.4.

BRUCELLOSIS (INFECTION WITH *B. ABORTUS, B. MELITENSIS* AND *B. SUIS*)

SUMMARY

Description of the disease: *Brucellosis is the generic name used for the animal and human infections caused by several species of the genus Brucella, mainly Brucella abortus, B. melitensis and B. suis. Infection with Brucella in cattle is usually caused by B. abortus, less frequently by B. melitensis, and occasionally by B. suis. Brucella melitensis is the main causative agent of infection with Brucella in sheep and goats. Brucella melitensis and B. abortus may also infect other species, including camels. Infection with Brucella in pigs is due to B. suis biovars 1–3, but the disease caused by biovar 2 differs in its host range and its limited geographical distribution. In some areas, B. suis infection has become established in wild pigs. Clinically, infection with Brucella in animals is characterised by one or more of the following signs: abortion, infertility, retained placenta, orchitis, epididymitis and, rarely, arthritis, with excretion of the organisms in uterine discharges, milk, urine and semen. Unequivocal diagnosis depends on the isolation of Brucella from abortion material, udder secretions or from tissues removed at post-mortem. Brucella abortus, B. melitensis and B. suis are highly pathogenic for humans, and potentially contaminated tissues, cultures and materials must be handled under appropriate containment conditions.*

Detection of the agent: *Indication of Brucella is provided by the demonstration of Brucella-like organisms in abortion material or vaginal discharge using modified acid-fast staining, and is considered presumptive, especially if supported by serological tests. Polymerase chain reaction (PCR) methods are additional means for detection of the presence of Brucella DNA in a sample. Whenever possible, Brucella spp. should be isolated by culturing samples from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes and male and female reproductive organs. Species and biovars can be identified by phage lysis, and by cultural, biochemical and serological tests. PCR can provide the basis of complementary identification and typing methods based on specific genomic sequences.*

Serological and cellular immunity tests: *Serological tests indicate exposure to Brucella species, but cannot identify the aetiological agent to the species level. The buffered Brucella antigen tests (rose bengal test and buffered plate agglutination test), the complement fixation test, the enzyme-linked immunosorbent assays (ELISA) or the fluorescence polarisation assay, are suitable tests for screening of herds/flocks and individual small ruminants, camelids and bovines (cattle and buffaloes). However, no single serological test is appropriate in each animal species and all epidemiological situations, and some of these tests are not adequate for diagnosing brucellosis in pigs. Therefore, the reactivity of samples that are positive in screening tests should be assessed using an established confirmatory or complementary strategy. The indirect ELISA or milk ring test performed on bulk milk samples is effective for screening and monitoring dairy cattle. The brucellin skin test can be used in unvaccinated ruminants, camels and swine as either a screening or a confirmatory herd test when positive serological reactors occur in the absence of obvious risk factors.*

Requirements for vaccines and diagnostic biologicals: *Brucella abortus strain 19 and B. melitensis strain Rev.1 remain the reference vaccines for the control of Brucella infections in cattle and in sheep and goats, respectively, with which any other vaccines should be compared. Both should be prepared from adequately derived seed cultures. The rough B. abortus strain RB51 vaccine has also become the official vaccine for prevention of B. abortus infection in cattle in some countries. No suitable vaccines exist for the control of Brucella infection in swine. Brucellin preparations must be free of smooth lipopolysaccharide, and antigens for serological tests must be prepared from smooth*

B. abortus strain 1119-3 or 99 and, in the case of indirect ELISA, from smooth B. melitensis strain 16M as well. Vaccines and brucellin preparations must comply with relevant standards.

A. INTRODUCTION

Brucellosis is the generic name used for the animal and human infections caused by several species of the genus *Brucella*, mainly *Brucella abortus, B. melitensis, B. suis* and *B. canis.* Infection of sheep with *B. ovis* is described separately in Chapter 3.8.7 *Ovine epididymitis* (*Brucella ovis*).

Causal pathogens: Genetic and immunological evidence indicates that all members of the *Brucella* genus are closely related. Nevertheless, based on relevant differences in host preference and epidemiology displayed by the major variants, as well as molecular evidence of genomic variation, the International Committee on Systematics of Prokaryotes, Subcommittee on the Taxonomy of *Brucella* took a clear position in 2005 on a return to pre-1986 *Brucella* taxonomic opinion; the consequences of this statement imply the re-approval of the six classical *Brucella* nomenspecies with their corresponding recognised biovars, although both opinions remain valid. The classical names related to the six *Brucella* nomenspecies are validly published in the Approved Lists of Bacterial Names, 1980, and the designated type strains are attached to these validly published names: *B. abortus, B. melitensis, B. suis, B. neotomae, B. ovis* and *B. canis*. The first three of these are subdivided into biovars based on cultural and serological properties (see Tables 2 and 3). Strains of *Brucella* have been isolated from marine mammals and classified into two new species: *B. ceti* and *B. pinnipedialis* (Foster *et al*., 2007). A new species, named *B. microti,* was also isolated from the common vole (*Microtus arvalis*) as well as from foxes, soil and frogs raised for human consumption in Europe (Scholz *et al*., 2008). Novel isolates from human breast implant infection, from baboons that had delivered stillborn offspring, and from foxes have also been described, although the natural reservoir of these isolates remains uncertain. While limited isolates of each new type have been described, they have been formally published as the tenth, eleventh, and twelfth *Brucella* species, *B. inopinata*, *B. papionis* and *B. vulpis* respectively (Scholz *et al*., 2010; 2016; Whatmore *et al*., 2014). Finally, various strains isolated from rodents, foxes reptiles, fish and frogs were characterised as atypical *Brucella* strains distinct from the currently described species. They have not yet been approved as new *Brucella* species.

Brucella is a member of the *Brucellaceae* family, in the order Rhizobiales, class Alphaproteobacteria. It shows close genetic relatedness to some plant pathogens and symbionts of the genera *Agrobacterium* and *Rhizobium*, as well as animal pathogens (*Bartonella*) and opportunistic or soil bacteria (e.g. *Ochrobactrum*).

1. Description of the disease

1.1. Infection with *Brucella* in cattle

Infection with *Brucella* in cattle is usually caused by biovars (bv.) of *Brucella abortus*. In some countries, particularly in southern Europe, Africa and western Asia, where cattle are kept in close association with sheep or goats, infection can also be caused by *B. melitensis* (Verger, 1985). Occasionally, *B. suis* may cause infections in cattle. The disease is global in distribution but a number of countries are considered free from both *B. abortus* and *B. melitensis*. For up-to-date information, consult WOAH WAHIS interface[1].

Young animals and non-pregnant females usually show no signs of the disease. Following infection with *B. abortus* or *B. melitensis*, pregnant adult females develop a placentitis usually resulting in abortion between the fifth and ninth month of pregnancy. Even in the absence of abortion, profuse excretion of the organism occurs in the placenta, fetal fluids and vaginal discharges. The mammary gland and associated lymph nodes may also be infected, and organisms may be excreted in the milk. Colostrum originating from infected dams is a source of infection in the newborn population. Subsequent pregnancies are usually carried to term, but uterine and mammary infection recurs, with reduced numbers of organisms in afterbirth products and milk. In acute infections, the organism is present in most major body lymph nodes. Adult male cattle may develop orchitis/epididymitis and brucellosis may be a cause of infertility in both sexes. *Brucella abortus* can be shed in semen, seminal fluid and urine. Hygromas, usually involving leg joints, are a common manifestation of brucellosis in some tropical countries and may be the only obvious indicator of infection; the hygroma fluid is often infected with *Brucella*.

1 https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/

1.2. Infection with *Brucella* in sheep and goats

Infection with *Brucella* in sheep and goats (excluding *B. ovis* infection) is primarily caused by *B. melitensis*. Sporadic infections caused by *B. abortus* or *B. suis* have been observed in sheep and goats, but such cases are extremely rare. Infection with *Brucella* in sheep and goats is widespread although a number of countries are believed to be free from the agent. For up-to-date information, consult WOAH WAHIS interface. Pathologically and epidemiologically, *B. melitensis* infection in sheep and goats is very similar to *B. abortus* infection in cattle. In most circumstances, the primary routes of transmission of *Brucella* are the placenta, fetal fluids and vaginal discharges expelled by infected ewes and goats at and up to several months following abortion or parturition. Shedding of *Brucella* is also common in udder secretions and semen, and *Brucella* may be isolated from various tissues, such as lymph nodes from the head, spleen and organs associated with reproduction (uterus, epididymides and testes), and from arthritic lesions (Alton *et al*., 1988).

1.3. Infection with *Brucella* in pigs

Infection with *Brucella* in pigs is primarily caused by biovars 1, 2 or 3 of *B. suis*. Sporadic infections caused by *B. abortus* or *B. melitensis* have been also observed in pigs, but such cases are rare. The disease occurs in many countries where pigs are raised. Generally, the prevalence is low, but in some regions, such as South America and south-east Asia, the prevalence may be much higher. Porcine brucellosis may be a serious but presently unrecognised problem in some countries. *Brucella suis* bv. 1 infection has been reported from feral pigs in some of the southern states of the United States of America (USA), in parts of Australia and several other countries in Oceania. In these countries, a number of human infections have been reported from people who hunt and handle material taken from feral pigs. The disease is generally transmitted by consumption of feed contaminated by birth or abortion products and uterine discharges. Pigs will instinctively eat aborted fetuses and placental membranes. Transmission during copulation also occurs frequently, and *B. suis* excretion in semen has implications for those practising artificial insemination. In pigs, as in ruminants, after the initial bacteraemia, *B. suis* colonises the reproductive tract of either sex. In females, placentas and fetuses are invaded, while in males, invasion occurs in one or more of the following: testes, prostate, epididymides, seminal vesicles or bulbo–urethral glands. In males the lesions, which are most often unilateral, start with a hyperplasia that may progress to abscess formation; the final stage is characterised by sclerosis and atrophy. The most common manifestation of brucellosis in female pigs is abortion, occurring at any time during pregnancy, but most frequently between day 50 and 110 of gestation. Vaginal discharge is not often evident, and, in chronically infected herds, infertility rather than abortion is the most relevant clinical sign of the disease. In males, brucellosis is more likely to be persistent, with lesions in the genital tract often leading to interference with sexual activity, which can be temporary or permanent. The boar may excrete *Brucella* in the semen without any apparent abnormality in the sex organs or interference with sexual activity. In both sexes, arthritis may occur in various joints, there may be swollen joints and tendon sheaths, lameness and, occasionally, posterior paralysis or spondylitis. A significant proportion of both male and female pigs will recover from the infection, often within 6 months, but many will remain permanently infected (Olsen *et al*., 2012).

Infection caused by *B. suis* bv. 2 differs from infection caused by bv. 1 and bv. 3 in its host range, distribution, and in pathogenicity. Historically, the geographical distribution of *B. suis* bv. 2 has been in a broad range between Scandinavia and the Balkans. The prevalence in wild boars appears to be high throughout continental Europe (EFSA, 2009). In outbreaks in Europe, wild boars were implicated as the source of transmission of bv. 2 to outdoor reared pigs, and are considered as the main wild reservoir of this infection (EFSA, 2009). *Brucella suis* bv. 2 causes miliary lesions, particularly in reproductive tissues, that often become purulent. To date, *B. suis* bv. 2 has rarely been reported as the cause of human brucellosis. However, *B. suis* bv. 2 infections have been reported in immuno-compromised hunters, who had been extensively exposed through gutting or skinning boars or hares. Moreover, rare cases of *B. suis* bv. 2 infection without clinical signs have been reported in Europe in cattle or sheep exposed to infected wild boars.

1.4. Infection with *Brucella* in other domestic, captive–wild or wild species

Infection with *B. abortus* or *B. melitensis* has been reported in the one-humped camel (*Camelus dromedarius*) and the two-humped camel (*C. bactrianus*), as well as in the South American camelids: llama (*Lama glama*), alpaca (*Vicugna pacos*), guanaco (*Lama guanicoe*), and vicuña (*Vicugna vicugna*), and is related to contact with large and small ruminants infected with *B. abortus* or *B. melitensis.*

In addition, brucellosis has been observed in the domestic buffalo (*Bubalus bubalis*), American and European bison (*Bison bison* and *B. bonasus*, respectively), yak (*Bos grunniens),* elk/wapiti (*Cervus canadensis, sika deer* (*C. nippon*), African buffalo (*Syncerus caffer*) and various antelope species. The clinical manifestations of brucellosis in these animals are similar to those seen in cattle, sheep and goats.

Brucella melitensis infection in wild ruminants may occur when these species are in close contact with sheep and goats in enzootic areas. The manifestations of brucellosis in these animals are similar to those in cattle or sheep and goats. However, in several wild ruminant species (e.g. chamois [*Rupicapra rupicapra*], Alpine ibex [*Capra ibex*] and the Iberian wild goat [*Capra pyrenaica*]), purulent or calcified arthritis and orchitis as well as uveitis and neurological signs have been reported. These species are considered as dead-end carriers, and the disease usually disappears naturally as soon as *Brucella* infection has been eradicated from domestic livestock, unless anthropogenic effects take place. Nevertheless three reservoirs are currently described in wild ruminants: *B. abortus* in bison in the Yellowstone area of North America, *B. melitensis* in alpine ibex and *B. abortus* in wood bison in Wood Buffalo National Park in Canada. There have also been sporadic reports of *B. melitensis* isolation from dogs, especially from contact with infected sheep or goats, or ingestion of placenta or aborted fetuses.

There are two different types of epidemiological situation with regard to *B. suis* infection in other non-porcine species. In the first case, *B. suis* infection occurs in animals that are not the natural host of the particular infection through the ingestion of contaminated materials or by co-habitation with infected natural hosts. For example, Arctic foxes and wolves may contract *B. suis* bv. 4 from reindeer; dogs and rodents, such as rats and mice, may acquire other *B. suis* biovars by cohabitation with infected hosts; cattle and horses may become infected by cohabitation or interaction with infected swine. The infecting bacteria are invariably the well-defined biovars of the natural host species. In the second case, wildlife species are natural hosts for *B. suis* or *B. suis*-like infections. One example is the so-called murine brucellosis of the Commonwealth of Independent States (CIS) and the Baltic countries, where small rodents are infected with *B. suis* bv. 5.

In addition to wild boar, the European hare (*Lepus europaeus*) is also considered to be a reservoir for *B. suis* bv. 2 and has been implicated as a possible source of transmission to domestic livestock. The disease in the European hare is characterised by the formation of nodules, varying in size from that of a millet seed to a cherry or even larger; these often become purulent. Such nodules may occur in almost any location, sometimes subcutaneously or intramuscularly, in the spleen, liver or lung and in the reproductive organs of either sex. The body condition of the hare may be unaffected. Other species may also become infected by cohabitation with *B. suis* bv. 2 infected swine, wild boars or hares. Gutting or skinning wild boars in cattle sheds could be a route of transmission to cattle.

Brucella suis bv. 4 causes a serious zoonotic disease in wild or domesticated reindeer or caribou (*Rangifer tarandus* and its various subspecies) throughout the Arctic region, including Siberia, Canada and Alaska. *Rangifer tarandus* is very susceptible to *B. suis* infection, which causes fever, depression and various local signs, such as abortion, retained placentas, metritis, sometimes with blood-stained discharge, mastitis, bursitis and orchitis. Transmission to humans may be by direct contact or through consumption of raw milk and other inadequately heated products from reindeer, bone marrow in particular.

1.5. Zoonotic risk and biosafety requirements

Some *Brucella* species, most notably *B. melitensis, B. abortus, B. suis* and, probably to a lesser extent *B. canis* are readily transmissible to humans, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculo-skeletal, cardiovascular, and central nervous systems. Precautions should be taken to prevent human infection. Infection is acquired by the oral, respiratory, or conjunctival routes. Ingestion of raw milk products constitutes the main risk to the general public where the disease is endemic. There is an occupational risk to veterinarians, abattoir workers and farmers who handle infected animals/carcasses and aborted fetuses or placentas. Brucellosis is also one of the most easily acquired laboratory infections, and all laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Specific recommendations have been made for the biosafety precautions to be observed with *Brucella*-infected materials (for further details see Alton *et al*., 1988;

Joint FAO/WHO Expert Committee on Brucellosis, 1986; WHO, 1953; WHO, 2004; Chapter 1.1.3 *Transport of biological materials*).

B. DIAGNOSTIC TECHNIQUES

***Table 1. Test methods available for the diagnosis of infection with* Brucella abortus, melitensis *or* suis**

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection[a]	Contribute to eradication policies[b]	Confirmation of suspect or clinical cases[c]	Herd/flock prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Staining methods	–	–	–	+	–	–
Culture	–	–	–	+++	–	–
PCR[d]	–	–	–	+/++	–	–
Detection of immune response						
BBAT (RBT or BPAT)	+++	++	+++	+	+++	–
FPA	++	++	+	++	++	–
CFT	++	++	+++	++	+++	–
I-ELISA	+++	++	+++	++	+++	–
C-ELISA	++	+	+	+	++	–
BST	++	–	+	+++	++	–
SAT	++	+	+	–	+	–
NH and cytosol protein-based tests[e]	–	–	+	++	–	–
Bulk milk tests[f] Milk I-ELISA or Milk ring-test	+++	–	+++	+	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; BBAT = buffered *Brucella* antigen tests (i.e. RBT [rose bengal test] and BPAT [buffered plate agglutination test]); FPA = fluorescence polarisation assay; CFT = complement fixation test; I- or C-ELISA = indirect/competitive enzyme-linked immunosorbent assay; BST = brucellin skin test; SAT = serum agglutination test; NH = native hapten

[a]This applies only to herds/flocks, countries or zones free from infection with *Brucella*.

[b]To increase the efficiency of eradication policies in infected herds/flocks, it is recommended to associate tests in parallel so as to increase the sensitivity of the diagnosis, i.e. two serological tests at least, e.g. BBAT or FPA and CFT or I-ELISA. The sensitivity is further increased by parallel testing by both serology and BST.

[c]In low-prevalence or almost-free zones, the predictive value of positive results to serological tests may be very low. In such situations, agent identification is usually needed to confirm clinical cases.
In infected herds/flocks, a positive result to any serological test may be considered as confirmation of a clinical case. Any reactor in any serological test should be considered to be infected even in the absence of clinical signs.
In low-prevalence or almost-free zones, singleton serological reactors may be confirmed by culture (or PCR) or BST.
In free countries or zones, suspect animals are those positive to both a screening and a confirmatory serological test (tests in series) and may be confirmed by culture (or PCR) and/or BST.

[d]False-positive results may occur.

[e]In zones where subcutaneous S19 or Rev.1 vaccination is practised, this test may help in differentiating antibodies due to vaccination from those due to infection.

[f]Dairy cattle only.

All cases of abortion as well as orchitis in cattle, sheep and goats, camels and pigs, should be considered as suspected brucellosis and should be investigated through the herd/flock history and submission of specimens for laboratory testing. The clinical signs are not pathognomonic and unequivocal diagnosis of *Brucella* infections can be made only by the isolation and identification of *Brucella*, but in situations where bacteriological examination is not practicable, diagnosis must be based on molecular or immunological methods.

1. Detection of the agent

Classically a bacterial culture is identified as *Brucella* by growth characteristics (see Section B.1.3 *Identification and typing*) although unequivocal identification as *Brucella* is now possible through various molecular approaches.

All samples from suspect cases should be cooled (4°C) immediately after they are taken, and transported to the laboratory by the most rapid means. If they are to spend more than 12 hours in transit, all samples apart from vaginal swabs, should be frozen (–20°C). On arrival at the laboratory, samples that are not to be cultured immediately should be frozen (Alton *et al*., 1988). In all cases, the shorter the shipment and storage time, the higher is the probability of *Brucella* isolation, especially in cases where the initial amount of *Brucella* is low in the sample. No specific transport medium has been demonstrated to improve *Brucella* survival in animal samples.

1.1. Staining methods

Brucella are coccobacilli or short rods measuring from 0.6 to 1.5 μm long and from 0.5 to 0.7 μm wide. They are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident. *Brucella* are nonmotile. They do not form spores; pili or true capsules are not produced. *Brucella* are Gram negative and usually do not show bipolar staining. They are resistant to decolourisation by weak acids and thus stain red by the Stamp's modification of the Ziehl–Neelsen's method (Alton *et al*., 1988). With this method, in smears of organs or biological fluids previously fixed with heat or ethanol, *Brucella* organisms stain red against a blue background. A fluorochrome or peroxidase-labelled antibody conjugate-based technique could also be used. The presence of intracellular, weakly acid-fast organisms of *Brucella* morphology or immuno-specifically stained organisms is presumptive evidence of brucellosis. However, these methods are not feasible or have a low sensitivity in milk and dairy products where *Brucella* are often present in small numbers, and interpretation is frequently impeded by the presence of fat globules. Care must be taken as well in the interpretation of positive results in the Stamp's method because other organisms that cause abortions, e.g. *Chlamydia abortus* or *Coxiella burnetii,* may be difficult to differentiate from *Brucella* organisms in these preparations. The results, whether positive or negative, should be confirmed by culture.

Polymerase chain reaction (PCR) methods can also be used to demonstrate the agent in various biological samples (Bricker, 2002; Whatmore & Gopaul, 2011), but the sensitivity of these approaches may be low with respect to classical bacteriology because of limitations around sample volume. Molecular tests may detect infection where poor sample storage means bacteria are no longer viable.

1.2. Collection of samples and culture

Bacteriological isolation is slow, expensive and cumbersome, but it should be performed whenever possible to confirm the disease and to determine the *Brucella* species involved. It also enables emerging epidemiological approaches such as high throughput sequencing to be applied. Although often considered not sensitive, it can be very effective when the type and number of samples, their adequate storage, amount seeded and the culture media used are optimised.

1.2.1. Basal media

Direct isolation and culture of *Brucella* are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants. However, the use of liquid media may be recommended for voluminous samples or for the purpose of enrichment. A wide range of commercial dehydrated basal media is available, e.g. *Brucella* medium base, tryptose (or trypticase)–soy agar (TSA). The addition of 2–5% bovine or equine serum is necessary for the growth of strains such as *B. abortus* bv. 2, and many laboratories systematically add serum to basal media, such as blood agar base or Columbia agar, with excellent results. Other satisfactory media, such as serum–dextrose agar (SDA) or glycerol–dextrose agar, can be used (Alton *et al*., 1988). SDA is usually preferred for

observation of colonial morphology. A non-selective, biphasic medium, known as Castañeda's medium, is recommended for the isolation of *Brucella* from blood and other body fluids or milk, where enrichment culture is advised. Castañeda's medium is used because brucellae tend to dissociate in broth medium, interfering with biotyping by conventional bacteriological techniques.

1.2.2. Selective media

All the basal media mentioned above can be used for the preparation of selective media. Appropriate antibiotics are added to suppress the growth of organisms other than *Brucella*.

The most widely used selective medium is the modified Farrell's medium (FM) (Stack *et al*., 2002), added to 1 litre of agar: polymyxin B sulphate (5000 units = 5 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); nystatin (100,000 units); vancomycin (20 mg). A corresponding freeze-dried antibiotic supplement is available commercially. However, nalidixic acid and bacitracin, at the concentration used in FM, have inhibitory effects on some *B. abortus*, *B. melitensis* and *B. suis* strains. Accordingly, the simultaneous use of FM and the less selective Thayer–Martin's modified (mTM) culture media has been considered the strategy of choice for *Brucella* primary isolation from field veterinary samples. However, the mTM is not translucent because of the haemoglobin contained as a basal component, being thus unsuitable for the direct observation of colonial morphology, probably the most practical procedure for the presumptive identification of *Brucella* (Alton *et al*., 1988).

A selective and translucent culture medium (named CITA) was formulated by De Miguel *et al*. (2011). For its preparation, blood agar base is used as a basal component, supplemented with 5% sterile calf serum and containing vancomycin (20 mg/litre), colistin methanesulfonate (7.5 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU)]/litre), and amphotericin B (4 mg/litre). This antibiotic mixture can be prepared as follows: weigh vancomycin, colistin and nystatin in the same 50 ml sterile container, then rehydrate the mixture with 10 ml of a 1:1 solution of absolute methanol in sterile purified water. Weigh then nitrofurantoin in a sterile tube and dissolve it with 1 ml of 0.1 M NaOH solution (sterilised previously by filtration through a 0.22 μm filter). Finally, weigh 10 mg of amphotericin B in a 20 ml sterile container and dissolve with 1 ml dimethyl sulphoxide. Once fully dissolved (5–10 minutes are required), add 9 ml of 10 mM sterile phosphate-buffered saline (PBS) (pH=7.2 ± 0.2). The final concentration of amphotericin B would be 1 mg/ml; a total of 4 ml of this solution are required for 1 litre of medium. The remaining Amphotericin B suspension can be kept at 5°C ± 3°C for several days for further uses. This CITA medium inhibits most contaminant microorganisms but allows simultaneously the growth of all *Brucella* species and is more sensitive than both mTM and Farrell's media for isolating all smooth *Brucella* species from field samples, being thus the selective medium of choice for overall *Brucella* isolation, although the maximal diagnostic sensitivity is obtained using both FM and CITA simultaneously (De Miguel *et al*., 2011).

A modified *Brucella* selective medium (named MBS) has been developed to select *B. abortus* strains, including the RB51 vaccine strain more effectively than previously described media but needs further validation studies to confirm performance (Her *et al*., 2010).

Contrary to the situation with several *B. abortus* biovars as well as *B. ovis*, the growth of *B. melitensis* or *B. suis* is not dependent on an incubating atmosphere containing 5–10% CO_2 (Table 2), but such a CO_2 enriched-atmosphere is optimal for the culture of all *Brucella*.

As the number of *Brucella* organisms is likely to be lower in milk, colostrum and some tissue samples than in abortion material, enrichment can be advisable. In the case of milk, results can be improved by centrifugation and culture from both the cream and the pellet, but strict safety measures should be implemented in this case to avoid aerosols. A more practical way to increase the sensitivity of milk culture while avoiding the risks of centrifugation is increasing the number of both FM and CITA culture plates per milk sample tested (two plates per udder quarter should be a minimum), each plate being inoculated with ca. 0.5 ml of milk. Enrichment can be carried out in liquid medium consisting of serum–dextrose broth, tryptose broth or trypticase–soy broth (TSB) or *Brucella* broth supplemented with an antibiotic mixture of at least amphotericin B (1 μg/ml), and vancomycin (20 μg/ml) (all final concentrations). The enrichment medium should be incubated at 37°C ± 2°C in air supplemented with 5–10% (v/v) CO_2 for up to 6 weeks, with weekly subcultures on to solid FM and CITA selective media. If preferred, a biphasic system of

solid and liquid selective medium in the same bottle (Castañeda's method) may be used to minimise subculture. A selective biphasic medium composed of the basal Castañeda's medium with the addition of the following antibiotics to the liquid phase, is sometimes recommended for isolation of *Brucella* in milk (quantities are per litre of medium): polymyxin B (sulphate) (6000 units = 6 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); amphotericin B (1 mg); vancomycin (20 mg); D-cycloserine (100 mg).

***Table 2. Differential characteristics of species of the genus* Brucella**

Species	Colony morphology[b]	Serum requirement	Lysis by phages[a]					Oxidase	Urease activity	Preferred host
			Tb		Wb	Iz_1	R/C			
			RTD[c]	10^4 RTD	RTD	RTD	RTD			
B. abortus	S	–[d]	+	+	+	+	–	(+)[e]	(+)[f]	Cattle and other *Bovidae*
B. melitensis	S		–	–	(–)[g]	+	–	(+)	+[h]	Sheep and goats
B. suis	S	–	–	+	(+)[i]	(+)[i]	–	+	+[j]	Bv. 1: swine Bv. 2: swine, hare Bv. 3: swine Bv. 4: reindeer Bv. 5: rodents
B. neotomae	S	–	–[k]	+	+	+	–	–	+[j]	Desert wood rat[l]
B. ovis	R	+	–	–	–	–	+	–	–	Sheep
B. canis	R	–	–	–	–	–	+	+	+[j]	Dogs
B. ceti	S	ND	(–)		(+)	(+)	–	(+)	+	Cetaceans
B. pinnipedialis	S	ND	(–)		(+)	(+)	–	(+)	+[h]	Pinnipeds
B. microti	S	–	–	+	+	+	ND	+	+[h]	Unknown[n]
B. inopinata	S	ND	–	ND	ND	ND	ND	ND	+[j]	Unknown
B. papionis	S		PL[m]	PL[m]	+	ND	–	–	+[j]	Unknown
B. vulpis	S		+		+	+	–	–	+	Unknown

From Alton *et al.* (1988), Joint FAO/WHO Expert Committee on Brucellosis (1986), Whatmore (2009), Whatmore *et al.* (2014) and Scholz *et al* (2016).

(+)/(–) Most isolates positive/negative

a Phages: Tbilisi (Tb), Weybridge (Wb), Izatnagar1 (Iz_1) and R/C

b Normally occurring phase: S: smooth, R: rough

c RTD: routine test dilution

d *B. abortus* bv. 2 generally requires serum for growth on primary isolation

e Some African isolates of *B. abortus* bv. 3 are negative

f Intermediate rate, except strain 544 and some field strains that are negative

g Some isolates are lysed by Wb

h Slow rate, except some strains that are rapid

l Some isolates of *B. suis* bv. 2 are not or only partially lysed by phage Wb or Iz_1

j Rapid rate

k Minute plaques

l *Neotoma lepida*

m Partial lysis

n strains isolated from many wild mammals (rodent, fox, wild boar), amphibians and environment

ND Not determined

All culture media used should be subjected to quality control with the reference strains to show that it performs properly. The use of a small inoculum of fastidious strains, such as *B. abortus* bv. 2, *B. ovis* or *B. suis* bv. 2, is preferred.

On suitable solid media, colonies of *B. abortus*, *B. melitensis* and *B. suis* can be clearly visible after a 3- to 4-day incubation period. After 4-days' incubation, *Brucella* colonies are round, 1–2 mm in diameter, with smooth margins. They are translucent and a pale honey colour when plates are viewed through a transparent medium. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker. Smooth (S) *Brucella* cultures have a tendency to undergo variation during growth, especially with subcultures, and to dissociate to rough (R) forms. Colonies are then much less transparent, have a more granular, dull surface, and range in colour from matt white to brown in reflected or transmitted light. Checking for dissociation is easily tested by crystal violet staining: rough colonies stain red/violet and smooth colonies do not uptake dye or stain pale yellow. If the colonies are smooth, they should be checked against antiserum to smooth *Brucella*, or, if available, against anti-A and -M monospecific sera. In the case of non-smooth colonies, isolates should be checked with antiserum to *Brucella* R antigen. Changes in the colonial morphology are generally associated with changes in virulence, serological properties or phage sensitivity. Typical colonial morphology and positive agglutination with specific *Brucella* antiserum, followed by the oxidase and urease tests (see Tables 2 and 3), allow preliminary identification of the isolate as *Brucella*. However, it is recommended that subsequent confirmation and typing is performed by a reference laboratory.

1.2.3. Collection and culture of samples

For the diagnosis of animal brucellosis by cultural examination, the choice of samples usually depends on the clinical signs observed. The most valuable samples include vaginal secretions (swabs), aborted fetuses (stomach contents, spleen and lung), fetal membranes, and milk, semen and arthritis or hygroma fluids. From animal carcasses, the preferred tissues for culture are those of the reticulo-endothelial system (i.e. head, mammary and genital lymph nodes and spleen), the pregnant or early post-parturient uterus, and the udder. Growth normally appears after 3–4 days, but cultures should not be discarded as negative until 7–10 days have elapsed. When *Brucella* are present in small numbers, isolation from such samples is very unlikely so enrichment culture is advised and molecular detection may be considered as a parallel complementary diagnostic to increase sensitivity.

1.2.3.1. Tissues

Samples are removed aseptically with sterile instruments. The tissue samples are prepared by removal of extraneous material (e.g. fat), cut into small pieces, and macerated using a paddle blender or tissue grinder with a small amount of sterile PBS, before being inoculated on to solid media or enrichment broth.

1.2.3.2. Vaginal discharge

A vaginal swab taken after abortion or parturition is an excellent source for the recovery of *Brucella* and far less risky for the personnel than abortion material. The swab is then streaked directly onto solid media.

1.2.3.3. Milk

Milk culture can be particularly valuable for screening individual animals or herds for the presence of *Brucella*. Samples of milk must be collected cleanly after washing, drying and disinfecting the teats. Personal protection must be worn throughout. It is essential that samples should contain milk from all quarters, and 10–20 ml of milk should be taken from each teat, changing or disinfecting the gloves from one animal to the next to avoid cross-contamination of the samples. The first streams are discarded and the sample is milked directly into a sterile vessel or container. Care must be taken to avoid contact between the milk and the milker's hands. The milk can be centrifuged and the cream and deposit are spread on solid selective medium, either separately or mixed or streaked directly as indicated above. If *Brucella* are present in bulk milk samples, their numbers are usually low, and isolation from such samples is very unlikely.

Table 3. Differential characteristics of the biovars of* Brucella *species

Species	Biovar	CO_2 requirement	H_2S production	Growth on dyes[a]		Agglutination with monospecific sera			Reference strain		
				Thionin	Basic Fuchsin	A	M	R	Strain	ATCC	NCTC
B. abortus	1	(+)[b]	+	–	+	+	–	–	544	23448	10093
	2	(+)[b]	+	–	–	+	–	–	86/8/59	23449	10501
	3[c]	(+)[b]	+	+	+	+	–	–	Tulya	23450	10502
	4	(+)[b]	+	–	(+)	–	+	–	292	23451	10503
	5	–	–	+	+	–	+	–	B3196	23452	10504
	6[c]	–	(–)	+	+	+	–	–	870	23453	10505
	9	+/–	+	+	+	–	+	–	C68	23455	10507
B. melitensis	1	–	–	+	+	–	+	–	16M	23456	10094
	2	–	–	+	+	+	–	–	63/9	23457	10508
	3	–	–	+	+	+	+	–	Ether	23458	10509
B. suis	1	–	+	+	(–)	+	–	–	1330	23444	10316
	2	–	–	+	–	+	–	–	Thomsen	23445	10510
	3	–	–	+	+	+	–	–	686	23446	10511
	4	–	–	+	(–)	+	+	–	40	23447	11364
	5	–	–	+	–	–	+	–	513	ND	11996
B. neotomae		–	+	–[d]	–	+	–	–	5K33	23459	10084
B. ovis		+	–	+	(–)	–	–	+	63/290	25840	10512
B. canis		–	–	+	(–)	–	–	+	RM6/66	23365	10854
B. ceti		(–)	–	(+)	(+)	+	(–)	–	B1/94 *BCCN 94-74*	ND	12891
B. pinnipedialis		(+)	–	+	(+)	(+)	(–)	–	B2/94 *BCCN 94-73*	ND	12890
B. microti		–	–	+	+	(–)	(+)	–	CCM4915 *BCCN 07-01* *CAPM 6434*	ND	ND
B. inopinata		–	+	+	+	–	+[e]		BO1 *BCCN 09-01* *CAPM 6436*	ND	ND
B. papionis		–	–	–	–	+	–	–	F8/08-60 CIRMBP 0958	ND	13660
B. vulpis		–	–	+	+	+	–		F60 BCCN 09-2 DSM 101715	ND	ND

From Alton *et al.* (1988), Joint FAO/WHO Expert Committee on Brucellosis (1986), Whatmore (2009), Whatmore *et al.* (2014), and Scholz *et al.* (2016).

(+)/(–) Most isolates positive/negative

a Dye concentration in serum dextrose agar: 20 μg/ml

b Usually positive on primary isolation

c For more certain differentiation of bv. 3 and 6, thionin at 40 μg/ml is used in addition: bv. 3 = +, bv. 6 = –

d Growth at a concentration of 10 μg/ml thionin

e Weak agglutination

ND Not determined

1.2.3.4. Dairy products

Dairy products, such as cheese, should be cultured on the media described above. As these materials are likely to contain small numbers of organisms, enrichment culture with selective media is advised. Samples need to be carefully homogenised before culture, after they have been ground in a tissue grinder or macerated and pounded in a 'paddle blender or an electric blender with an appropriate volume (avoiding over-dilution) of sterile PBS. Superficial strata (rind and underlying parts) and the core of the product should be cultured. As brucellae grow, survive or disappear quite rapidly, their distribution throughout the different parts of the product varies according to the local physico-chemical conditions linked to specific process technologies.

1.2.3.5. Arthritis/hygroma fluids – abscesses content

Such samples must be collected aseptically and spread directly on solid selective media.

All the above samples should be cooled (4–10°C) immediately after sampling and transported to the laboratory in the fastest way. Otherwise, the samples should be frozen to avoid viability losses. On arrival at the laboratory, milk and tissue samples and other biological liquids should be frozen if they are not to be cultured immediately.

1.2.3.6. Blood culture

Culture from blood can be attempted although bacteraemia in livestock animals is generally considered short lived or intermittent so the approach is not widely used. Direct culturing of anti-coagulant treated blood (sodium citrate or heparin, except EDTA [ethylene diamine tetra-acetic acid]) may be performed on selective or non-selective agar to obtain results in a shorter time (4–7 days; Alton, 1988). Alternatively an appropriate non-selective media, such as TSB, can be inoculated with blood and subcultured at weekly intervals onto selective Farrell's media for up to 4 weeks.

1.2.3.7. Animal passage

Although used historically, use of laboratory animals should be avoided unless absolutely necessary, but may sometimes provide the only means of detecting the presence of *Brucella,* especially when samples have been shown to be heavily contaminated or are likely to contain a low number of *Brucella* organisms. Animal inoculation may be intravenously or intraperitoneally in mice or intra-muscularly, subcutaneously or intraperitoneally in guinea-pigs. This work must be carried out under appropriate biosafety conditions as outlined in chapter 1.1.4. The spleens of inoculated animals are cultured at 7 days (mice) or 3–6 weeks (guinea-pigs) after inoculation. Serum samples can be collected by intra-cardiac puncture before necropsy from guinea-pigs and subjected to buffered *Brucella* antigen tests (BBAT); a positive serological result is highly suggestive of brucellosis (Alton *et al*., 1988).

1.3. Identification and typing

Any colonies showing the characteristic *Brucella* morphology should be examined using a Gram-stained-smear. As the serological properties, dyes and phage sensitivity are usually altered in the non-smooth phases, attention to the colonial morphology is essential in the typing tests described below. The recommended methods for observing colonial morphology are Henry's method by obliquely reflected light, the acriflavine test described by Braun & Bonestell, or White & Wilson's crystal violet method of staining colonies (Alton *et al*., 1988).

Identification of *Brucella* organisms to species and biovar level can be carried out by a combination of the following tests: organism morphology after Gram or Stamp's staining, direct observation of colonial morphology, growth characteristics, urease and oxidase tests, and the slide agglutination test with a polyclonal anti-*Brucella* serum. Species and biovar identification requires elaborate tests (such as phage lysis and agglutination with anti-A, -M or -R monospecific sera), the performance of which should be left to reference laboratories with accredited expertise in these methods. The simultaneous use of several phages e.g. Tbilisi (Tb), Weybridge (Wb), Izatnagar$_1$ (Iz$_1$) and R/C provides a phage-typing system that, in experienced hands, allows a practical identification of the *Brucella* species. However, several characteristics, for example added CO_2 requirement for growth, production of H_2S (detected by lead

acetate papers), and growth in the presence of basic fuchsin and thionin, are revealed by routine tests that can be performed in moderately equipped non-specialised laboratories (see Tables 2 and 3). While the technique remains useful particularly at the species level, the value of the biovar designations as epidemiological markers is increasingly questioned as, notably in the case of *B. melitensis* and some *B. abortus* biovars, molecular evidence has shown biovars do not correspond to meaningful genetic divisions.

MALDI-TOF (matrix assisted laser desorption ionisation time of flight) is increasingly used in diagnostic microbiology and has been applied to identification of *Brucella*. While application of MALDI-TOF appears effective for genus identification the close genetic relationship among *Brucella* species, and limitations in commercial database coverage, has not yet enabled robust and unambiguous discrimination of *Brucella* species.

For the maintenance of *B. abortus, B. melitensis* or *B. suis* strains as well as for sending them to a reference laboratory for typing, it is essential that only smooth colonies be selected. Cultures may be maintained for short periods at 5°C ± 3°C, but for longer periods they should be lyophilised or stored in a screw-capped tube at a temperature ≤ –16°C in tryptose broth with 15% (v/v) glycerol. For shipment, cultures should be lyophilised and sealed in ampoules packed in screw-capped canisters or subcultured onto appropriate nutrient agar slopes contained in screw-capped bottles. The strains could also be sent suspended in transport media (e.g. Amies), but this could cause dissociation.

For transporting *Brucella* cultures, the caps of the bottles or canisters should be screwed tightly down and sealed with PVC (polyvinyl chloride) tapes. Bottles should be wrapped in absorbent paper or cotton wool, sealed in polyethylene bags and packed into a rigid container (triple packaging) in accordance with the requirements of the International Air Transport Association (IATA) for shipping dangerous goods (IATA, 2021). These regulations are summarised in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*, and they must be followed.

1.4. Nucleic acid recognition methods

The PCR, including the real-time format, provides an additional means of detection and identification of *Brucella* sp. (Bricker, 2002; Lopez-Goni *et al*., 2011; Ocampo-Sosa *et al*., 2005; Whatmore & Gopaul, 2011). Despite the high degree of DNA homology within the genus *Brucella*, several historical molecular methods including PCR, PCR restriction fragment length polymorphism (RFLP) and Southern blot, allowed, to a certain extent, the differentiation of *Brucella* species and some of their biovars (for a review see Bricker, 2002; Moreno *et al*., 2002; Whatmore & Gopaul, 2011).

The first species-specific multiplex PCR assay for the differentiation of *Brucella* was described by Bricker & Halling (1994). The assay, named AMOS-PCR, was based on the polymorphism arising from species-specific localisation of the insertion sequence IS711 in the *Brucella* chromosome, and comprised five oligonucleotide primers that could identify without differentiating *B. abortus* bv. 1, 2 and 4 but could not identify *B. abortus* bv. 3, 5, 6, and 9. Modifications to the assay have been introduced over time to improve performance, and additional strain-specific primers were incorporated for identification of the *B. abortus* vaccine strains, and other biovars and species (Ocampo-Sosa *et al*., 2005). A multiplex PCR assay (Bruce-ladder) has been proposed for rapid and simple one-step identification of *Brucella* (Lopez-Goni *et al*., 2011). The major advantage of this assay over previously described PCRs is that it can identify and differentiate in a single step most *Brucella* species as well as the vaccine strains *B. abortus* strain 19 (S19), *B. abortus* RB51 and *B. melitensis* Rev.1. In contrast to other PCRs, Bruce-ladder is also able to detect DNA from *B. neotomae, B. pinnipedialis* and *B. ceti*. An update to the original Bruce-ladder PCR protocol has been described. This updated version (Bruce-ladder v2.0), that has been validated in several laboratories, is also able to discriminate between *B. suis* and *B. canis*, and allows the differentiation of *B. microti* as does a modification reported by Kang *et al.* (2011). Similarly, another updated multiplex PCR assay (Suis-ladder), has been developed for fast and accurate identification of *B. suis* strains at the biovar level (Lopez-Goni *et al*., 2011).

Alternative approaches allowing identification of all *Brucella* species, *B. suis* biovars and vaccine strains based on single nucleotide polymorphism (SNP) discrimination by either primer extension or real-time PCR or the ligase-chain-reaction have been described. These tests are rapid, simple, unambiguous, and based on a robust population genetic analysis that helps ensure the species/biovar specificity of markers used (Whatmore & Gopaul, 2011).

A number of other methods adding useful epidemiological information have also been described and are widely used. These include multilocus sequencing schemes (Whatmore & Foster, 2021) and several typing schemes based on the use of MLVA (multiple locus variable number of tandem repeats analysis) (Le Fleche *et al*., 2006; Scholz & Vergnaud, 2013; Whatmore & Foster, 2021). Depending on the particular markers chosen, these methods allow isolates to be identified at species level and provide epidemiological information at the subspecies level. Finally, whole genome sequence-based approaches are rapidly becoming tools for routine surveillance and outbreak detection for several infectious diseases including brucellosis. Through various analytical approaches being developed, including SNP-based approaches or gene-by-gene comparison through core-genome multilocus sequence typing (cgMLST), it is already clear that whole genome sequence approaches will offer the ultimate level of epidemiological precision. These tools will become more widely accessible with time and ultimately greatly inform understanding of local and international brucellosis epidemiology.

1.5. Identification of vaccine strains

Vaccine strains *B. abortus* S19, *B. melitensis* Rev.1 and *B. abortus* RB51 may be identified using specific PCRs (Kang *et al*., 2011; Lopez-Goňi *et al*., 2011), or by their growth characteristics in culture.

Brucella abortus S19 has the typical properties of a bv. 1 strain of *B. abortus*, but does not require CO_2, does not grow in the presence of benzyl-penicillin (3 μg/ml = 5 IU/ml), thionin blue (2 μg/ml), or i-erythritol (1 mg/ml) (all final concentrations), and presents a high L-glutamate use (Alton *et al*., 1988).

Brucella melitensis strain Rev.1 has the typical properties of a bv. 1 strain of *B. melitensis*, but develops smaller colonies on solid media, does not grow in the presence of basic fuchsin, thionin (both at 20 μg/ml) or benzyl-penicillin (3 μg/ml), but does grow in the presence of streptomycin at 2.5 or 5 μg/ml (5 IU/ml) (Alton *et al*., 1988).

Brucella abortus strain RB51 can be distinguished from its *B. abortus* biovar 1 smooth counterparts by its rough morphology and growth in presence of rifampicin (250 μg per ml of media).

2. Serological tests

No single serological test is appropriate in all epidemiological situations and all animal species; all tests have limitations especially when testing individual animals. Consideration should be given to all factors that impact on the relevance of the test method and test results to a specific diagnostic interpretation or application. In epidemiological units where vaccination with smooth *Brucella* is practised, and depending on the vaccination method (dose/route) used, positive serological reactions may be expected among the vaccinated animals because of antibodies cross-reacting with wild strain infection. Moreover, a number of bacteria, in particular *Yersinia enterocolitica* O:9, may induce antibody responses that cause false positive serological reactions (FPSR) in brucellosis tests, impeding accurate serological diagnosis. FPSR may occur in all animal species at variable rates according to the time and the region.

The serum agglutination test (SAT) is generally regarded as being unsatisfactory for the purposes of international trade. The complement fixation test (CFT) is more specific than the SAT, and has also a standardised system of unitage, but can be impacted by anti-complementary activity. The diagnostic performance characteristics of some enzyme-linked immunosorbent assays (ELISAs) and the fluorescence polarisation assay (FPA) are comparable with or better than that of the CFT, and as they are technically simpler to perform and more robust, their use may be preferred. The diagnostic performance of these tests has been compared in cattle, small ruminants and swine.

For the control of brucellosis at the national or local level, BBATs (the rose bengal test [RBT] and the buffered plate agglutination test [BPAT]), ELISA and FPA, are considered as suitable screening tests. Depending on the purpose of testing, positive reactors could be retested using a suitable confirmatory or complementary method.

In other species, for example, buffaloes (*Bubalus bubalis*), American and European bison (*Bison bison, Bison bonasus*), yak (*Bos grunniens*), elk/wapiti (*Cervus elaphus*), camels (*Camelus bactrianus* and *C. dromedarius*), and South American camelids, *Brucella* sp. infection follows a course similar to that in cattle. The same serological procedures may be used for these animals, but each test should be validated for its fitness in the corresponding animal species.

2.1. Reference sera

WOAH reference standards are those against which all other standards are compared and standardised. These reference standards are all available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.

These sera have been developed and designated by WOAH as International Standard Sera[2]. The use of these reagents promotes international harmonisation of diagnostic testing and antigen standardisation:

i) For RBT, CFT, SAT and milk ring test (MRT), WOAH International Standard Serum (WOAHISS, previously named the WHO Second International standard anti-Brucella abortus Serum; WHO, 1953) is used. This serum is of bovine origin and contains 1000 IU (SAT) and 1000 ICFTU (international complement fixation test units).

ii) For indirect ELISA (I-ELISA), competitive or blocking ELISA (C-ELISA) and FPA in cattle, three WOAH ELISA Standard Sera are available for use. These are also of bovine origin and consist of a strong positive ($WOAHELISA_{SP}SS$), a weak positive ($WOAHELISA_{WP}SS$) and a negative ($WOAHELISA_{N}SS$) standard.

iii) For I-ELISA, C-ELISA and FPA in sheep and goats, the International standard anti-*Brucella melitensis* Serum (ISaBmS) is used (McGiven *et al*., 2011).

iv) For I-ELISA, C-ELISA and FPA in pigs, there is no WOAH International Standard serum available at present. However, an EU standard for porcine I-ELISA and C-ELISA, EUPigBSS, is available[3].

2.2. Production of antigens

Brucella abortus strain 99 (Weybridge) (S99)[3] or *B. abortus* strain 1119-3 (USDA) (S1119-3)[4] should always be used for the production of antigens for the BBATs, SAT, CFT and FPA. These *B. abortus* strains can be also used as a source of soluble antigen extracts (smooth lipopolysaccharide [S-LPS] or O-polysaccharide [OPS]) for the ELISAs or the Native Hapten tests, but *B. melitensis* strain 16M is also suitable for such a purpose. It should be emphasised that antigen made with any of the two *B. abortus* or *B. melitensis* 16M strains is used to test for any infections due to smooth *Brucella* species.

The strains must be completely smooth and should not auto-agglutinate in saline and 0.1% (w/v) acriflavine. They must be pure cultures and conform to the characteristics of CO_2-independent strains of *B. abortus* bv. 1 or *B. melitensis* bv. 1. The original seed cultures should be propagated to produce a seed lot that must conform to the properties of these strains, and should be preserved by lyophilisation or by freezing in liquid nitrogen.

For antigen production, the seed culture is used to inoculate a number of potato-infusion agar slopes that are then incubated at 37°C ± 2°C for 48 hours. SDA and TSA, to which 5% equine or new-born calf serum or 0.1% yeast extract may be added, are satisfactory solid media provided a suitable seed is used as recommended above. The growth is checked for purity, resuspended in sterile PBS, pH 6.4, and used to seed layers of potato-infusion agar or glycerol-dextrose agar in Roux flasks. These are then incubated at 37°C ± 2°C for 72 hours with the inoculated surface facing down. Each flask is checked for purity by Gram staining samples of the growth, and the organisms are harvested by adding 50–60 ml phenol saline (0.5% phenol in 0.85% sodium chloride solution) to each flask. The flasks are gently agitated, the suspension is decanted, and the organisms are killed by heating at 80°C for 90 minutes. Following a viability check, the antigen is stored at 5°C ± 3°C.

Alternatively, the cells may be produced by batch or continuous culture in a fermenter, using a liquid medium containing (per litre of purified water) D-glucose (30 g), a high-grade peptone (30 g), yeast extract (Difco) (10 g), sodium dihydrogen phosphate (9 g) and disodium hydrogen phosphate (3.3 g). The initial pH is 6.6 (± 0.2), but this tends to rise to pH 7.2 (± 0.2) during the growth cycle. Care should be taken to check batches of peptone and yeast extract for capacity to produce good growth without formation of abnormal or dissociated cells. Vigorous aeration and stirring is required during growth, and adjustment

2 Obtainable from the WOAH Reference Laboratory for brucellosis in the United Kingdom (see online list of WOAH Reference Laboratories for address: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

3 Obtainable from the WOAH Reference Laboratory for brucellosis in France.

4 Obtainable from the United States Department of Agriculture (USDA), National Veterinary Services Laboratories (NVSL) 1800 Dayton Road, Ames, Iowa, United States of America.

to pH 7.2 (± 0.2) by the addition of sterile 0.1 M HCl may be necessary. The seed inoculum is prepared as described above. The culture is incubated at 37°C ± 2°C for 48 hours. Continuous culture runs can be operated for much longer periods, but more skill is required to maintain them. In-process checks should be made on the growth from either solid or liquid medium to ensure purity, an adequate viable count and freedom from dissociation to rough forms. Cells for use in the preparation of all antigens should be checked for purity and smoothness at the harvesting stage.

The culture is harvested by centrifugation to deposit the organisms, which are resuspended in phenol saline. The organisms are killed by heating at 80°C for 90 minutes and are stored at 5°C ± 3°C. They must form stable suspensions in physiological saline solutions and show no evidence of auto-agglutination. A viability check must be performed on the suspensions and no growth must be evident after 10 days of incubation at 37°C ± 2°C. The packed cell volume (PCV) of the killed suspensions can be determined by centrifuging 1 ml volumes in Wintrobe tubes at 3000 ***g*** for 75 minutes.

2.3. Buffered *Brucella* antigen tests (BBAT)

2.3.1. Rose bengal test

This test is a simple spot agglutination test using antigen stained with rose bengal and buffered to a low pH, 3.65 ± 0.05 (Morgan *et al*., 1969).

2.3.1.1. Antigen production

Antigen for the RBT is prepared by depositing killed *B. abortus* S99 or S1119-3 cells by centrifugation at 23,000 ***g*** for 10 minutes (or 14,000 ***g*** for 40 minutes) at 5°C ± 3°C, and uniformly resuspending in sterile phenol saline (0.5%) at the rate of 1 g to 22.5 ml. (Note: if sodium carboxymethyl cellulose is used as the sedimenting agent during preparation of the cell concentrate, insoluble residues must be removed by filtering the suspension through an AMF-CUNO Zeta-plus prefilter [Type CPR 01A] before staining.) In case of production of cells in liquid media (e.g. in bioreactor), it is possible to concentrate the bacterial suspension (up to 40 times) before centrifugation, using a tangential flow filtration system (TFFS) with 0.1 μm cassette and doing three washes (1:3 ratio) with sterile phenol saline (0.5%). To every 35 ml of this suspension, 1 ml of 1% (w/v) rose bengal (CI No. 45440) in sterile purified water is added, and the mixture is stirred for 2 hours at room temperature. The mixture is filtered through sterile cotton wool, and centrifuged at 10,000 ***g*** to deposit the stained cells, which are then uniformly resuspended at the rate of 1 g cells to 7 ml of diluent (21.1 g of NaOH dissolved in 353 ml of sterile phenol saline, followed by 95 ml of lactic acid, and adjusted to 1056 ml with sterile phenol saline). The colour of this suspension should be an intense pink and the supernatant of a centrifuged sample should be free of stain; the pH should be 3.65 ± 0.05. After filtration through cotton wool, the suspension is filtered twice through a Sartorius No. 13430 glass fibre prefilter, adjusted to a PCV of approximately 8%, pending final standardisation against serum standardised against the WOAHISS, and stored at 5°C ± 3°C in the dark. The antigen should be stored as recommended by the manufacturer. It should not be frozen.

2.3.1.2. Antigen standardisation

When used in the standard test procedure, the RBT antigen should give a clearly positive reaction with 1/45 dilution, but not 1/55 dilution, of the WOAHISS diluted in 0.5% phenol saline or normal saline.

Additional checks may be performed with the ISaBmS. The highest dilution (in negative goat serum) of this standard that must give a positive result and the lowest dilution (in negative goat serum) that must simultaneously give a negative result have been established at 1/16 and 1/200, respectively (McGiven *et al*., 2011).

It is also be advisable to compare the reactivity of new and previously standardised batches of antigen using a panel of well-defined reference sera.

However the above standardisation against the WOAHISS is probably a cause of the reduced sensitivity of some RB antigen batches for diagnosing *B. melitensis* infection in small ruminants and of the discrepancies with the CFT (Blasco *et al*., 1994a). When testing small ruminants, the discrepancies with the CFT can be minimised by using three volumes

of serum and one volume of antigen (e.g. 75 µl and 25 µl, respectively) in place of an equal volume of each as mentioned in the standard test procedure. However, this modification of the RBT should not be recommended for testing cattle and pig sera.

2.3.1.3. Test procedure

i) Bring the serum samples and antigen to room temperature (22°C ± 4°C); only sufficient antigen for the day's tests should be removed from the refrigerator.

ii) Place 25–30 µl of each serum sample on a white tile, enamel or plastic plate, or in a WHO haemagglutination plate.

iii) Shake the antigen bottle well, but gently, and place an equal volume of antigen near each serum spot.

iv) Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen thoroughly (using a clean glass or plastic rod for each test) to produce a circular or oval zone approximately 2 cm in diameter.

v) The mixture is agitated gently for 4 minutes at room temperature (22°C ± 4°C) on a rocker or three-directional agitator (if the reaction zone is oval or round, respectively).

vi) Read for agglutination immediately after the 4-minute period is completed. Any visible coloured agglutination is considered to be a positive reaction. A control serum that gives a minimum positive reaction should be tested before each day's tests are begun to verify the sensitivity of test conditions.

The RBT is very sensitive. However, like all other serological tests, it could sometimes give a positive result in cattle because of *B. abortus* S19 vaccination or FPSR. The same phenomenon occurs in small ruminants or pigs affected by FPSR and in small ruminants vaccinated with *B. melitensis* Rev.1. Therefore positive reactions should be investigated using suitable confirmatory or complementary strategies (including epidemiological investigation). Conversely, false-negative reactions occur rarely. Nevertheless RBT appears to be adequate as a screening test for detecting infected herds or to guarantee the absence of infection in brucellosis-free herds or flocks.

2.3.2. Buffered plate agglutination test

2.3.2.1. Antigen production

Antigen for the BPAT is prepared from *B. abortus* S1119-3 according to the procedure described by Angus & Barton (1984).

Two staining solutions are required: brilliant green (2 g/100 ml) and crystal violet (1 g/100 ml) both certified stains dissolved in purified water. Once prepared, the two solutions should be stored separately for a period of 24 hours, and then mixed together in equal volumes in a dark bottle and stored in a refrigerator for a period of not less than 6 months before use. The mixed stain may only be used between 6 and 12 months after initial preparation.

Buffered diluent is prepared by slowly dissolving sodium hydroxide (150 g) in 3–4 litres of sterile phenol saline. Lactic acid (675 ml) is added to this solution, and the final volume is adjusted to 6 litres by adding sterile phenol saline. The pH of the solution should be 3.65 ± 0.05.

Brucella abortus S1119-3 packed cells are diluted to a concentration of 250 g/litre in phenol saline; 6 ml of stain is added per litre of cell suspension, and the mixture is shaken thoroughly before being filtered through sterile absorbent cotton. The cells are centrifuged at 10,000 ***g*** at 5°C ± 3°C, and the packed cells are then resuspended at a concentration of 50 g/100 ml in buffered diluent (as described above). This mixture is shaken thoroughly for 2 hours, and is then further diluted by the addition of 300 ml of buffered diluent per 100 ml of suspended cells (i.e. final concentration of 50 g packed cells/400 ml buffered diluent). The mixture is stirred at room temperature for 20–24 hours before the cell concentration is adjusted to 11% (w/v) in buffered diluent. This suspension is stirred overnight before testing. Pending final

quality control tests, the antigen is stored at 5°C± 3°C until required for use. The antigen should not be frozen.

The pH of the buffered plate antigen should be 3.70 ± 0.03 and the pH of a serum–antigen mixture at a ratio of 8:3 should be 4.02 ± 0.04. The 11% stained-cell suspension should appear blue–green. Each batch of buffered plate antigen should be checked by testing at least 10 weakly reactive sera and comparing the results with one or more previous batches of antigen. If possible, the antigen batches should be compared with the standard antigen prepared by the NVSL, USDA (see footnote 4 for address). There is, however, no international standardisation procedure established for use with either the WOAHISS or with the ISaBmS.

2.3.2.2. Test procedure

i) Bring the serum samples and antigen to room temperature (22°C ± 4°C); only sufficient antigen for the day's tests should be removed from the refrigerator.

ii) Shake the sample well. Place 80 µl of each serum sample on a glass plate marked in 4 × 4 cm squares

iii) Shake the antigen bottle well, but gently, and place 30 µl of antigen near each serum spot.

iv) Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen.

v) Thoroughly (using a clean glass or plastic rod for each test) to produce a circular zone approximately 3 cm in diameter.

vi) After the initial mixing, the plate should be rotated three times in a tilting motion to ensure even dispersion of the reagents, and then incubated for 4 minutes in a humid chamber at ambient temperature.

vii) The plate should be removed and rotated as above, and then returned for a second 4-minute incubation.

viii) Read for agglutination immediately after the 8-minute period is completed. Any visible reaction is considered to be positive. A control serum that gives a minimum positive reaction should be tested before each day's tests are begun to verify the sensitivity of test conditions.

Like the RBT, the test is very sensitive in cattle, especially for detection of vaccine-induced antibody, and positive samples should be retested using a confirmatory or complementary test(s). False-negative reactions may occur, usually due to prozoning, which may be overcome by diluting the serum or retesting after a given time. While the BPAT has been extensively used with apparent good results in small ruminants and pigs in some countries, its diagnostic value in these species has not been reported at international level.

2.4. Complement fixation test

The CFT is widely used but it is complex to perform, and requires good laboratory facilities and adequately trained staff to accurately titrate and maintain the reagents. There are numerous variations of the CFT in use, but this test is most conveniently carried out in a microtitre format. Warm or cold fixation may be used for the incubation of serum, antigen and complement: either 37°C ± 2°C for 30 minutes or 5°C ± 3°C for 14–18 hours. A number of factors affect the choice of the method: anti-complementary activity in serum samples of poor quality is more evident with cold fixation, while fixation at 37°C ± 2°C increases the frequency and intensity of prozones, and a number of dilutions must be tested for each sample.

Several methods have been proposed for the CFT using different concentrations of fresh or preserved sheep red blood cells (SRBCs) (a 2%, 2.5% or 3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-SRBC serum diluted to contain several times (usually from two to five times) the minimum concentration required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea-pig complement. The latter is independently titrated (in the presence or absence of antigen according to the method) to determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in a unit volume of a standardised suspension; these are defined

as the 50% or 100% haemolytic unit of complement/minimum haemolytic dose (C'H or MHD_{50} or C'H or MHD_{100}), respectively. It is generally recommended to titrate the complement before each set of tests, a macromethod being preferred for an optimal determination of $C'H_{50}$. Usually, 1.25–2 $C'H_{100}$ or 5–6 $C'H_{50}$ are used in the test.

Barbital (veronal) buffered saline is the standard diluent for the CFT. This is prepared from tablets available commercially; otherwise it may be prepared from a stock solution of sodium chloride (42.5 g), barbituric acid (2.875 g), sodium diethyl barbiturate (1.875 g), magnesium sulphate (1.018 g), and calcium chloride (0.147 g) in 1 litre of purified water, diluted by the addition of four volumes of 0.04% gelatine solution before use. However, this buffer contains barbituric derivatives that are no longer available in several countries. Satisfactory results may be also obtained with a barbituric-free solution of sodium chloride 0.85% containing calcium and magnesium, prepared by adding 1 ml of a stock solution of 1 M magnesium chloride and 0.3 M calcium chloride (anhydrous $MgCl_2$: 9.5 g $CaCl_2$: 3.7 g; purified water: up to 100 ml) (stored in small amounts at 5°C ± 3°C) to 1 litre of saline solution (Alton *et al*., 1988). The pH is critical and must be strictly adjusted to 7.35 (± 0.05). The replacement of the veronal buffer by this barbituric-free buffer has been validated in the WOAH Brucellosis Reference Laboratory in France.

2.4.1. Antigen production

Numerous variations of the test exist but, whichever procedure is selected, the test must use an antigen that has been prepared from an approved smooth strain of *B. abortus*, such as S99 or S1119-3, and standardised against the WOAHISS. Antigen for the CFT can be prepared following specialised procedures (Alton *et al*., 1988) or a whole cell antigen can be used after diluting the stock suspension such that the PCV of the concentrated antigen suspension for CFT is approximately 2% before standardisation against the WOAHISS.

2.4.2. Antigen standardisation

The antigen should be standardised to give 50% fixation at a dilution of 1/200 of the WOAHISS and must also show complete fixation at the lower serum dilutions, because too weak (or too strong) a concentration of antigen may not produce 100% fixation at the lower dilutions of serum. When two dilutions of antigen are suitable, the more concentrated antigen suspension must be chosen in order to avoid prozone occurrence. The appearance of the antigen, when diluted 1/10 must be that of a uniform, dense, white suspension with no visible aggregation or deposit after incubation at 37°C ± 2°C for 18 hours. It must not produce anti-complementary effects at the working strength for the test. The antigen is stored at 5°C ± 3°C and should not be frozen.

2.4.3. Test procedure (example)

The undiluted test sera and appropriate working standards should be inactivated for 30 minutes in a water bath at 60°C ± 2°C. If previously diluted with an equal volume of veronal buffered saline, these sera could be inactivated at 58°C ± 2°C for 50 minutes. Usually, only one serum dilution is tested routinely (generally 1/4 or 1/5 depending on the CF procedure chosen), but serial dilutions are recommended for trade purposes and when clinical signs have been reported in order to detect prozone.

Using standard 96-well microtitre plates with round (U) bottoms, the technique is usually performed as follows:

i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first, second and third rows. The first row is an anti-complementary control for each serum. Volumes of 25 µl of CFT buffer are added to the wells of the first row (anti-complementary controls) to compensate for lack of antigen. Volumes of 25 µl of CFT buffer are added to all other wells except those of the second row. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the third row onwards; 25 µl of the resulting mixture in the last row are discarded.

ii) Volumes of 25 µl of antigen, diluted to working strength, are added to each well except in the first row.

iii) Volumes of 25 µl of complement, diluted to the number of units required, are added to each well.

iv) Control wells containing:

a) diluent only,

b) complement + diluent,

c) antigen + complement + diluent,

are set up to contain 75 µl total volume in each case.

A control serum that gives a minimum positive reaction should be tested in each set of tests to verify the sensitivity of test conditions.

v) The plates are incubated at 37°C ± 2°C for 30 minutes or at 5°C ± 3°C overnight, and a volume (25 or 50 µl according to the technique) of sensitised SRBCs is added to each well. The plates are re-incubated at 37°C ± 2°C for 30 minutes.

vi) The results are read after the plates have been centrifuged at 1000 ***g*** for 10 minutes at 5°C ± 3°C or left to stand at 5°C ± 3°C for 2–3 hours to allow unlysed cells to settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The absence of anti-complementary activity is checked for each serum in the first row.

vii) Standardisation of results of the CFT

A unit system that is based on the WOAHISS exists for the standardisation of results. This serum contains 1000 ICFTU per ml. If this serum is tested in a given method and gives a titre of, for example 200 (50% haemolysis), then the factor for an unknown serum tested by that method can be found from the formula: 1000 × 1/200 × titre of test serum = number of ICFTU of antibody in the test serum per ml. The WOAHISS contains specific IgG; national standard sera should also depend on this isotype for their specific complement-fixing activity. Difficulties in standardisation arise because different techniques selectively favour CF by different immunoglobulin isotypes. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test to use the same method in order to obtain the same level of sensitivity. To facilitate comparison between countries, results should always be expressed in ICFTUs, calculated in relation to those obtained in a parallel titration with a standard serum, which in turn may be standardised against the WOAHISS.

viii) Interpretation of the results: Sera giving a titre equivalent to 20 ICFTU/ml or more are considered to be positive.

Animals that have been vaccinated with *B. abortus* S19 or *B. melitensis* Rev.1 between 3 and 6 months are usually considered to be infected if the sera give positive fixation at a titre of 30 or greater ICFTU/ml when the animals are tested at an age of 18 months or older.

This procedure is an example, other volumes and quantities of reagents could be chosen provided that the test is standardised against the WOAHISS as described above and the results expressed in ICFTU/ml.

The CFT is usually very specific but less sensitive than RBT and ELISA, particularly in the case of swine, as swine complement interacts with guinea-pig complement to produce a pro-complementary activity that reduces the sensitivity. Thus, the CFT has a reduced sensitivity for diagnosing *B. suis* infection, is not capable of eliminating the FPSR problem, and can be recommended only as a complementary test in swine. Moreover, like most serological tests, the CFT can be positive in ruminants after *B. abortus* S19 or *B. melitensis* Rev.1 vaccination and it is not specific enough in presence of FPSR. Therefore, CFT results should be investigated using suitable confirmatory or complementary strategies.

2.5. Enzyme-linked immunosorbent assays

2.5.1. Indirect ELISA

Numerous variations of the I-ELISA have been described for cattle, small ruminants, camelids and pigs employing different antigen preparations, antiglobulin-enzyme conjugates, and substrate/

chromogens. *Brucella abortus* strain 99 (Weybridge) (S99)[3] or *B. abortus* strain 1119-3 (USDA) (S1119-3)[4] should be used for production of these antigens, but *B. melitensis* strain 16M can also be suitable for such a purpose. Several commercial I-ELISAs using whole cell, S-LPS or the OPS as antigens that have been validated in extensive field trials are available and are in wide use. Nevertheless, the technique used and the interpretation of results must have been validated in accordance with the principles laid down in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*. The OPS antibody epitopes from these strains have also been exactly replicated through chemical synthesis and successfully used as antigens in ELISA, but these assays await full validation.

2.5.1.1. Test standardisation of the I-ELISA (EU, 2008; McGiven *et al.*, 2011)

2.5.1.1.1. Infection with *Brucella* in cattle

i) A 1/2 pre-dilution of the WOAHELISA$_{WP}$SS or a 1/16 pre-dilution of the WOAHELISA$_{SP}$SS made up in a negative bovine serum (or in a negative pool of bovine sera) must give a positive reaction;

and

ii) A 1/8 pre-dilution of the WOAHELISA$_{WP}$SS or a 1/64 pre-dilution of the WOAHELISA$_{SP}$SS made up in a negative bovine serum (or in a negative pool of bovine sera) must give a negative reaction;

and

iii) The WOAHELISA$_{N}$SS must always give a negative reaction.

2.5.1.1.2. Infection with *Brucella* in sheep and goats:

i) A 1/64 pre-dilution of the lSaBmS made up in a negative goat serum (or in a negative pool of goat sera) must give a positive reaction;

and

i) A 1/750 pre-dilution of the lSaBmS made up in a negative goat serum (or in a negative pool of goat sera) must give a negative reaction;

and

i) The above-mentioned negative goat serum (or negative pool of goat sera) must always give a negative reaction.

2.5.1.1.3. Infection with *Brucella* in pigs and camelids:

i) In the absence of appropriate international standard sera the test should be duly validated and the cut-off established in the test population with appropriate validation techniques (see chapter 1.1.6).

The I-ELISAs that use S-LPS or OPS as antigens are highly sensitive for the detection of anti-*Brucella* antibodies in cattle, small ruminants and pigs, but are not capable of fully resolving the problem of differentiating between antibodies resulting from *B. abortus* S19 and *B. melitensis* Rev.1 vaccination. The *B. abortus* RB51 vaccine may also interfere in S-LPS-based I-ELISAs.

Positive reactions should be investigated using suitable confirmatory or complementary strategies as for BBAT and CFT.

Using I-ELISA standardised against WOAH standard sera described above, the diagnostic sensitivity should be equal to or greater than that of the BBATs (RBT/BPAT) or the CFT in the testing of infected cattle, small ruminants and pigs. However, the specificity would usually be lower (EFSA, 2006; 2009; Greiner *et al.,* 2009; Gusi *et al.,* 2019).

The problem of FPSR may be reduced but not fully resolved, in pigs in particular, by performing I-ELISAs using extracts from rough strains of *Brucella*. Most FPSR are a result of cross reaction with the OPS portion of the S-LPS molecule; cross-reaction among core epitopes is less frequent but does exist. The use of chaotropic I-ELISAs or procedures using heterologous extracts or

Brucella cytosolic proteins as antigens does not resolve this problem, at least in cattle (Munoz *et al*., 2005). Moreover, in the context of FPSR, the most specific diagnostic procedure remains the brucellin skin test (see Section B.3.1 below).

Monoclonal, polyclonal antiglobulin or protein G or AG enzyme conjugates may be used depending on availability and performance requirements. A monoclonal antibody (MAb) specific for the heavy chain of bovine IgG_1 may provide some improvement in specificity at the possible cost of some loss of sensitivity while a protein G or AG enzyme conjugate may provide a reagent useful for testing a variety of mammalian species.

Several commercial I-ELISAs are available. Some protocols are less sensitive or less specific than others; therefore results obtained from different assays are not always comparable. I-ELISA for diagnosing anti-*Brucella* antibodies in small ruminants and pigs is essentially the same as that described for cattle, but the cut-off should have been properly established for these species using the appropriate validation techniques (see chapter 1.1.6), and, moreover, I-ELISA for sheep and goats should be standardised against the lSaBmS (McGiven *et al*., 2011).

Whatever the I-ELISA format used:

i) A positive and a negative control are included in each plate. OD (optical density) ranges to be obtained with these two controls must be established to define the criteria for validating each plate results. The OD of the positive control is the one with which the OD of each test serum is compared to establish the final result (negative or positive).

ii) An additional positive serum (internal control) must be included in each plate to validate the repeatability of the test from plate to plate and from day to day.

2.5.2. Competitive ELISA

Several variations of the C-ELISA, using S-LPS or OPS as antigens, have been described for cattle, small ruminants, camelids and pigs employing different antiglobulin-enzyme conjugates, substrate or chromogens and antigens prepared from different smooth *Brucella* strains. Nevertheless, the technique used and the interpretation of results must have been validated in accordance with the principles laid down in chapter 1.1.6.

2.5.2.1. Test standardisation of the C-ELISA (EU, 2008; McGiven *et al.*, 2011)

2.5.2.1.1. Infection with *Brucella* in cattle:

i) A 1/2 pre-dilution of the $ELISA_{WP}SS$ or a 1/16 pre-dilution of the $WOAHELISA_{SP}SS$ made up in a negative bovine serum (or in a negative pool of bovine sera) must give a positive reaction;

and

ii) A 1/8 pre-dilution of the $WOAHELISA_{WP}SS$ or a 1/64 pre-dilution of the $WOAHELISA_{SP}SS$ made up in a negative bovine serum (or in a negative pool of bovine sera) must give a negative reaction;

and

iii) The $WOAHELISA_{N}SS$ must always give a negative reaction.

2.5.2.1.2. Infection with *Brucella* in sheep and goats:

i) A 1/8 pre-dilution of the lSaBmS made up in a negative goat serum (or in a negative pool of goat sera) must give a positive reaction;

and

ii) a 1/300 pre-dilution of the lSaBmS made up in a negative goat serum (or in a negative pool of goat sera) must give a negative reaction;

and

iii) the above-mentioned negative goat serum (or negative pool of goat sera) must always give a negative reaction.

2.5.2.1.3. Infection with *Brucella* in pigs:

i) In the absence of an international standard serum for porcine brucellosis, the test should be duly validated and the cut-off established in the test population with appropriate validation techniques (see chapter 1.1.6).

Several commercial C-ELISAs are available. Some protocols are less sensitive or less specific than others, therefore results obtained from different assays are not always comparable. The cut-off should have been properly established using the appropriate validation techniques (see chapter 1.1.6).

The C-ELISA using an MAb specific for one of the epitopes of the *Brucella* sp. OPS has been shown in cattle, sheep and swine to have usually, but not always, higher specificity but lower sensitivity than the BBAT or I-ELISA (Munoz *et al*., 2005; 2012; Nielsen *et al*., 1995; Praud *et al*., 2012; Stack *et al*., 1999).

The C-ELISA may reduce but not fully eliminate the reactions caused by antibodies produced in response to vaccination. It is highly probable that the improved specificity is due to a reduction in sensitivity of the C-ELISA compared with BBAT and I-ELISA. Therefore, the results from C-ELISA reactions should not be considered in isolation but alongside suitable confirmatory or complementary strategies as for RBT, BBAT, CFT and I-ELISA.

The choice of MAb and its more or less high and unique specificity and affinity will have a distinct influence on the diagnostic performance characteristics of the assay. As with any MAb-based assays, the universal availability of the MAb or the hybridoma must also be considered with respect to international acceptance and widespread use.

Whatever the C-ELISA format used:

i) A positive and a negative control are included in each plate. OD ranges to be obtained with these two controls must be established to define the criteria for validating each plate results. The OD of the positive control is the one with which the OD of each test serum is compared to establish the final result (negative or positive).

ii) An additional positive serum (internal control) must be included in each plate to validate the repeatability of the test from plate to plate and from day to day.

2.6. Fluorescence polarisation assay

The FPA is a simple technique for measuring antigen/antibody interaction. It is a homogeneous assay in which analytes are not separated and it is therefore very rapid. However, unlike another homogeneous method (e.g. RBT) a blank/background read is required for each sample before adding the antigen. Thus it is a two-step assay.

For the diagnosis of brucellosis, a small molecular weight fragment (average 22 kD) of the OPS of *B. abortus* strain *B. abortus* 1119-3 S-LPS is labelled with fluorescein isothiocyanate (FITC) and used as the antigen. Once the blank/background read is performed (2–3 minutes), this antigen is added to diluted serum and a measure of the antibody content is obtained in about 2 minutes after the addition of antigen using a fluorescence polarisation analyser (FPM) (Nielsen *et al*., 1996).

2.6.1. Antigen production (example)

OPS from 5 g dry weight (or 50 g wet weight) of *B. abortus* S1119-3 is prepared by adding 400 ml of 2% (v/v) acetic acid, autoclaving the suspension for 15 minutes at 121°C and removing the cellular debris by centrifugation at 10,000 ***g*** for 10 minutes at 5°C ± 3°C. The supernatant solution is then treated with 20 g of trichloroacetic acid to precipitate any proteins and nucleic acids. The precipitate is again removed by centrifugation at 10,000 ***g*** for 10 minutes at 5°C ± 3°C. The supernatant fluid is dialysed against at least 100 volumes of purified water and freeze dried; 3 mg of OPS are dissolved in 0.6 ml of 0.1 M sodium hydroxide (4 g NaOH/litre) and incubated at 37°C ± 2°C for 1 hour, followed by the addition of 0.3 ml of FITC isomer 1 at a concentration of 100 mg/ml in dimethyl sulphoxide and a further incubation at 37°C ± 2°C for 1 hours. The conjugated OPS is applied to a 1 × 10 cm column packed with DEAE (diethylaminoethyl) Sephadex A 25 equilibrated

in 0.01 M phosphate buffer, pH 7.4 ± 0.2. The first fraction (after 10–15 ml of buffer) is bright green, after which the buffer is switched to 0.1 M phosphate, pH 7.4 ± 0.2. This results in the elution of 10–15 ml of buffer followed by 25–40 ml of green fluorescent material. The latter material is the antigen used in the FPA. Antigen preparation may be scaled up proportionally.

The amount of antigen used per test is determined by diluting the material derived above until a total fluorescence intensity of 250,000–300,000 is achieved using the FPM.

The antigen can be stored as a liquid for several years at 5°C ± 3°C in a dark bottle or it may be freeze-dried in dark bottles. Labelled antigen may be obtained from a limited number of commercial sources.

2.6.2. Test standardisation of the FPA (EU, 2008; McGiven *et al.*, 2011)

2.6.2.1. Infection with *Brucella* in cattle

i) the WOAHELISA$_{WP}$SS and WOAHELISA$_{SP}$SS consistently give a positive reaction;

and

ii) a 1/8 pre-dilution of the WOAHELISA$_{WP}$SS or a 1/64 pre-dilution of the WOAHELISA$_{SP}$SS made up in a negative bovine serum (or in a negative pool of bovine sera) must give a negative reaction;

and

iii) the WOAHELISA$_{N}$SS must always give a negative reaction.

2.6.2.2. Infection with smooth *Brucella* in sheep and goats

i) a 1/16 pre-dilution of the ISaBmS made up in a negative goat serum (or in a negative pool of goat sera) must give a positive reaction;

and

ii) a 1/200 pre-dilution of the ISaBmS made up in a negative goat serum (or in a negative pool of goat sera) must give a negative reaction;

and

iii) the above-mentioned negative goat serum (or negative pool of goat sera) must always give a negative reaction.

2.6.2.3. Infection with *Brucella* in pigs

i) In the absence of international standard serum for porcine brucellosis the test should be duly validated and the cut-off established in the test population with appropriate validation techniques (see chapter 1.1.6).

The diagnostic sensitivity and specificity of the FPA for bovine brucellosis are almost identical to those of the C-ELISA. The FPA for diagnosing anti-*Brucella* antibodies in small ruminants and swine is essentially the same as that described for cattle, but the cut-off should have been properly established for these species using the appropriate validation techniques (see chapter 1.1.6), and the test should be standardised against the corresponding international Standards, as mentioned above.

The FPA is capable of reducing but not fully eliminating the reactions due to residual antibody produced in response to vaccination (Nielsen *et al*., 1996). Moreover, the specificity of FPA in FPSR conditions is currently unknown in cattle and small ruminants, but it has been clearly shown that it does not resolve the FPSR problem in swine (Praud *et al*., 2012).

Accordingly, like all other serological tests, positive reactions should be investigated using suitable confirmatory and/or complementary strategies.

2.6.3. Test procedure (Nielsen *et al.*, 1996)

The FPA can be performed in glass tubes or a 96-well plate format.

Bovine sera are diluted 1/10 for the plate test or 1/100 for the tube test.

Sheep and goat as well as porcine sera are diluted 1/10 for the plate test or 1/25 (goat and porcine) and 1/40 (sheep) for the tube test.

The diluent used is 0.01 M Tris (1.21 g), containing 0.15 M sodium chloride (8.5 g), 0.05% Igepal CA630 (500 µl) (formerly NP40), 10 mM EDTA (3.73 g) per litre of purified water, pH 7.2 ± 0.2 (Tris buffer).

An initial reading to assess light scatter is obtained with the FPM after mixing. Suitably labelled titrated antigen is added, mixed and a second reading is obtained in the FPM about 2 minutes later.

A reading (in millipolarisation units, mP) over the established threshold level is indicative of a positive reaction.

A typical threshold level is 90–100 mP units, however, the test should be standardised locally against the corresponding WOAH reference standard sera (as mentioned above). A strong positive, a weak positive and a negative working standard serum (standardised against the above-mentioned WOAH reference standard sera) should be included.

2.6.3.1. Example for bovine sera

i) 1 ml of diluent is added to a 10 × 75 mm borosilicate glass tube followed by 10 µl of serum or 20 µl of EDTA-treated blood. For the 96-well format, 20 µl of serum is added to 180 µl of buffer. It is important to mix well. A reading is obtained on the FPM to determine light scatter.

ii) A volume of antigen, which results in a total fluorescence intensity of 250–300 × 10^3, is added to the tube and mixed well. This volume will vary from batch to batch, but is generally in the range of about 10 µl. A second reading is obtained on the FPM after incubation at room temperature (22°C ± 4°C) for approximately 2 minutes for serum and 15 seconds for EDTA-treated blood.

iii) A reading above the predetermined threshold is indicative of a positive reaction.

2.7. Serum agglutination test (cattle only)

The SAT has been used with success for many years in surveillance and control programmes for bovine brucellosis, particularly in northern Europe. Its specificity is significantly improved with the addition of EDTA to the antigen (MacMillan & Cockrem, 1985).

The antigen represents a bacterial suspension of *B. abortus* strain 99 or *B. abortus* strain 1119-3 in phenol saline (NaCl 0.85% [w/v] and phenol at 0.5% [v/v]). Formaldehyde must not be used. Antigens may be delivered in the concentrated state provided the dilution factor to be used is indicated on the bottle label. EDTA may be added to the antigen suspension to 5 mM final test dilution to reduce the level of false-positive results. Subsequently the pH of 7.2 ± 0.2 must be readjusted in the antigen suspension.

The WOAHISS contains 1000 IUs of agglutination. The antigen should be prepared without reference to the cell concentration, but its sensitivity must be standardised in relation to the WOAHISS in such a way that the antigen produces either 50% agglutination with a final serum dilution of 1/600 to 1/1000 or 75% agglutination with a final serum dilution of 1/500 to 1/750. It may also be advisable to compare the reactivity of new and previously standardised batches of antigen using a panel of defined sera.

The test is performed either in tubes or in microplates. The mixture of antigen and serum dilutions should be incubated for 16–24 hours at 37°C ± 2°C. If the test is carried out in microplates, the incubation time can be shortened to 6 hours. At least three dilutions must be prepared for each serum in order to refute prozone negative responders. Dilutions of suspect serum must be made in such a way that the reading of the reaction at the positivity limit is made in the median tube (or well for the microplate method).

Interpretation of results: The degree of *Brucella* agglutination in a serum must be expressed in IU per ml. A serum containing 30 or more IU per ml is considered to be positive.

2.8. Native hapten and cytosol protein-based tests (ruminants only)

In cattle, native hapten tests[5] are highly specific in *B. abortus* S19 vaccination contexts, and have been used successfully in combination with the RBT as a screening test. The optimal sensitivity (close to that of CFT but significantly lower than that of RBT and S-LPS based I-ELISAs) is obtained in a reverse radial immunodiffusion (RID) system in which the serum diffuses into a hypertonic gel containing the polysaccharide, but the double gel diffusion assay is also useful (Munoz *et al*., 2005). Calves vaccinated subcutaneously with the standard dose of *B. abortus* S19 at 3-5 months of age are negative usually by 2 months after vaccination, and adult cattle vaccinated subcutaneously 4–5 months previously with the reduced dose of *B. abortus* S19 do not give positive reactions unless the animals become infected and shed the vaccine strain in their milk. The conjunctival vaccination (both in young and adults) reduces significantly the time to obtain a negative response in native hapten tests. A characteristic of the native hapten tests is that a positive result correlates with *Brucella* shedding as shown in experimentally and in naturally infected cattle (Jones *et al*., 1980). In case of FPSR caused by *Yersinia enterocolitica* O:9 and FPSR of unknown origin in cattle, gel precipitation tests using native hapten or *Brucella* cytosol proteins usually give negative results (Munoz *et al*., 2005).

These native hapten tests are also of interest in sheep and goats as they are very specific for discriminating the serological responses of infected animals (positive) from those induced in *B. melitensis* Rev.1 vaccinated animals (usually negative after a given time post-vaccination). The optimal diagnostic sensitivity (around 90%) is obtained in the double gel diffusion or RID tests for sheep and goats, respectively.

2.9. Milk tests

An efficient means of screening dairy herds is by testing milk from the bulk tank. When a positive test result is obtained, all cows contributing milk should be blood tested. The milk I-ELISA is a sensitive and specific test, and is particularly valuable for testing large herds. The MRT is a suitable alternative if the milk I-ELISA is not available. However, the MRT is not suitable in milk from small ruminants.

2.9.1. Milk I-ELISA (cattle and sheep and goats only)

As with the serum I-ELISA, many variations of the milk I-ELISA are possible. Several commercial I-ELISAs are available that have been validated in extensive field trials and are in wide use. In the interests of international harmonisation, the three WOAH ELISA Standard Sera should be used by national reference laboratories to check or standardise a particular test for use in cattle.

2.9.1.1. Test standardisation of the Milk I-ELISA in dairy cattle

The milk I-ELISA for cattle should be standardised such that the WOAH ELISA strong positive standard when diluted 1/125 in negative serum and further diluted 1/10 in negative milk consistently tests positive (EU, 2008).

Bulk milk samples are generally tested at much lower dilutions than sera, i.e. undiluted to 1/2 to 1/10 in diluent buffer, with the remainder of the assay being similar to that described for serum.

FPSR may be observed in milk I-ELISA, but usually less frequently than with blood tests.

The I-ELISA for diagnosing anti-*Brucella* antibodies in sheep or goat milk is essentially the same as that described for cattle, but the cut-off should be properly established for these species using the appropriate validation techniques (see chapter 1.1.6). However there are no international standardisation recommendations of the milk I-ELISA against the corresponding ISaBmS.

5 The detailed procedure can be obtained from the Brucellosis Laboratory, Centro de Investigación y Tecnología Agroalimentaria / Gobierno de Aragón, Avenida Montañana 930, 50059 Zaragoza, Spain.

2.9.2. Milk ring test (cattle only)

In lactating cattle, the MRT can be used for screening herds for brucellosis.

In large herds (> 100 lactating cows), the sensitivity of the test becomes less reliable. The MRT may be adjusted to compensate for the dilution factor from bulk milk samples from large herds. The samples are adjusted according to the following formula: herd size < 150 animals use, 1 ml bulk milk; 150–450 animals, use 2 ml milk sample, 451–700 animals, use 3 ml milk sample.

False-positive reactions may occur in cattle vaccinated less than 4 months prior to testing, in samples containing abnormal milk (such as colostrum) or in cases of mastitis. Therefore, it is not recommended to use this test in very small farms where these problems have a greater impact on the test results.

2.9.2.1. Antigen production

MRT antigen is prepared from concentrated, killed *B. abortus* S99 or S1119-3 cell suspension, grown as described previously. It is centrifuged at, for example, 23,000 ***g*** for 10 minutes at 5°C ± 3°C, followed by resuspension in haematoxylin-staining solution. Various satisfactory methods are in use; one example is as follows: 100 ml of 4% (w/v) haematoxylin (CI No. 75290) dissolved in 95% ethanol is added to a solution of ammonium aluminium sulphate (5 g) in 100 ml of purified water and 48 ml of glycerol; 2 ml of freshly prepared 10% (w/v) sodium iodate is added to the solution. After standing for 30 minutes at room temperature (22°C ± 4°C), the deep purple solution is added to 940 ml of 10% (w/v) ammonium aluminium sulphate in purified water. The pH of this mixture is adjusted to 3.1 ± 0.2, and the solution must be aged by storage at room temperature in the dark for 45–90 days.

Before use, the staining solution is shaken and filtered through cotton wool. The packed cells are suspended in the staining solution at the rate of 1 g per 30 ml stain, and held at room temperature (22°C ± 4°C) for 48 hours (some laboratories prefer to heat at 80°C for 10 minutes instead). The stained cells are then deposited by centrifugation, and washed three times in a solution of sodium chloride (6.4 g), 85% lactic acid (1.5 ml) and 10% sodium hydroxide (4.4 ml) in 1.6 litres of purified water, final pH 3.0 ± 0.2. The washed cells are resuspended at the rate of 1 g in 27 ml of a diluent consisting of 0.5% phenol saline, adjusted to pH 4.0 ± 0.2 by the addition of 0.1 M citric acid (approximately 2.5 ml) and 0.5 M disodium hydrogen phosphate (approximately 1 ml) and maintained at 5°C± 3°C for 24 hours. The mixture is filtered through cotton wool, the pH is checked, and the PCV is determined and adjusted to approximately 4%.

2.9.2.2. Antigen standardisation

The antigen should be standardised against the WOAHISS so that a 1/500 dilution is positive and 1/1000 dilution is negative. The sensitivity of the new batch should be compared as well with a previously standardised batch using a panel of samples of varying degrees of reaction prepared by diluting a positive serum in milk.

The antigen should be stored as recommended by the manufacturer but usually at 5°C ± 3°C.

The pH of the antigen should be 3.5 (± 0.2) and its colour should be dark blue. A little free stain in the supernatant of a centrifuged sample is permissible. When diluted in milk from a brucellosis-free animal, the antigen must produce a uniform coloration of the milk layer with no deposit and no coloration of the cream layer.

2.9.2.3. Test procedure

The test is performed on bulk tank milk samples. If necessary, samples could be pre-treated with preservative (0.1% formalin or 0.02% bronopol) for 2–3 days at 5°C ± 3°C prior to use.

i) Bring the milk samples and antigen to room temperature (22°C ± 4°C); only sufficient antigen for the day's tests should be removed from the refrigerator.

ii) Gently shake the antigen bottle well.

iii) The test is performed by adding 30–50 µl of antigen to a 1–2 ml volume of whole milk (the volume of milk may be increased for bulk samples from larger herds – see above).

iv) The height of the milk column in the tube must be 20–25 mm. The milk samples must not have been frozen, heated, subjected to violent shaking or stored for more than 72 hours.

v) The milk/antigen mixtures are normally incubated at 37°C ± 2°C for 1 hour, together with positive and negative working standards. However, overnight incubation at 5°C± 3°C increases the sensitivity of the test and allows for easier reading.

vi) A strongly positive reaction is indicated by formation of a dark blue ring above a white milk column. Any blue layer at the interface of milk and cream should be considered to be positive as it might be significant, especially in large herds.

vii) The test is considered to be negative if the colour of the underlying milk exceeds that of the cream layer.

viii) When the MRT is adjusted for large herd sizes (2 or 3 ml of milk used), 0.1 ml of pooled negative cream is added to the test tube and is followed by 30–50 µl of the ring test antigen. After mixing, the test is incubated and read in the same manner as the unadjusted MRT. The negative pooled cream is collected from the separation of composite, unpasteurised milk from a brucellosis negative herd of 25 or more cows.

2.10. Serological tests in wildlife

Serological investigations in wild species are usually carried out for screening purposes. In these particular circumstances, adequate specificity is of paramount importance. The RBT can be recommended as a general purpose diagnostic test in all wildlife species. The CFT could also be recommended for such purpose, but the selection of the complement inactivation temperature and the cut-off titres have not been properly documented in all wildlife species. Both tests require the use of high quality serum samples that are not easy to obtain in wildlife studies. When poor quality serum samples are tested in both RBT and CFT, the results are frequently uninterpretable. The I- and C-ELISAs appear to be useful for epidemiological sero-surveys in wild animals as both are generally more reliable than both RBT and CFT, and, moreover, can be used with poor quality and haemolysed sera (Stack *et al*., 1999). Another advantage of the ELISAs is that if serum is not available, it is possible to test meat juice samples. Attention must be paid to the conjugate used in the I-ELISA as it must have a satisfactory affinity for the corresponding antibody isotypes of the wild species under study. However, in wild species, the interpretation of ELISA results may be problematic, due to the lack of validation studies. Whenever possible, the cut-off of ELISAs should be properly established for the particular species using the appropriate validation techniques (see chapter 1.1.6). Nevertheless, where positive or doubtful serological results are found, a bacteriological investigation should be conducted, when possible, to clarify the diagnosis.

3. Tests for cellular immunity

3.1. Brucellin skin test

An alternative immunological assay is the brucellin skin test, which can be used for screening unvaccinated herds, provided that a purified (free of S-LPS) and standardised antigen preparation is used. The brucellin skin test has a very high specificity, such that serologically negative unvaccinated cattle that are positive reactors to the brucellin test should be regarded as infected animals (Pouillot *et al*., 1997). The brucellin skin test also has a high sensitivity for the diagnosis of *B. melitensis* infection in small ruminants and, in the absence of vaccination, is considered one of the most specific diagnostic tests.

Brucellin was developed for use in ruminants, but is also effective for confirming the disease at the herd level in pigs. Field trials have also shown its good sensitivity in *Brucella*-infected pigs (Dieste-Perez *et al*., 2014). Also, results of this test may aid the interpretation of serological reactions thought to be FPSR due to infection with cross-reacting bacteria, as FPSR affected animals always give negative results in the skin test (Dieste-Perez *et al*., 2014; Pouillot *et al*., 1997). Preliminary studies have suggested that brucellin can be used as a confirmatory diagnostic test in camels (Khalafalla *et al.,* 2020).

Animals vaccinated with *B. melitensis* Rev.1, *B. abortus* S19 or RB51 can give positive results in this test for years (Pouillot *et al*., 1997; De Massis *et al.*, 2005; 2015). Therefore this test cannot be recommended either as the sole diagnostic test or for the purposes of international trade in areas where *Brucella* vaccines are used. Moreover, not all infected animals react, therefore this test alone cannot be recommended as an individual diagnostic test or for the purposes of international trade. However, due to its high specificity and its adequate sensitivity at the herd or flock level, it can be recommended for herd/flock surveillance in brucellosis-free areas.

It is essential to use a standardised, defined brucellin preparation that does not contain S-LPS antigen, as this may provoke nonspecific inflammatory reactions or interfere with subsequent serological tests.

Although the brucellin test is probably the most specific indirect assay for diagnosing brucellosis (in unvaccinated animals), the final diagnosis should not be made solely on the basis of positive intradermal reactions given by a few animals in the herd, and should be supported by a complementary diagnostic test. The intradermal inoculation of brucellin induces a temporary anergy in the cellular immune response, at least in some animal species (Blasco *et al*., 1994b). Therefore an interval of 6 weeks is generally recommended between two tests on the same animal.

3.1.1. Test procedure

3.1.1.1. Infection with *Brucella* in cattle

i) A volume of 0.1 ml of brucellin (2000 Units/ml) is injected intra-dermally into the caudal fold, the skin of the flank, or the side of the neck.

ii) The test is read after 48–72 hours.

iii) The skin thickness at the injection site is measured with vernier callipers before injection and at re-examination.

iv) A strong positive reaction is easily recognised by local swelling and induration. However, borderline reactions require careful interpretation. Skin thickening of 1.5–2 mm would be considered as a positive reaction.

3.1.1.2. Infection with *Brucella* in sheep and goats

i) A volume of 0.1 ml of brucellin (2000 Units/ml) is injected intra-dermally into the lower eyelid.

ii) The test is read after 48 hours.

iii) Any visible or palpable reaction of hypersensitivity, such as an oedematous reaction leading to an elevation of the skin or thickening of the eyelid (≥ 2 mm), should be interpreted as a positive reaction.

3.1.1.3. Infection with *Brucella* in pigs

As a diagnostic agent in pigs, 0.1 ml of the allergen suspension (2000 Units/ml) is injected intra-dermally into the skin at the base of the ear or preferably, next to the base of the tail. The latter appears more practical and less hazardous. The reaction is assessed by visual inspection and palpation of the inoculated area after 48 hours and a positive reaction is characterised by erythema of non-pigmented skin and an oedematous swelling. In some cases, there may also be some haemorrhage/necrosis.

3.2. Interferon gamma release assay

The interferon gamma release assay (IGRA) involves stimulation of lymphocytes in whole blood with a suitable antigen such as brucellin. The resulting gamma interferon production is detected through a capture ELISA. This test could be useful in the discrimination of brucellosis from FPSR but more specific antigens are still needed and the protocol needs to be standardised and properly validated in the different animal species and epidemiological conditions. For the moment, a fully validated protocol is not available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Vaccines

As mentioned previously, brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed. Laboratory manipulation of live cultures of *Brucella,* including vaccine strains, is hazardous and must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4). The S19, RB51 and Rev.1 vaccines have some virulence for humans, and a hazard warning should be included on the label of the final containers. Medical advice should be sought in the event of accidental inoculation or exposure (see Section C.1.2.3.2.3 Precautions) (Ashford *et al*., 2004; Joint FAO/WHO Expert Committee on Brucellosis, 1986; USDA, 2003).

1.1. Background

1.1.1. *Brucella abortus* strain 19 vaccine

A widely used vaccine for the prevention of brucellosis in cattle is *B. abortus* S19, which remains the reference vaccine with which any other vaccines must be compared. It is used as a live vaccine and is normally given to female calves aged between 3 and 6 months as a single subcutaneous dose of 5–8 × 10^{10} viable organisms. A reduced dose of from 3 × 10^{8} to 5 × 10^{9} organisms can be administered subcutaneously to adult cattle, but some animals can develop persistent antibody titres and may abort and excrete the vaccine strain in the milk. Alternatively, the vaccine can be administered to cattle of any age as either one or two doses of 5 × 10^{9} viable organisms, given by the conjunctival route. This vaccination procedure induces protection against both *B. abortus* (Nicoletti *et al*., 1978) and *B. melitensis* (Jimenez de Bagües *et al*., 1991) without a persistent antibody response and reduces the risks of abortion and excretion in milk when vaccinating adult cattle.

Brucella abortus S19 vaccine induces good immunity to moderate challenge by virulent *B. abortus* or *B. melitensis* organisms. The vaccine must be prepared from USDA-derived seed (see footnote 4 for address) and each batch must be checked for purity (absence of extraneous microorganisms), viability (live bacteria per dose) and smoothness (determination of dissociation phase). Seed lots for *B. abortus* S19 vaccine production should be regularly tested for residual virulence and immunogenicity in mice.

Control procedures for this vaccine are detailed in Section C.1.2.2.3 *In-process controls*.

1.1.2. *Brucella abortus* strain RB51 vaccine

Since 1996, *B. abortus* strain RB51 has become the official vaccine for prevention of brucellosis in cattle in several countries. However there is a disagreement over the protective performance of strain RB51 compared with strain S19 in cattle (Moriyon *et al*., 2004). Each country uses slightly different methods to apply this vaccine. In the USA (a country that was almost free of bovine brucellosis before RB51 was introduced), calves are vaccinated subcutaneously between the ages of 4 and 12 months with 1–3.4 × 10^{10} viable organisms. Vaccination of cattle over 12 months of age is carried out only under authorisation from the State or Federal Animal Health Officials, and the recommended dose is 1–3 × 10^{9} viable organisms (USDA, 2003). In other countries, it is recommended to vaccinate cattle as calves (4–12 months of age) with a 1–3.4 × 10^{10} dose, with revaccination from 12 months of age onwards with a similar dose to elicit a booster effect and increase immunity.

However, it has been reported that full doses of *B. abortus* strain RB51 when administered intravenously in cattle induce severe placentitis and placental infection in most vaccinated cattle, and that there is excretion in milk in a relevant number of vaccinated animals. Field experience also indicates that it can induce abortion and increased perinatal mortality if applied to pregnant cattle. These observations have led to the recommendation to avoid vaccination of pregnant cattle with *B. abortus* RB51. One way to reduce the side effects of *B. abortus* RB51 is to reduce the dose. When using the reduced dose of this vaccine (1 × 10^{9} colony-forming units [CFU]), on late pregnant cattle, no abortions or placentitis lesions have been reported, but the vaccine strain can be shed by a significant proportion of vaccinated animals. However, this reduced dose does not protect against *B. abortus* when used as a vaccine in calves, and the protection against *B. abortus*

is only moderate when used as an adult vaccine. The protection conferred by *B. abortus* RB51 against *B. melitensis* infection in cattle is unknown.

Control procedures for this vaccine are detailed in Section C.1.2.2.3.

1.1.3. *Brucella melitensis* strain Rev.1 vaccine

It is not infrequent to isolate *B. melitensis* in cattle in countries with a high prevalence of this infection in small ruminants (Verger, 1985). There has been some debate on the protective efficacy of *B. abortus* S19 against *B. melitensis* infection in cattle, but there is published evidence proving that this vaccine is able to control *B. melitensis* in cattle. It has been hypothesised that *B. melitensis* Rev.1 should be a more effective vaccine than *B. abortus* S19 in these conditions. However there is very little information related to this issue (Joint FAO/WHO Expert Committee on Brucellosis, 1986), and no experiments have been reported showing the efficacy of Rev.1 against *B. melitensis* infection in cows. Moreover, the safety of Rev.1 vaccine is practically unknown in cattle. Accordingly, until the safety of Rev.1 in cattle of different physiological status and efficacy studies against *B. melitensis* under strictly controlled conditions are performed, this vaccine should not be recommended for use in cattle.

Brucella melitensis Rev.1 is the most widely used vaccine for the prevention of brucellosis in sheep and goats, and, despite its drawbacks, remains the reference vaccine with which any other vaccines should be compared. By contrast, the rough *B. abortus* RB51 vaccine is not effective against *B. melitensis* infection in sheep. The Rev.1 vaccine is used as a freeze-dried suspension of live *B. melitensis* Rev.1 strain for the immunisation of sheep and goats. It should be given to lambs and kids aged between 3 and 5 months as a single subcutaneous or conjunctival inoculation, 5 months being the upper time limit to minimise the antibody response to make this vaccination compatible with further serological testing. No matter the inoculation route, the standard dose must be between 0.5×10^9 and 2.0×10^9 viable organisms. The reduced doses confer a significantly lower protection than the standard doses, and should not be recommended for vaccinating sheep and goats. The subcutaneous vaccination induces long-lasting serological responses, causing strong interferences in serological tests and should not be recommended for use in combined eradication programmes. However, when this vaccine is administered conjunctivally at the standard dose, it produces a similar protection without inducing a persistent antibody response, thus facilitating the application of eradication programmes combined with vaccination. Care must be taken when using *B. melitensis* Rev.1 vaccine to avoid the risk of contaminating the environment or causing human infection. In many developing countries and endemic areas, vaccination of the whole population has to be considered as the best option for the control of the disease (Blasco, 1997). However, Rev.1 vaccine is known to often cause abortion and excretion in milk when animals are vaccinated during pregnancy, either with a full or reduced dose (Blasco, 1997). These side-effects are considerably reduced when adult animals are vaccinated conjunctivally (full dose) during lambing/kidding, lactation or before mating. Therefore, when mass vaccination is the only means of controlling the disease, a vaccination campaign should be recommended using the standard dose of Rev.1 administered by the conjunctival route when the animals are not pregnant or during the late lambing/kidding and pre-breeding season (Blasco, 1997).

The subcutaneous vaccination of young animals and the vaccination of adult animals, even at reduced doses, may lead to long-term persistence of vaccinal antibodies in a significant proportion of Rev.1 vaccinated animals that creates serious interferences in the serological diagnosis of brucellosis. As indicated above, conjunctival vaccination minimises these problems (particularly when the upper limit of age for vaccination is 5 months) and thus it is the method of choice for combined eradication programmes. Therefore, the serological diagnosis of brucellosis should take into account the vaccinal state of the flock and the overall frequency distribution of antibody titres detected in the group of animals tested.

Control procedures for this vaccine are detailed in Section C.1.2.2.3.

1.1.4. Vaccination in pigs

Attempts have been made to develop a suitable vaccine to immunise pigs against *B. suis*, but none has been found fully effective. The live vaccine strain *B. suis* strain 2 (S2), produced in China

(People's Rep. of) by serial transfer of a virulent *B. suis* biovar 1 strain isolated from swine origin, has been widely used in that country (in pigs and other species), but efficacy data against *B. suis* infection under strictly controlled conditions are not available.

It is also reported that *B. abortus* strain RB51 vaccine is ineffective for the protection of swine against exposure to *B. suis* (Stoffregen *et al*., 2006).

1.1.5. Vaccination in other species

In wild species, differences in vaccine effects in wild and domestic species have been observed, particularly in the American bison (*Bison bison*) and elk (*Cervus canadensis*) regarding *B. abortus* (National Academies of Sciences, 2020) and in Alpine ibex using Rev.1 vaccination (Ponsart *et al*., 2019). The Rev.1 study suggests that in ibex, the Rev.1 vaccine strain persists longer at higher bacteriological levels (CFUs) than in goats. Given this discrepancy, it is very difficult to assess vaccine efficacy in wild species based on experiments done in domestic species. Injection route, type and dose of vaccines should be systematically assessed including an infection challenge.

1.2. Outline of production and requirements

1.2.1. Characteristics of the seed

1.2.1.1. Biological characteristics of the master seed

Brucella abortus S19 original seed for vaccine production must be obtained from the USDA (see footnote 4 for address), and used to produce a seed lot that is preserved by lyophilisation or by freezing at liquid nitrogen temperature. The properties of this seed lot must conform to those of a pure culture of a CO_2-independent *B. abortus* bv. 1 that is also sensitive to benzyl-penicillin (3 μg [5 IU]/ml), thionin blue (2 μg/ml) and i-erythritol (1 mg/ml), and that displays minimal pathogenicity for guinea-pigs.

Brucella abortus RB51 original seed for vaccine production is available commercially. It is also obtainable from the USDA (see footnote 4 for address). *Brucella abortus* RB51 has the normal properties of a bv. 1 strain of *B. abortus*, but is 100% in the rough phase and does grow in the presence of rifampicin (250 μg/ml).

Brucella melitensis strain Rev.1 original seed for vaccine production can be obtained commercially. A European reference Rev.1 strain that possesses the characteristics of the Rev.1 original seed is also obtainable from the WOAH Reference Laboratory for Brucellosis in France. Strain Rev.1 must conform to the characteristics of *B. melitensis* bv. 1, except that it should grow more slowly. Additionally, when incubated in air (atmospheres containing CO_2 alter the results) at 37°C± 2°C, it should grow on agar containing streptomycin (2.5 μg/ml), and it should be inhibited by the addition to a suitable culture medium of sodium benzyl-penicillin (3 μg [5 IU]/ml), thionin (20 μg/ml) or basic fuchsin (20 μg/ml).

PCR and molecular techniques have been used to further characterise the S19, RB51 or Rev.1 vaccines (see Section B.1.4).

The specific requirements for S19 and Rev.1 vaccine production recommend that each seed lot (i.e. the culture used to inoculate medium for vaccine production) should be no more than three passages removed from an original seed culture and that the harvest of a vaccine lot should be no more than three passages from a seed lot or an original seed. The original seed culture should always be checked for the absence of dissociation before use. The recommended method for preparing seed material is given in Alton *et al*. (1988).

1.2.1.2. Quality criteria

Brucella abortus S19 and RB51 as well as *B. melitensis* Rev.1 master seeds should be checked for purity, identity and, where appropriate, smoothness or roughness. S19 and Rev.1 seed lots must also conform to the characteristics of residual virulence and immunogenicity in mice of the original seed.

1.2.1.2.1. Purity

Tests for purity and freedom from contamination of biological materials may be found in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

1.2.1.2.2. Safety

The S19 and Rev.1 vaccines show reduced virulence, but should keep a minimal virulence to be efficient (see Section C.1.2.1.2.3 *Potency*). However a safety test is not routinely done. If desired, when a new manufacturing process is started and when a modification in the innocuousness of the S19 and Rev.1 vaccine preparations is expected, it may be performed on cattle (S19) and sheep and goats (Rev.1). This control should be done as follows: the test uses 12 female calves or sheep/goats respectively, aged 4–6 months. Six young females are injected with one or three recommended doses. Each lot of six young females are kept separately. All animals are observed for 21 days. No significant local or systemic reaction should occur. If, for a given dose and route of administration, this test gives good results on a representative batch of the vaccine, it does not have to be repeated routinely on seed lots or vaccine lots prepared with the same original seed and with the same manufacturing process. A safety test on S19/Rev.1 vaccine may also be performed in guinea-pigs. Groups of at least ten animals are given intramuscular injections of doses of vaccine diluted in PBS, pH 7.2 ± 0.2, to contain 5×10^9 viable organisms. The animals should show no obvious adverse effects and there must be no mortality.

If this safety test has been performed with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

If a safety test for RB51 is desired, 8- to 10-week-old female Balb/c mice can be injected intraperitoneally with 1×10^8 CFUs and the spleens cultured at 6 weeks post-inoculation. Spleens should be free from RB51 and the mice should not develop anti-OPS antibodies.

1.2.1.2.3. Potency

i) S19 vaccine

An S19 vaccine is effective if it possesses the characteristics of the S19 original strain, i.e. if it is satisfactory with respect to identity and smoothness. Moreover, it should have been produced with a given seed lot with adequate immunogenicity and residual virulence (Grillo *et al*., 2000).

a) Identity

Brucella abortus present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: *B. abortus* S19 has the normal properties of a *B. abortus* bv. 1 strain of, but does not require CO_2 for growth, does not grow in the presence of benzyl-penicillin (3 μg/ml = 5 IU/ml), thionin blue (2 μg/ml), and i-erythritol (1 mg/ml) (all final concentrations). PCR and molecular techniques have been described to identify the S19 vaccine strain (see Section B.1.4).

b) Smoothness (determination of dissociation phase)

The S19 vaccine reconstituted in purified water is streaked across six agar plates (serum–dextrose agar or trypticase–soy agar (TSA) with added serum 5% [v/v] or yeast extract 0.1 % [w/v) in such a manner that the colonies will be close together in certain areas, while semi-separated and separated in others. Slight differences in appearance are more obvious in adjacent than widely separated colonies. Plates are incubated at 37°C ± 2°C for 5 days and examined by obliquely reflected light (Henry's method) before and after staining (three plates) with crystal violet (White & Wilson's staining method).

Appearance of colonies before staining: S colonies appear round, glistening and blue to blue–green in colour. R colonies have a dry, granular appearance and are dull yellowish–white in colour. Mucoid colonies (M) are transparent and greyish in colour and can be distinguished by their slimy consistency when touched with a loop. Intermediate colonies (I), which are the most difficult to classify, have an appearance intermediate between S and R forms: they are slightly opaque and more granular than S colonies.

Appearance of colonies after staining with crystal violet: S colonies do not take up the dye. Dissociated colonies (I, M, or R) are stained various shades of red and purple and the surface may show radial cracks. Sometimes a stained surface film slips off a dissociated colony and is seen adjacent to it.

The colony phase can be confirmed by the acriflavine agglutination test (Alton *et al*., 1988). S colonies remain in suspension, whereas R colonies are agglutinated immediately and, if mucoid, will form threads. Intermediate colonies may remain in suspension or a very fine agglutination may occur.

At least 99% of cells in seed lots should be in the smooth phase.

c) Residual virulence (50% persistence time or 50% recovery time) (Grillo *et al*., 2000; Pouillot *et al*., 2004)

1) Prepare adequate suspensions of either the *B. abortus* S19 seed lot to be tested (test vaccine) and the S19 original seed culture (as a reference strain). For this, harvest 24–48 hours' growth of each strain in sterile buffered saline solution (BSS: NaCl 8.5 g; KH_2PO_4 1.0 g; K_2HPO_4 2.0 g; purified water 1000 ml; pH 6.8 ± 0.2) and adjust the suspension in BSS to 10^9 CFU/ml using a spectrophotometer (0.170 OD when read at 600 nm). The exact number of CFU/ml should be checked afterwards by plating serial tenfold dilutions on to adequate culture medium (blood agar base or TSA are recommended).

2) Inject subcutaneously 0.1 ml (10^8 CFU/mouse) of the suspension containing the test vaccine into each of 32 female CD1 mice, aged 5–6 weeks. Carry out, in parallel, a similar inoculation in another 32 mice using the suspension containing the S19 reference strain. The original seed S19 strain, which has been shown satisfactory with respect to immunogenicity and/or residual virulence, can be obtained from USDA (see footnote 4 for address).

3) Kill the mice by cervical dislocation, in groups of eight selected at random 3, 6, 9 and 12 weeks later.

4) Remove the spleens and homogenise individually and aseptically with a glass grinder (or in adequate sterile bags with a paddle blender) in 1 ml of sterile BSS.

5) Spread each whole spleen suspension *in toto* onto several plates containing a suitable culture medium and incubate in standard *Brucella* conditions for 5–7 days (lower limit of detection: 1 bacterium per spleen). An animal is considered infected when at least 1 CFU is isolated from the spleen.

6) Calculate the 50% persistence time or 50% recovery time (RT_{50}) by a statistical method specifically developed for RT_{50} calculations[6]. For this, determine the number of cured mice (no colonies isolated in the spleen) at each slaughtering point time (eight mice per point) and calculate the percentage of cured accumulated mice over time, by the Reed and Muench method (see ref. cited in Grillo *et al*., 2000). The function of distribution of this percentage describes a sigmoid curve, which must be linearised for calculating the RT_{50} values, using the computerised PROBIT procedure of the statistical package.

6 To obtain details of the software contact the WOAH Reference Laboratory for brucellosis in France.

7) Compare statistically the parallelism (intercept and slope) between the distribution lines obtained for both tested and reference S19 strains using the software specifically designed for this purpose. Two RT_{50} values can be statistically compared exclusively when they come from parallel distribution lines. If parallelism does not exist, the residual virulence of the tested strain should be considered inadequate, and discarded for vaccine production.

8) If the parallelism is confirmed, compare statistically the RT_{50} values obtained for both tested and reference S19 strains using the software specifically designed for this purpose. To be accepted for vaccine production, the RT_{50} obtained with the tested strain should not differ significantly from that obtained with the reference S19 strain (RT_{50} and confidence limits are usually around 7.0 ± 1.3 weeks).

The underlying basis of the statistical procedure for performing the above residual virulence calculations have been described in detail (see ref. cited in Grillo *et al*., 2000). Alternatively, the statistical calculations described in steps 6 to 8 above can be avoided by an easy-to-use specific HTML-JAVA script program (Rev2) available free from the WOAH Reference Laboratory in France (Pouillot *et al*., 2004).

If this test has been performed with good results on a representative seed lot, it does not have to be repeated routinely on other seed lots and vaccine batches prepared from the same seed strain and using the same manufacturing process.

d) Immunogenicity in mice (Grillo *et al*., 2000)

This test uses three groups of six female CD1 mice, aged 5–7 weeks that have been selected at random.

1) Prepare and adjust spectrophotometrically the vaccine suspensions as indicated above.

2) Inject subcutaneously a suspension containing 10^5 CFU (in a volume of 0.1 ml/mouse) of the vaccine to be examined (test vaccine) into each of six mice of the first group.

3) Inject subcutaneously a suspension containing 10^5 CFU of live bacteria of a reference S19 vaccine into each of six mice of the second group. The third group will serve as the unvaccinated control group and should be inoculated subcutaneously with 0.1 ml of BSS.

4) The exact number of CFU inoculated should be checked afterwards by plating serial tenfold dilutions onto adequate culture medium (blood agar base or TSA are recommended).

5) All the mice are challenged 30 days after vaccination (and immediately following 16 hours starvation), intraperitoneally with a suspension (0.1 ml/mouse) containing 2×10^5 CFU of *B. abortus* strain 544 (CO_2-dependent), prepared, adjusted and retrospectively checked as above.

6) Kill the mice by cervical dislocation 15 days later.

7) Each spleen is excised aseptically, the fat is removed, and the spleen is weighed and homogenised.

8) Alternatively, the spleens can be frozen and kept at ≤ –16°C for 24 hours to 7 weeks.

9) Each spleen is homogenised aseptically with a glass grinder (or in adequate sterile bags in Stomacher) in nine times its weight of BSS, pH 6.8 ± 0.2 and three serial tenfold dilutions (1/10, 1/100 and 1/1000) of each homogenate made in the same diluent. Spread 0.2 ml of each dilution by quadruplicate in agar plates and incubate two of the plates in a 10% CO_2 atmosphere (allows the growth of both vaccine and challenge strains) and the other two

plates in air (inhibits the growth of the *B. abortus* 544 CO_2-dependent challenge strain), both at 37°C± 2°Cfor 5 days.

10) Colonies of *Brucella* (*B. abortus* 544) should be enumerated on the dilutions corresponding to plates showing fewer than 300 CFU. When no colony is seen in the plates corresponding to the 1/10 dilution, the spleen is considered to be infected with five bacteria. These numbers of *Brucella* per spleen are first recorded as X and expressed as Y, after the following transformation: Y = log (X/log X). Mean and standard deviation, which are the response of each group of six mice, are then calculated.

11) The conditions of the control experiment are satisfactory when: i) the response of unvaccinated mice (mean of Y) is at least of 4.5; ii) the response of mice vaccinated with the reference S19 vaccine is lower than 2.5; and iii) the standard deviation calculated on each lot of six mice is lower than 0.8.

12) Carry out the statistical comparisons (the least significant differences test is recommended) of the immunogenicity values obtained in mice vaccinated with the S19 strain to be tested with respect to those obtained in mice vaccinated with the reference vaccine and in the unvaccinated control group. The test vaccine would be satisfactory if the immunogenicity value obtained in mice vaccinated with this vaccine is significantly lower than that obtained in the unvaccinated controls and, moreover, does not differ significantly from that obtained in mice vaccinated with the reference vaccine (for detailed information on this procedure, see the WOAH Reference Laboratory in France).

If this test has been performed with good results on a representative seed lot, it does not have to be repeated routinely on other seed lots and vaccine batches prepared from the same seed strain and with the same manufacturing process.

ii) RB51 vaccine

An RB51 vaccine seed lot must possess the characteristics of the RB51 original strain, i.e. if it is satisfactory with respect to identity, roughness and potency.

a) Identity

The reconstituted RB51 vaccine should not contain extraneous microorganisms. *Brucella abortus* present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: *B. abortus* RB51 has the normal properties of a *B. abortus* bv. 1 strain, but is 100% in the rough phase and does grow in the presence of rifampicin (250 μg/ml). PCR and molecular techniques have been described to further characterise the RB51 vaccine strain (see Section B.1.4).

b) Roughness (determination of dissociation phase)

The same technical procedures indicated for S19 vaccine (see Section C. 1.2.1.2.3.i *S19 vaccine* above) have to be applied for RB51. 100% of the RB51 cells must be in the rough phase. Additionally, for RB51, all colonies should be negative on dot-blot assays with MAbs specific for the OPS antigen.

c) Potency

As dosage (CFU) of the master seed was correlated to protection as part of registration of RB51 for use cattle in the USA, *in-vivo* potency tests are not routinely conducted for serials of the RB51 vaccine. In the USA, plate counts of viable organisms have been approved and used as a measure of potency (this approach is identical to the potency test for S19 vaccine in the USA (USDA, 2003). Rough vaccines for brucellosis have been discussed in some detail (Moriyon *et al*., 2004).

iii) Rev.1 vaccine

A Rev.1 vaccine is efficient if it possesses the characteristics of the Rev.1 original strain, i.e. if it is satisfactory with respect to identity and smoothness. Moreover, it should have been produced with a given seed lot with adequate immunogenicity, and residual virulence (Grillo *et al*., 2000).

a) Identity

Brucella melitensis present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: when incubated in air at 37°C ± 2°C, Rev.1 strain is inhibited by addition to the suitable culture medium of 3 µg (5 IU) per ml of sodium benzyl-penicillin, thionin (20 µg/ml) or basic fuchsin (20 µg/ml); the strain grows on agar containing 2.5 µg per ml of streptomycin. PCR and molecular techniques have been described to further characterise the Rev.1 vaccine strain (see Section B.1.4).

b) Smoothness (determination of dissociation phase)

The same technical procedures indicated for S19 vaccine (Section C. 1.2.1.2.3.i above) have to be applied for Rev.1.

Sometimes, slight and difficult to observe differences can be seen in the size of Rev.1 colonies. The small colonies (1–1.2 mm in diameter) are typical for Rev.1, but larger Rev.1 colonies can appear depending on the medium used, the amount of residual moisture in the incubator atmosphere, and the presence or absence of CO_2. The frequency of variation in colony size occurs normally at a ratio of 1 large to 10^3 small colonies. Both Rev.1 variants are of the S (smooth) type. To avoid an increase in this colony size variation along successive passages, it is important to always select small colonies for preparation of seed lots.

At least 99% of cells in seed lots should be in the smooth phase.

c) Residual virulence (50% persistence time or 50% recovery time) (Grillo *et al*., 2000)

The same technical procedures indicated for RT_{50} calculation of S19 vaccine (see above) have to be applied for Rev.1, except that *B. abortus* S19 seed lot to be tested (test vaccine) and the S19 original seed culture (used as a reference strain), respectively, are replaced by the corresponding *B. melitensis* Rev.1 seed lot to be tested (test vaccine) and the *B. melitensis* Rev.1 original seed culture as the reference strain. For the reference original Rev.1 strain, RT_{50} and confidence limits are around 7.9 ± 1.2 weeks. A given Rev.1 vaccine seed lot or batch should keep similar residual virulence to be acceptable.

If this test has been done with good results on a representative seed lot, it does not have to be repeated routinely on seed lots and vaccine batches prepared from the same seed strain and with the same manufacturing process.

d) Immunogenicity in mice

The same technical procedures indicated for immunogenicity calculation of S19 vaccine (see above) have to be applied for Rev.1, except that *B. abortus* S19 seed lot to be tested (test vaccine) and the *B. abortus* S19 original seed culture (used as a reference strain), respectively, are replaced by the corresponding *B. melitensis* Rev.1 seed lot to be tested (test vaccine) and the *B. melitensis* Rev.1 original seed culture as the reference strain.

Conditions of the control experiment are satisfactory when: i) the response in unvaccinated mice (mean of Y) is at least of 4.5; ii) the response in mice vaccinated with the reference Rev.1 vaccine is lower than 2.5; and iii) the standard deviation calculated on each lot of six mice is lower than 0.8.

If this test has been done with good results on a representative seed lot, it does not have to be repeated routinely on seed lots and vaccine batches prepared from the same seed strain and with the same manufacturing process.

1.2.1.3. Validation as a vaccine

Numerous independent studies have confirmed the value of S19 as a vaccine for protecting cattle from brucellosis, and it has been the vaccine used (in combination with serological testing and culling) to eradicate bovine brucellosis in most currently free countries. The organism behaves as an attenuated strain when given to sexually immature cattle. In rare cases, when standard doses are applied subcutaneously, it may produce localised infection in the genital tract particularly in males. For this reason, vaccination of males is contra-indicated. Antibody responses persisting for 6 months or longer are likely to occur in a substantial proportion of cattle that have been vaccinated subcutaneously with the standard dose as adults. Some of the cattle vaccinated subcutaneously as calves may later develop arthropathy, particularly of the femoro-tibial joints. The vaccine is safe for most animals if administered to calves between 3 and 6 months of age. It may also be used in adult animals at a reduced dose, with the advantage of reducing the diagnostic interferences. It produces lasting immunity to moderate challenge with virulent *B. abortus* strains, but the precise duration of this immunity is not well known. The length of protection of this vaccine against *B. melitensis* infection in cattle is also unknown. The vaccine strain is stable and reversion to virulence is extremely rare. It has been associated with the emergence of i-erythritol-using strains when inadvertently administered to pregnant animals. The organism behaves as an attenuated strain in mice, and even large inocula are rapidly cleared from the mice tissues.

Reports from both experimental challenge studies and field studies remain controversial as far as the value of *B. abortus* strain RB51 in protecting cattle from brucellosis is concerned (see above). The organism is attenuated in calves but can cause safety problems in adults. *Brucella abortus* strain RB51 contains minimally expressed OPS but there is no serological conversion in both standard RBT and CFT in vaccinated animals. In addition, it has also been claimed that RB51 does not induce detectable antibodies, using current OPS-based testing procedures (USDA, 2003). However, the presence of common core epitopes in both smooth and rough *Brucella* does not allow always the antibody response to RB51 to be distinguished from that induced by field smooth *Brucella* strains, no matter which S-LPS or OPS-based ELISA is used (Gusi *et al*., 2019). The efficacy of RB51 against *Brucella* infection in cattle is controversial (Moriyon *et al*., 2004), but it is claimed that it protects against moderate challenge with virulent *B. abortus*, although the precise duration of this protection is unknown. The efficacy of this vaccine against *B. melitensis* infection in cattle is also unknown. The vaccine is very stable and no reversion to smoothness has been described *in vivo* or *in vitro*. The organism behaves as an attenuated strain in a variety of animals including mice where it is rapidly cleared from the tissues.

Numerous independent studies have confirmed the value of *B. melitensis* strain Rev.1 as a vaccine for protecting sheep and goats from brucellosis. Its virulence is unchanged after passage through pregnant sheep and goats. However, abortions and excretion in milk may result when the Rev.1 vaccine is inoculated into pregnant ewes and goats. The vaccine-induced abortions are not avoided using reduced doses and doses as low as 10^6, used either subcutaneously or conjunctivally in pregnant animals, have been proven to induce abortions and milk excretion of the vaccine strain (Blasco, 1997).

1.2.2. Method of manufacture

1.2.2.1. Procedure

Production of *Brucella* live vaccines is based on the seed-lot system described above (Section B.2.2) for BBAT and CFT antigens.

For the production of S19 vaccine, the procedures described above can be used, except that the cells are collected in PBS, pH 6.3 ± 0.2, and deposited by centrifugation or by the addition of sodium carboxymethyl cellulose at a final concentration of 1.5 g/litre. The yield from one fermenter run or the pooled cells from a batch of Roux flask cultures that have been

inoculated at the same time from the same seed lot constitutes a single harvest. More than one single harvest may be pooled to form a final bulk, which is used to fill the final containers of a batch of vaccine. Before pooling, each single harvest must be checked for purity, cell concentration, dissociation and identity. A similar range of tests must be done on the final bulk, which should have a viable count of between 8 and 24 × 10^9 CFU/ml. Adjustments in concentration are made by the addition of PBS for vaccine to be dispensed in liquid form, or by the addition of stabiliser for lyophilised vaccine. If stabiliser is to be used, loss of viability on lyophilisation should be taken into account, and should not be in excess of 50%. The final dried product should not be exposed to a temperature exceeding 35°C during drying, and the residual moisture content should be 1–2%. The contents must be sealed under vacuum or dry nitrogen immediately after drying, and stored at 5°C ± 3°C.

The production process for *B. abortus* strain RB51 is very similar to the one used for S19.

For the production of *B. melitensis* strain Rev.1 vaccine, the procedures described above for antigens (Alton *et al*., 1988) can be used except that the cells are collected in a freeze-drying stabiliser and deposited by centrifugation. The yield from one fermenter run or the pooled cells from a batch of Roux flask cultures inoculated on the same occasion from the same seed lot constitutes a single harvest. More than one single harvest may be pooled to form the final bulk that is used to fill the final containers of a batch of vaccine. Before pooling, each single harvest must be checked for purity, cell concentration, dissociation and identity. The volume of the final bulk is adjusted by adding sufficient stabiliser so that a dose contains an appropriate number of viable organisms. After adjusting the cell concentration of the final bulk, tests for identity, dissociation and absence of contaminating organisms are conducted (see below).

1.2.2.2. Requirements for ingredients

Strains should be cultured in a suitable medium.

Brucella abortus S19 and *B. melitensis* strain Rev.1 for vaccine production is grown on medium free from serum or other animal products, under conditions similar to those described above for *B. abortus* S99 or S1119-3 (Alton *et al*., 1988). The phenol saline is replaced by a freeze-drying stabiliser.

Brucella abortus strain RB51 follows similar culture methods.

Serum–dextrose agar, and trypticase–soy agar, to which 5% serum or 0.1% yeast extract may be added, are among the solid media that have been found to be satisfactory for propagating the Rev.1 strain (Alton *et al*., 1988). However, Rev.1 strain does not grow well on potato agar and generally needs 3–5 days to grow.

For all vaccines, the organisms are not killed but are stored at 5°C ± 3°C while quality control examinations are carried out as described below.

For preparation of the lyophilised vaccine, a stabiliser containing 2.5% casein digest, e.g. Tryptone (Oxoid), 5% sucrose and 1% sodium glutamate, dissolved in purified water and sterilised by filtration is recommended.

Antimicrobial preservatives must not be used in live *B. abortus* strain S19 or RB51 and *B. melitensis* strain Rev.1 vaccines.

1.2.2.3. In-process controls

Brucella abortus S19 and RB51 as well as *B. melitensis* Rev.1 vaccines should be checked for purity, identity and, where appropriate, smoothness or roughness during preparation of the single harvests. The cell concentration of the bulks should also be checked. This can be done by opacity measurement, but a viable count must be performed on the final filling lots. The identity of these should also be checked by agglutination tests with antiserum to either *Brucella*-A, -R or -M antigen respectively.

The viable count of the final containers should not be less than the recommended doses (see above).

For S19 and Rev.1, at least 99% of cells in seed lots and 95% of cells in final lots should be in the smooth phase, while 100% of the RB51 cells must be in the rough phase. Additionally, for RB51, all colonies should be negative on dot-blot assays with monoclonal antibodies specific for the OPS antigen.

For S19 and Rev.1, the immunogenicity and the residual virulence (50% persistence time or 50% recovery time) in the mice model should also be determined on representative seed lots. If these tests have been done with good results on a representative seed lot, it does not have to be repeated routinely on seed lots and vaccine batches prepared from the same seed strain and with the same manufacturing process.

1.2.2.4. Final product batch tests

With freeze-dried vaccine, the control tests should be conducted on the vaccine reconstituted in the form in which it will be used (same diluent).

1.2.2.4.1. Purity

Tests for purity and freedom from contamination of biological materials may be found in chapter 1.1.9.

1.2.2.4.2. Identity

See Section C.1.2.1.2.3 *Potency*.

1.2.2.4.3. Safety

See Section C.1.2.1.2.2 *Safety*.

1.2.2.4.4. Batch potency

i) Potency

For S19 and Rev.1 vaccines, potency can also be determined on the final lyophilised product. The procedure is as described above (identity; smoothness; residual virulence and immunogenicity checks; see Section C.1.2.1.2.3.). If residual virulence and immunogenicity checks have been performed with good results on a representative batch of the test vaccine, they do not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

ii) Enumeration of live bacteria

Batches should also be checked for the number of viable organisms. The same procedure may be applied for the S19, Rev.1 and RB51 vaccine batches. Inoculate each of at least five plates of tryptose, serum-dextrose or other suitable agar medium with 0.1 ml of adequate dilutions of the vaccine spread with a sterile glass, wire or plastic spreader. CFU per vaccine volume unit are enumerated.

Suitable CFU counts are the following:

S19:

a) $0.5–1 \times 10^{11}$ CFU (standard dose; subcutaneous route);

b) $0.5–5 \times 10^{9}$ CFU (reduced dose; subcutaneous route);

c) 5×10^{9} CFU (reduced dose; conjunctival route).

Rev.1:

a) $0.5–2 \times 10^{9}$ CFU (standard dose, subcutaneous or conjunctival route).

RB51:

a) 1–3.4 × 10^{10} CFU (standard dose; subcutaneous route).

1.2.3. Requirements for regulatory approval

1.2.3.1. Manufacturing process

For regulatory approval of the vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see above) should be submitted to the authorities. This information should be provided from three consecutive vaccine batches with a volume of not less than 1/3 of the typical industrial batch volume.

1.2.3.2. Safety requirements

1.2.3.2.1. Target and non-target animal safety

For the potential side-effects of the *Brucella* vaccines according to the status of the animals, see Section C.1.1. *Background*.

1.2.3.2.2. Reversion-to-virulence

Brucella abortus S19 and *B. melitensis* Rev.1 vaccines prepared from seed stock from appropriate sources are stable in characteristics, provided that the in-process and batch control requirements described above are fulfilled, and show no tendency to reversion to virulence.

Brucella abortus strain RB51 has shown no tendency to revert to virulent smooth organisms after many passages *in vitro* or *in vivo*. This is probably due to the nature and place of the mutations found in this strain. *Brucella abortus* strain RB51, despite carrying, among other unknown mutations, an IS711-disrupted *wbo*A (a putative glycosyl-tranferase gene), accumulates low amounts of cytoplasmic M-like OPS.

1.2.3.2.3. Precautions

Brucella abortus S19 and RB51, as well as *B. melitensis* Rev.1, although attenuated strains, are still capable of causing disease in humans. Accordingly cell cultures and suspensions must be handled under appropriate conditions of biohazard containment (see chapter 1.1.4). Reconstitution and subsequent handling of the reconstituted vaccine should be done with care to avoid accidental injection or eye or skin contamination. Vaccine residues and injection equipment should be decontaminated with a suitable disinfectant (phenolic, iodophor or aldehyde formulation) at recommended concentration. Medical advice should be sought in the event of accidental exposure. The efficacy of the antibiotic treatment of infections caused by S19, RB51 or Rev.1 in humans has not been fully established. However, it must be reiterated that, while the S19 strain carries no particular antibiotic resistance compared with other *Brucella* field strains, Rev.1 and RB51 strains are respectively streptomycin- and rifampicin-resistant.

Vaccine should be identified as pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection or exposure to vaccine (including aerosols) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

1.2.3.3. Efficacy requirements

Potency can also be determined on the final batch, but if safety/efficacy tests have been performed with good results on a representative seed lot or a batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

1.2.3.4. Duration of immunity

Vaccinating calves with a full dose of S19 vaccine is considered to give long-lasting immunity, and subsequent doses are not recommended. However, there is scanty evidence for this and revaccination within 6–12 months could be advisable in endemic areas.

The duration of immunity induced by RB51 vaccine in cattle is unknown, whatever the dose applied and the age at vaccination. Revaccination within 6–12 months has been proposed for boosting immunity in endemic areas.

It is accepted that subcutaneous or conjunctival vaccination with standard doses of Rev.1 confers a solid and durable immunity in sheep and goats. However, growing field evidence shows that the immunity conferred declines with time, and revaccination within 6–12 months could be advisable in endemic areas.

1.2.3.5. Stability

Brucella abortus S19 and *B. melitensis* Rev.1 vaccines prepared from seed stock from appropriate sources are stable in characteristics, provided that the in-process and batch control requirements described above are fulfilled, and show no tendency to reversion to virulence. The lyophilised vaccine shows a gradual loss of viable count, but should retain its potency for the recommended shelf life. Allowance for this phenomenon is normally made by ensuring that the viable count immediately following lyophilisation is well in excess of the minimum requirement. Maintenance of a cold chain during distribution of the vaccine will ensure its viability.

Brucella abortus strain RB51 has shown no tendency to revert to virulent smooth organisms after many passages *in vitro* or *in vivo*. This is probably due to the nature and place of the mutations found in this strain. *Brucella abortus* strain RB51, despite carrying, among other unknown mutations, an IS711-disrupted *wbo*A (a putative glycosyl-tranferase gene), accumulates low amounts of cytoplasmic M-like OPS.

2. Diagnostic biologicals: brucellin

2.1. Background

Brucellin-INRA is an LPS-free extract from rough *B. melitensis* B115, and a single inoculation of this preparation does not provoke formation of antibodies reactive in BBAT, CFT or ELISAs. However, as this rough strain contains *Brucella* OPS sugars in the cytosol extract, the repeated inoculation of brucellin could elicit antibodies, interfering with other diagnostic tests. For this reason, cytosolic protein extracts have been obtained from rough *B. abortus* mutants, defective in genes strictly necessary to synthesise perosamine, and therefore unable to generate OPS antibody response in sheep.

2.2. Outline of production and requirements

2.2.1. Characteristics of the seed

2.2.1.1. Biological characteristics of the master seed and quality criteria

Production of brucellin-INRA is based on a seed-lot system as described for antigens and vaccines. The original seed *B. melitensis* strain B115 for brucellin production[5] should be propagated to produce a seed lot, which should be preserved by lyophilisation or freezing at liquid nitrogen temperature. It should conform to the properties of a pure culture of a rough strain of *B. melitensis* and must not produce smooth *Brucella* LPS. It should produce reasonable yields of a mixture of protein antigens reactive with antisera to smooth and rough *Brucella* strains.

2.2.1.2. Quality criteria

Brucella melitensis B115 seed should be checked for purity.

Tests for purity and freedom from contamination of biological materials may be found in chapter 1.1.9.

2.2.1.3. Validation as an *in-vivo* diagnostic reagent

Laboratory and field studies in France have confirmed that brucellin-INRA is safe, non-toxic and specific in action. The preparation contains 50–75% proteins, mainly of low molecular weight, and 15–30% carbohydrate. It does not contain S-LPS antigens, does not provoke inflammatory responses in unsensitised animals, and it is not in itself a sensitising agent. After a single inoculation, it does not induce detectable antibodies in the standard serological tests for brucellosis. More than 90% of small ruminants infected with *B. melitensis* manifest delayed hypersensitivity to brucellin-INRA at some stage. The preparation is not recommended as a diagnostic agent for individual animals, but can be useful when used for screening herds. It is given to small ruminants in 100-µg doses by the intradermal route, and provokes a local delayed hypersensitivity reaction visible at 48–72 hours in sensitised animals. Positive reactions can be given by vaccinated as well as by infected animals (Pouillot *et al*., 1997).

2.2.2. Method of manufacture (Alton *et al*., 1988)

2.2.2.1. Procedure and requirements for ingredients

Brucellin is produced from *B. melitensis* strain B115 according to Alton *et al*., 1988.

2.2.2.2. In-process control

The crude brucellin extract should be checked for sterility after acetone extraction, to ensure killing of *Brucella* cells, and again at the end of the process to check possible contamination. The pH and protein concentration should be determined, and control tests (as described in Section C.2.2.2.3 below) should be performed on the bulk material before filling the final containers.

2.2.2.3. Final product batch tests

i) Sterility

Brucellin preparations should be checked for sterility as described in chapter 1.1.9.

ii) Safety

Brucellin preparations should also be checked for abnormal toxicity. Doses equivalent to 20 cattle doses (2 ml) should be injected intraperitoneally into a pair of normal guinea-pigs that have not been exposed previously to *Brucella* organisms or their antigens. Five normal mice are also inoculated subcutaneously with 0.5 ml of the brucellin (2000 U/ml) to be examined. Animals are observed for 7 days, and there should be no local or generalised reaction to the injection.

Dermo-necrotic capacity is examined by intradermal inoculation of 0.1 ml of the product to be tested into the previously shaved and disinfected flank of three normal albino guinea-pigs that have not been exposed previously to *Brucella* organisms or their antigens. No cutaneous reaction should be observed.

Absence of allergic and serological sensitisation is checked by intradermal inoculation of three normal albino guinea-pigs, three times every 5 days, with 0.1 ml of a 1/10 dilution of the preparation to be examined. A fourth similar injection is given, 15 days later, to the same three animals and to a control lot of three guinea-pigs of the same weight that have not been injected previously. The animals should not become seropositive to the standard tests for brucellosis (RBT, CFT) when sampled 24 hours after the last injection, and should not develop delayed hypersensitivity responses.

iii) Batch potency

The potency of brucellin preparations is determined by intradermal injection of graded doses of brucellin into guinea-pigs that have been sensitised by subcutaneous inoculation

of 0.5 ml of reference brucellin[7] in Freund's complete adjuvant from 1 to 6 months previously (the use of a live *Brucella* strain, for example Rev.1 strain, is possible provided that it produces the same level of sensitisation). The erythematous reactions are read and measured at 24 hours and the titre is calculated by comparison with a reference brucellin[8]. This method is only valid for comparing brucellin preparations made according to the same protocol as the sensitising allergen. Initial standardisation of a batch of allergen and the sensitisation and titration in ruminants is described (Alton *et al*., 1988).

2.2.3. Requirements for regulatory approval

2.2.3.1. Manufacturing process

For regulatory approval of the brucellin, all relevant details concerning manufacture of the product and quality control testing (see above) should be submitted to the regulatory authorities in accordance with their requirements. This information should be provided from three consecutive product batches with a volume of not less than 1/3 of the typical industrial batch volume.

2.2.3.2. Safety requirements

i) Target and non-target animal safety

No side-effects of the brucellin have ever been reported in animals.

ii) Reversion-to-virulence

Not applicable.

iii) Precautions

Brucellin is not toxic. Nevertheless it may provoke severe hypersensitivity reactions in sensitised individuals who are accidentally exposed to it. Care should be taken to avoid accidental injection or mucosal contamination. Used containers and injection equipment should be carefully decontaminated or disposed of by incineration in a suitable disposable container.

Brucellin should be identified as potentially harmless for practitioners. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection or mucosal exposure with warnings included on the product label/leaflet so that the practitioner is aware of any danger.

2.2.3.3. Efficacy requirements

Potency must be determined on the final product. The procedure is as described above.

2.2.3.4. Duration of sensitivity

Duration of sensitivity is uncertain. Individual animals vary considerably in the degree of hypersensitivity manifested to brucellin. Animals in the very early stages of infection, or with long-standing infection, may not manifest hypersensitivity to intradermal injection.

2.2.3.5. Stability

The freeze-dried preparation retains full potency for several years. The liquid commercial preparation should retain potency for the recommended shelf-life.

7 An EU reference brucellin (2000 Units/ml) is obtainable from the WOAH Reference Laboratory for Brucellosis in France.
8 The statistical procedure can be obtained from the WOAH Reference Laboratory for Brucellosis in France.

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NB: There are WOAH Reference Laboratories for brucellosis (*Brucella abortus*, *B. melitensis* and *B. suis*) (please consult the WOAH Web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for these *Brucella* agents

NB: BOVINE BRUCELLOSIS FIRST ADOPTED IN 1990. BRUCELLOSIS IN SHEEP, GOATS AND SWINE FIRST ADOPTED IN 1991. CHAPTER FIRST ADOPTED WITH CURRENT TITLE IN 2016. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.1.5.

CRIMEAN–CONGO HAEMORRHAGIC FEVER

SUMMARY

Crimean–Congo haemorrhagic fever virus (*CCHFV*) *of the genus* Orthonairovirus *of the family* Nairoviridae *causes a zoonotic disease in many countries of Asia, Africa, the Middle East and south-eastern Europe. As the distribution of CCHFV coincides with the distribution of its main vector, ticks of the genus* Hyalomma*, the spread of infected ticks into new, unaffected areas facilitates the spread of the virus. The virus circulates in a tick–vertebrate–tick cycle, but can also be transmitted horizontally and vertically within the tick population.* Hyalomma *ticks infest a wide spectrum of different wildlife species, e.g. deer and hares, and free-ranging livestock animals, e.g. goat, cattle, and sheep. Many birds are resistant to infection, but ostriches appear to be more susceptible. Viraemia in livestock is short-lived, and of low intensity. These animals play a crucial role in the life cycle of ticks, and in the transmission and amplification of the virus and are, therefore, in the focus of veterinary public health. As animals do not develop clinical signs, CCHFV infections have no effect on the economic burden regarding livestock animal production. In contrast to animals, infections of humans can result in the development of a severe disease, Crimean–Congo haemorrhagic fever* (*CCHF*).

Every year, more than 1000 human CCHF cases are reported with case fatality rates of 5–80% depending on the virus strain and other local factors. The pathogenesis of the disease in humans is not well understood. Most people become infected by tick bites and by crushing infected ticks, but infection is also possible through contact with blood and other body fluids of viraemic animals, for example in slaughterhouses. As CCHFV also has the potential to be transmitted directly from human-to-human, nosocomial outbreaks have been reported.

There is no approved CCHF vaccine available and therapy is restricted to treatment of the symptoms. Health education and information on prevention and behavioural measures are most important in order to enhance public risk perception and, therefore, decrease the probability of infections. Thus the identification of endemic areas is crucial for focused and targeted implementation of public health measures. Serological screening of ruminants allows CCHFV-affected areas to be identified, as antibody prevalence in animals is a good indicator of local virus circulation. Treatment with tick repellents can be quite effective in reducing the tick infestation of animals. To protect laboratory staff, handling of CCHFV infectious materials should only be carried out at an appropriate biocontainment level.

Detection and identification of agent: *Only a single virus serotype is known to date although sequencing analysis indicates considerable genetic diversity. CCHFV has morphological and physiochemical properties typical of the family* Nairoviridae*. The virus has a single-stranded, negative-sense RNA genome consisting of three segments: L* (*large*)*, M* (*medium*) *and S* (*small*)*, each of which is contained in a separate nucleocapsid within the virion. The virus can be isolated from serum or plasma samples collected during the febrile or viraemic stage of infection, or from liver of infected animals. Primary isolations are made by inoculation of several tissue cultures, commonly African green monkey kidney* (*Vero*) *cells. For identification and characterisation of the virus, conventional and real-time reverse transcription polymerase chain reaction* (*PCR*) *can be used. As infections of animals remain clinically unapparent, the likelihood of isolating virus from a viraemic animal is very low.*

Serological tests: *Type-specific antibodies are demonstrable by indirect immunofluorescence test or by IgG-sandwich and IgM-capture enzyme-linked immunosorbent assay. Commercial test systems are available for animal health; in addition a few in-house systems have been published or kits are used replacing the conjugate provided in kit with one that is suitable for the animal species to be screened for CCHFV-specific antibodies.*

Requirements for vaccines: *There is no vaccine available for animals.*

A. INTRODUCTION

Crimean-Congo haemorrhagic fever (CCHF) is a zoonotic disease caused by a primarily tick-borne CCHF virus (CCHFV) of the genus *Orthonairovirus* of the family *Nairoviridae,* order *Bunyavirales*. CCHFV possesses a negative-sense RNA genome consisting of three segments, L (large), M (medium) and S (small) each contained in a separate nucleocapsid within the virion. All orthonairoviruses are believed to be transmitted by either ixodid or argasid ticks, and only three are known to be pathogenic to humans, namely CCHF, Dugbe and Nairobi sheep disease viruses (Swanepoel & Burt, 2004; Swanepoel & Paweska, 2011; Whitehouse, 2004). CCHFV can be grown in several tick cell lines derived from both a natural vector (*Hyalomma anatolicum*) and other tick species not implicated in natural transmission of the virus (Bell-Sakyi*et al*., 2012).

The virus from an outbreak of "Crimean haemorrhagic fever" in the Crimean Peninsula in 1944 was not isolated or characterised until 1967. "Congo haemorrhagic fever" virus, isolated from a patient in the former Zaire (now Democratic Republic of the Congo) in 1956, was shown in 1969 to be the same virus. As a consequence the names of both countries have been used in combination to describe the disease (Hoogstraal, 1979). Distribution of the virus reflects the broad distribution of *Hyalomma* ticks, the predominant vector of the virus (Avsic-Zupanc, 2007; Grard *et al*., 2011; Papa *et al*., 2011; Swanepoel & Paweska, 2011).

The natural cycle of CCHFV includes transovarial and transstadial transmission among ticks and a tick-vertebrate-tick cycle involving a variety of wild and domestic animals. Infection can also be transferred between infected and uninfected ticks during co-feeding on a host; so called 'non-viraemic transmission' phenomenon. *Hyalomma* ticks feed on a variety of domestic ruminants (sheep, goats, and cattle), and wild herbivores, hares, hedgehogs, and certain rodents. CCHFV infection in animals was reviewed by Nalca & Whitehouse (2007). Experimental infections of wild animals and livestock with CCHFV were reviewed by Spengler *et al. (*2016). Although animal infections are generally subclinical, the associated viraemia levels are sufficient to enable virus transmission to uninfected ticks (Swanepoel & Burt, 2004; Swanepoel & Paweska, 2011). Many birds are resistant to infection, but ostriches appear to be more susceptible than other bird species (Swanepoel *et al*., 1998). Although they do not appear to become viraemic, ground feeding birds may act as a vehicle for spread of CCHFV infected ticks. Results from serological surveys conducted in Africa and Eurasia indicate extensive circulation of the virus in livestock and wild vertebrates (Swanepoel & Burt, 2004).

Humans acquire infection from tick bites, or from contact with infected blood or tissues from livestock or human patients. After incubation humans can develop a severe disease with a prehaemorrhagic phase, a haemorrhagic phase, and a convalescence period. Haemorrhagic manifestations can range from petechiae to large haematomas. Bleeding can be observed in the nose, gastrointestinal system, uterus and urinary tract, and the respiratory tract, with a case fatality rate ranging from 5% to 80% (Ergonul, 2006; Yen *et al.,* 1985; Yilmaz *et al*., 2008).The severity of CCHF in humans highlights the impact of this zoonotic disease on public health. Although CCHFV has no economic impact on livestock animal production, the serological screening of animal serum samples for CCHFV-specific antibodies is very important. As seroprevalence in animals is a good indicator for local virus circulation, such investigations allow identification of high-risk areas for human infection (Mertens *et al.,* 2013). Slaughterhouse workers, veterinarians, stockmen and others involved with the livestock industry should be made aware of the disease. They should take practical steps to limit or avoid exposure of naked skin to fresh blood and other animal tissues, and to avoid tick bites and handling ticks. Experiences from South Africa demonstrated that the use of repellents on animals before slaughter could reduce the numbers of infected slaughterhouse workers (Swanepoel *et al.,* 1998). The treatment of livestock in general can reduce the tick density among these animals and thus reduce the risk of tick bite in animal handlers (Mertens *et al.,* 2013). Such tick control by the use of acaricides is possible to some extent, but may be difficult to implement under extensive farming conditions. Inactivated mouse brain vaccine for the prevention of human infection has been used on a limited scale in Eastern Europe and the former USSR (Swanepoel & Paweska, 2011). Progress in CCHFV vaccine development is being made with several different approaches trialled to overcome current challenges (Dowall *et al.,* 2017).

Infectivity of CCHFV is destroyed by boiling or autoclaving and low concentrations of formalin or beta-propriolactone. The virus is sensitive to lipid solvents. It is labile in infected tissues after death, presumably due to a fall in pH, but infectivity is retained for a few days at ambient temperature in serum, and for up to 3 weeks at 4°C. Infectivity is stable at temperatures below –60°C (Swanepoel & Paweska, 2011). CCHFV should be handled with appropriate biocontainment measures determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities* (Palmer, 2011; Whitehouse, 2004).

B. DIAGNOSTIC TECHNIQUES

Table 1. Diagnostic test formats for Crimean-Congo haemorrhagic fever virus infections in animals

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases in animals	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[a]						
Real-time RT-PCR	–	+++	–	+++[c]	+[b]	–
Virus isolation in cell culture	–	–	–	+[c]	–	–
Detection of immune response						
IgG ELISA	+++	+	–	+	+++	–
Competitive ELISA	+++	+	–	+	+++	–
IgM ELISA	–	++	–	++	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]RT-PCR is used for the screening of tick populations in the context of surveillance studies.
[c]Molecular testing/isolation can be used to confirm acute infection in rare cases in animals showing clinical signs as viraemia tends to be transient.

CCHFV infection causes only a mild fever in domestic and wild vertebrate animals with a detectable viraemia of up to 2 weeks (Gonzalez *et al.,* 1998; Gunes *et al.,* 2011). Similarly infected ostriches develop only low and short-lived viraemia and no clinical signs (Swanepoel & Burt, 2004). Therefore, recent infections in animals are rarely diagnosed and methods such as polymerase chain reaction (PCR), virus isolation in cell culture and IgM detection by enzyme-linked immunosorbent assay (ELISA) are mainly used in human CCHF diagnostics or in the special case that an animal has to be classified as CCHFV free. For prevalence analysis and for determination of whether CCHFV is circulating in a country, methods for the detection of IgG antibodies are preferred (Table 1). If there is any possibility or suspicion that diagnostic samples could be contaminated with CCHFV, they should be handled under an adequate biosafety level and all persons dealing with those samples should be aware of the possible risk and should use personal protective equipment to avoid human infections.

1. Detection and identification of the agent

For testing animals for viraemia, rapid diagnosis can be achieved by detection of viral nucleic acid in serum or plasma using conventional (Burt *et al.,* 1998) or real-time reverse transcription (RT-) PCR (Drosten *et al.,* 2002; Duh *et al.,* 2006; Koehler *et al.*, 2018; Negredo *et al.*, 2017; Sas *et al.*, 2018; Wolfel *et al.,* 2007), or by demonstration of viral antigen (Shepherd *et al.,* 1988). Specimens to be submitted for laboratory confirmation of CCHF include blood and liver samples. Because of the risk of laboratory-acquired infections, work with CCHFV should be conducted in appropriate biosafety facilities.

The virus can be isolated from serum and organ suspensions in a wide variety of cell cultures, including Vero, LLC-MK2, SW-13, BSR-T7/5, CER and BHK21 cells, and identified by immunofluorescence using specific antibodies. Isolation and identification of virus can be achieved in 1–5 days, but cell cultures lack sensitivity and usually only detect high concentrations of virus present in the blood.

1.1. Virus isolation in cell culture

CCHFV can be isolated in mammalian cell cultures. Vero cells are commonly used, usually yielding an isolate between 1 and 5 days post-inoculation (p.i). CCHFV is poorly cytopathic and thus infectivity is

titrated by demonstration of immunofluorescence in infected cells (Shepherd *et al*., 1986). SW-13 cell line has also been used extensively for virus isolation, producing plaques within 4 days (p.i.). Identification of a CCHFV isolate has to be confirmed by immunofluorescence or molecular techniques (Burt *et al*., 1998; Shepherd *et al*., 1986).

1.1.1. Test procedure

i) Susceptible cell lines include Vero-E6, BHK-21, LLC-MK2 and SW-13 cells. Inoculate 80% confluent monolayers of the preferred cell line with the specimen. The volume of specimen to be used depends on the size of the culture vessel (i.e. 25 cm^2 culture flask or 6- or 24-well tissue culture plate). The specimen volume should be sufficient to cover the cell monolayer. Samples of insufficient volumes can be diluted with tissue culture medium to prepare sufficient inoculation volume.

ii) Adsorb the specimen for 1 hour at 37°C.

iii) Remove inoculum. Add fresh tissue culture medium containing 2% fetal calf serum and other required additives, as per specific medium and cell line requirements.

iv) Incubate at 37°C and 5% CO_2 for 4–7 days.

v) Test supernatant for presence of CCHFV viral RNA using real-time RT-PCR as described below, or perform immunofluorescence assay on cell scrapings.

vi) Isolates of CCHFV from clinical specimens cause no microscopically recognisable cytopathic effects (CPE) in most of these cell lines.

1.2. Nucleic acid detection

Molecular-based diagnostic assays, such as RT-PCR, serve as the front-line tool in the diagnosis of CCHF, as well as other viral haemorrhagic fevers (Drosten *et al*., 2003). The benefit of molecular diagnostic assays is their rapidity compared to virus culture, often allowing a presumptive diagnosis to be reported within a few hours after receiving a specimen (Burt *et al*., 1998). The RT-PCR is a sensitive method for diagnosis, but because of the genetic diversity of CCHFV, there might be some challenges with regard to design of primers or probes that allow detection of all circulating strains of the virus. Indeed, based on geographical origin and phylogenetic analyses of the S gene segment, CCHFV has previously been classified into nine geographical clades – four predominantly diffused in Africa, three in Europe, and two in Asia. Several real-time RT-PCR assays that detect strains from different geographical locations have been evaluated (Gruber *et al.,* 2019). While some assays have been shown to be highly sensitive, detecting as little as 10 viral RNA copies per ml of plasma, it is necessary to combine at least two molecular assays to ensure detection of the different CCHFV clades (Gruber *et al.,* 2019). The best assay combination(s) with the best detection efficacy for each CCHFV clade, on the basis of all CCHFV sequences known at the time of the study, are shown in Table 2. In addition, a low-density macroarray has been extensively validated in clinical specimens collected from confirmed cases of CCHF over 20 years by a WHO reference laboratory. It was shown to detect as few as 6.3 genome copies per reaction (Wolfel *et al*., 2009).

Table 2. Molecular assay combinations for the detection of CCHFV-specific nucleic acid

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
Africa 1	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
Africa 2	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Fwd CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
Africa 3	Nested RT-PCR	Fwd CCHF1 (CTG-CTC-TGG-TGG-AGG-CAA-CAA) Rev CCHF2_5 (TGG-GTT-GAA-GGC-CAT-GAT-GTA-T) Nested Fwd CCHFn15 (AGG-TTT-CCG-TGT-CAA-TGC-AAA) Nested Rev CCHFn25 (TTG-ACA-AAC-TCC-CTG-CAC-CAG-T)
	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1– (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Nested Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Nested Rev CriCon2– (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
Africa 4	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Real-time RT-PCR	Fwd CCHF-III (CAA-GAG-GTA-CCA-AGA-AAA-TGA-AGA-AGG-C) Rev CCHF-III-r (GCC-ACG-GGG-ATT-GTC-CCA-AAG-CAG-AC) Probe CCHFprobe-1 (ATC-TAC-ATG-CAC-CCT-GCY-GTG-YTG-ACA) Probe CCHFprobe-2 (TTC-TTC-CCC-CAC-TTC-ATT-GGR-GTG-CTC-A)
Asia 1	Nested PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
	Real-time RT-PCR	Fwd CC1a_for (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Probe CCHF-01 (CAA-CAG-GCT-GCT-CTC-AAG-TGG-AG)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
Asia 2	Nested PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
	Sybrgreen Real-time RT-PCR	Fwd (GAT-GAG-ATG-AAC-AAG-TGG-TTT-GAA-GA) Rev (GTA-GAT-GGA-ATC-CTT-TTG-TGC-ATC-AT)
	RT-PCR	Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A) Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC) Rev 2 CCAS2 (CTA-ATC-ATG-TCT-GAC-AGC-ATC-TC)
Europe 1	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Nested RT-PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
Europe 2	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1– (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Rev CriCon2– (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
Europe 3	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
All	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1– (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Rev Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Nested Rev CriCon2– (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
	RT-PCR	Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A) Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC) Rev 2 CCAS2 (CTA-ATC-ATG-TCT-GAC-AGC-ATC-TC)
	Real-time RT-PCR	Fwd CC1a_for (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Probe CCHF-01 (CAA-CAG-GCT-GCT-CTC-AAG-TGG-AG)

(Data and table modified from Gruber *et al*. 2019)

2. Serological tests

Virus neutralisation assays, generally considered to be highly specific, are rarely used for CCHFV diagnosis. Members of the *Orthonairovirus* genus generally induce a weaker neutralising antibody response than members of other genera in the family *Nairoviridae.* Another drawback is the necessity to perform this assay in high biosafety containment because it uses live virus (Burt *et al*., 1994; Rodriguez *et al*., 1997).

Currently, there are only a few CCHFV commercial kits for IgM or IgG by ELISA or immunofluorescence (IFA). These are all designed for the human diagnostic market. However, it is possible to adapt these commercial ELISAs and IFAs for serological testing in animals. In addition, some in-house ELISAs have been published for the detection of CCHFV-specific antibodies in animals.

Diagnostic performance for humans have been compared between the methods using sensitivity, specificity, concordance and degree of agreement with particular focus on the phase of the infection (Emmerich *et al.,* 2021). Available serological test systems detect anti-CCHFV IgM and IgG antibodies accurately, but their diagnostic performance varies with respect to the phase of the infection. In the early and convalescent phases of infection, the sensitivity for detecting specific IgG antibodies differed for the ELISA. Both test systems based on immunofluorescence showed an identical sensitivity for detection of anti-CCHFV IgM antibodies in acute and convalescent phases of infection.

IgM antibodies in livestock (sheep, goat and cattle) can be detected by using an IgM-capture ELISA. IgG antibodies can be detected by an IgG-sandwich or indirect ELISA, and total antibodies can be detected by competition ELISA. The benefit of competitive ELISA is the capacity to investigate different animal species, because they are host species independent. Commercial kits for the detection of CCHFV-specific antibodies or the detection of viral antigen are available. The limiting factor for the replication of these protocols in other laboratories is the availability of antigens and (where relevant) specified monoclonal antibodies. Most of the tests described for livestock and wild animals have not undergone a formal validation process (Mertens *et al*., 2013). One of the biggest challenges for such validation studies is the availability of an adequate number of positive well characterised control samples.

For information on the availability of reference reagents for use in veterinary diagnostic laboratories, contact the WOAH Collaborating Centres for Zoonoses in Europe and in Asia-Pacific.

C. REQUIREMENTS FOR VACCINES

There is no vaccine available for animals.

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NB: At the time of publication (2023) there was no WOAH Reference Laboratory for Crimean–Congo haemorrhagic fever (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 2014. MOST RECENT UPDATES ADOPTED 2023.

CHAPTER 3.1.6.

ECHINOCOCCOSIS (INFECTION WITH *ECHINOCOCCUS GRANULOSUS* AND WITH *E. MULTILOCULARIS*)

SUMMARY

Human cystic echinococcosis, caused by Echinococcus granulosus sensu lato (s.l.)*, and alveolar echinococcosis, caused by* E. multilocularis*, are important public health threats in many parts of the world. Diagnosis of echinococcosis in dogs or other susceptible carnivores relies on the detection of adult cestodes of the* Echinococcus *genus or their eggs in the faeces or small intestine. Coproantigen and coproDNA assays have proven useful particularly for epidemiological screening programmes For the metacestode stage, diagnosis in humans is performed by imaging techniques supported by immunological tests, while in animals, diagnosis is based on post-mortem detection of the larval form that can infect almost any organ, particularly the liver and lungs, with subsequent species confirmation by polymerase chain reaction (PCR)* and DNA sequencing.

Detection of the agent: *It was previously accepted that there were five valid species of the genus* Echinococcus; *the current view however, informed by biology, epidemiology and particularly molecular genotyping, recommends the inclusion of at least nine species within the genus. All those species of* Echinococcus *known to cause cystic echinococcosis in the intermediate host may be referred to as* E. granulosus s.l.*, whereas genotypes G1,3 which are closely related are now referred to as* E. granulosus sensu stricto (s.s.)*. It is also widely believed that within* E. granulosus s.l., E. equinus (*G4*), E. ortleppi (*G5*) *and* E. canadensis (*G6, G7, G8, G10*) *should be considered as distinct species although there is still some debate as to whether* E. canadensis *represents more than one species. Larval forms of* E. granulosus s.l. *and* E. multilocularis *in intermediate hosts can be detected by macroscopic and microscopic examination of visceral organs. Special care has to be taken for a specific diagnosis of* E. granulosus *in instances where* Taenia hydatigena *in sheep is also a problem. Histological examination may confirm the diagnosis after formalin-fixed material is processed by conventional staining methods. The presence of a periodic-acid-Schiff positive, acellular laminated layer with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of metacestodes of* Echinococcus*. Genotyping via PCR/sequencing is the only method available to confirm the exact species of* Echinococcus *infecting animals. The small intestine is required at necropsy for the detection of adult* Echinococcus *spp. in definitive hosts* (*wild and domestic carnivores*)*. Handling infected material needs detailed safety precautions to avoid risk to the operator of contracting a potentially fatal disease.*

Coproantigen or CoproDNA tests: *Significant progress is being made in the development of immunological tests for the diagnosis of intestinal* Echinococcus *infections by use of coproantigen detection. The technique has been used successfully only in some countries for surveys of* E. granulosus *in dogs and is currently used in surveys for* E. multilocularis *in populations of dogs and foxes in high endemic areas. Coproantigen detection is possible in faecal samples collected from dead or living animals or from the environment. However, as these tests were developed based on adult worm antigens, false positives may occur. PCR DNA methods for the detection of* E. multilocularis *and more recently* E. granulosus *in definitive hosts have now been validated as diagnostic techniques.*

Serological tests: *Antibodies directed against oncosphere, cyst fluid and protoscolex antigens can be detected in the serum of infected dogs and sheep, but this approach is presently of limited practical use as it does not distinguish between current and previous infections. Moreover, analytical*

sensitivity and specificity can sometimes be poor as cross-reactivity between Echinococcus *and* Taenia *species also may occur.*

Requirements for vaccines: *A vaccine for* E. granulosus *s.s. based on the EG95 recombinant antigen has proven to be safe and effective in livestock. Commercial EG95 vaccines are available and are manufactured in Argentina, Morocco and China (People's Rep. of). The vaccines have gained regulatory approval in the countries of manufacture as well as a number of other countries. The EG95 vaccine was adopted in 2016 as a compulsory part of the national program for control of echinococcosis in China. No vaccine is available for Echinococcus infection in the parasites' definitive hosts.*

A. INTRODUCTION

The species within the genus *Echinococcus* are small (1–11 mm length) tapeworms of carnivores with a larval stage known as metacestode that proliferates asexually encysting in the internal organs of various mammals including humans. Until recently it was accepted that there were five morphologically distinct species in this genus: *Echinococcus granulosus s.l.*, *Echinococcus multilocularis*, *Echinococcus oligarthra*, *Echinococcus vogeli* and *Echinococcus shiquicus*. However, the current view, informed by biology, epidemiology and particularly molecular genotyping, recommends the inclusion of at least nine species within the genus. *Echinococcus granulosus s.l.*, formerly regarded as a single species with a high genotypic and phenotypic diversity, is now recognised as an assemblage of five cryptic species, which differ considerably in morphology, development, host specificity (including infectivity or pathogenicity for humans). This diversity is reflected in the mitochondrial and nuclear genomes. Based on phenotypic characters and gene sequences, *E. granulosus s.l.* has now been subdivided into *E. granulosus sensu stricto* (*s.s.*) (including the formerly identified genotypic variants G1, 3), *Echinococcus felidis* (the former 'lion strain'), *Echinococcus equinus* (the 'horse strain', genotype G4), *Echinococcus ortleppi* (the'cattle strain', genotype G5) and *Echinococcus canadensis*. The latter species, as recognised here, shows the highest diversity and is composed of the 'camel strain', genotype G6, the 'pig strain', genotype G7, and two 'cervid strains', genotypes G8 and G10 (Nakao *et al*., 2013; Romig *et al*., 2015). Studies performed on nearly complete mitochondrial genome and significantly long nuclear genetic DNA sequences suggest the previous G2 genotype be considered a microvariant of G3 (Kinkar *et al*., 2017) and *E. canadensis* be considered a cluster composed of two different species (Laurimae *et al.,* 2018).

Echinococcus granulosus (s.l.) has a global distribution; *E. multilocularis* occurs in wide areas of the northern hemisphere, *E. shiquicus* is found in the Tibetan plateau and *E. oligarthra* and *E. vogeli* are confined to Central and South America. Nearly all the originally described species are infective to humans causing various echinococcal diseases, although in the most recent taxonomic classification there is no evidence of *E. shiquicus* and *E. felidis* infections in humans (Ma *et al*., 2015). Human cystic echinococcosis (CE), caused by *E. granulosus s.l.,* and alveolar echinococcosis (AE), caused by *E. multilocularis*, are important public health threats in many parts of the world (WHO/WOAH, 2001) caused by ingestion of eggs derived directly or indirectly from definitive hosts. The strong zoonotic potential of *E. granulosus s.l.* is mainly related to *E. granulosus s.s.* (Alvarez Rojas *et al.,* 2014). Clinical specimens and eggs of *Echinococcus* spp. should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Table 1. Useful characteristics for identification of Echinococcus species (source: Xiao* et al.*, 2006)

	***E. granulosus* (*sensu lato*)**	***E. multilocularis***	***E. oligarthra***	***E. vogeli***	***E. shiquicus***
Distribution	Cosmopolitan	Holoarctic region	Neotropical region	Neotropical region	Tibet plateau
Definitive host	Wild and domestic carnivores	Foxes/dogs	Wild felids	Bush dog	Tibetan fox
Intermediate host	Ungulates	Microtine rodents	Neotropical rodents	Neotropical rodents	Plateau pika

	E. granulosus (*sensu lato*)	*E. multilocularis*	*E. oligarthra*	*E. vogeli*	*E. shiquicus*
Adult					
Body length (mm)	2.0–11.0	1.2–4.5	2.2–2.9	3.9–5.5	1.3–1.7
No. segments	2–7	2–6	3	3	2–3
Length of large hooks (μm)	25.0–49.0	24.9–34.0	43.0–60.0	49.0–57.0	20.0–23.0
Length of small hooks (μm)	17.0–31.0	20.4–31.0	28.0–45.0	30.0–47.0	16.0–17.0
No. testes	25–80	16–35	15–46	50–67	12–20
Position of genital pore					
a. Mature segment	Near to middle	Anterior to middle	Anterior to middle	Posterior to middle	Near to upper edge
b. Gravid segment	Posterior to middle	Anterior to middle	Near to middle	Posterior to middle	Anterior to middle
Gravid uterus	Branching laterally	Sac-like	Sac-like	Tubular	Sac-like
Metacestode	Unilocular cysts in viscera	Multilocular cysts in viscera	Unicystic cysts in muscles	Polycystic cysts in viscera	Unilocular cysts in viscera

1. *Echinococcus granulosus sensu lato* (*s.l.*)

The parasite is most frequently transmitted between the domestic dog and a number of domestic ungulate species. In countries where sheep farming plays an important role in the local economy, *E. granulosus s.s.* is maintained prevalently by a dog–sheep cycle. Sylvatic cycles involving different definitive and intermediate hosts (e.g. wolf or cervid) have been reported for *E. canadensis* (see Deplazes *et al.,* 2017 for further illustration) and for *E. felidis,* with lion or spotted hyena acting as definitive hosts. There is some intermediate host predilection in some strains – e.g. *E. equinus* in horses, *E. ortleppi* in cattle and *E. canadensis* in pigs, camels and cervids. The adult worm varies between 2 and 11 mm in length and usually possesses from two to seven segments, averaging from three to four segments. The penultimate segment is mature, and the genital pore normally opens posterior to the middle in both mature and gravid segments. The last (gravid) segment is usually more than half the length of the entire worm. There are rostellar hooks of various sizes on the protoscolex in two rows. The size of the hooks varies between 25 and 49 μm in the first row, and between 17 and 31 μm in the second row. The gravid uterus has well-developed sacculations.

The metacestode, known as hydatid, is a fluid-filled bladder that is typically unilocular, although communicating chambers may also occur. Growth is expansive, and newly formed hydatids (daughter cysts) inside and, occasionally, outside the cyst may be produced. Individual CE cyst may reach up to 30 cm in diameter and occur mainly in liver and lungs. Other internal organs are affected less frequently. The infection with this stage is referred to as cystic echinococcosis

2. *Echinococcus multilocularis*

The parasite is transmitted primarily between wild definitive hosts (e.g. foxes, *Vulpes vulpes, V. corsac*, *Alopex lagopus*) and small arvicolid rodents (voles and lemmings). The adult varies between 1.2 and 4.5 mm in length and usually possesses from two to six segments, with an average of four to five. The penultimate segment is characteristically mature, and the genital pore is anterior to the midline in both mature and gravid segments. The gravid uterus is sac-like. On the rostellum, the larger hooks of the first row vary in size between 24.9 and 34.0 μm and the smaller hooks of the inner row between 20.4 and 31.0 μm.

The metacestode is a multivesicular structure consisting of conglomerates of small vesicles, usually not exceeding a few millimetres in diameter. Unlike *E. granulosus*, the larval mass often contains a semisolid rather than a fluid matrix. It proliferates by exogenous budding, which results in infiltration of tissues. Infection with this stage is commonly referred to as alveolar echinococcosis.

This zoonotic parasite is found mainly in the Northern Hemisphere, and its life cycle is mainly maintained in wildlife (Deplazes *et al.,* 2017). Like *E. granulosus,* there are a number of genetic variants or haplotypes based on microsatellite EmsB and mitochondrial gene sequences. These are associated with different geographical regions and have been named the Asian, the Mongolian, the North American 1, the North American 2 and the European haplotypes. In Europe the prevalence of *E. multilocularis* in red foxes varied from zero to >10% in different countries, and over 50% in high endemic areas. *E. multilocularis* has also been detected in Arctic foxes (Deplazes *et al.,* 2017). Domestic dogs, raccoon dogs, golden jackals and wolves have also been shown to act as definitive hosts. Experimental studies indicate that domestic cats play an insignificant role in transmission (Kapel *et al.,* 2006). Rodents of the genus *Microtus, Arvicola, Myodes* and *Lemmus* are all known to be suitable intermediate hosts as are muskrats (*Ondatra zibethicus*), nutria/coypu (*Myocastor coypus*) and beaver (*Castor fiber*).

3. *Echinococcus oligarthra*

The parasite typically uses neotropical wild felids as definitive hosts (e.g. *Felis concolor, F. jaguarundi*) and large rodents (e.g. *Dasyprocta* sp., *Cuniculus paca*) as intermediate hosts. The adult varies between 2.2 and 2.9 mm in length, and normally possesses three segments, the penultimate of which is mature. The genital pore is anterior to the middle in mature segments and approximately at the middle in gravid segments. The gravid uterus is sac-like.

The metacestode is polycystic and fluid-filled with a tendency to become septate and multichambered. The rostellar hooks of the protoscolex vary in length between 25.9 and 37.9 μm. The hooks are described in more detail in the next section where they are also compared with those of *E. vogeli*. The single cyst may reach a diameter of approximately 5 cm. Predilection sites are internal organs and muscles. To date, there have only been a few reports of human disease. The parasite appears not to mature in dogs.

4. *Echinococcus vogeli*

The parasite typically uses the South American bush dog (*Speothus venaticus*) as a wild definitive host, but the domestic dog is susceptible, as are large rodents (e.g. *Cuniculus paca*) as intermediate hosts. The adult varies between 3.9 and 5.5 mm in length, and usually has three segments, the penultimate of which is mature. The genital pore is situated posterior to the middle in both the mature and gravid segments. The gravid uterus has no lateral sacculations and is characterised by being relatively long and tubular in form, compared with the other segments, which are sac-like.

The metacestode is similar to that of *E. oligarthra*. It has been reported that the two species can be distinguished by comparing differences in the dimensions and proportions of the rostellar hooks on the protoscolex. The hooks of *E. oligarthra* vary in length between 25.9 and 37.9 μm (average 33.4 μm) and between 22.6 and 29.5 μm (average 25.45 μm) for large and small hooks, respectively. Those of *E. vogeli* vary between 19.1 and 43.9 μm (average 41.64 μm) and between 30.4 and 36.5 μm (average 33.6 μm) for the large and small hooks, respectively. The hook-guard for *E. oligarthra* also divides the hook 50:50, compared with 30:70 for *E. vogeli.*

Echinococcus vogeli is a zoonotic agent with approximately 200 human cases in total reported in South America. The infection caused by the larval stage of this species may be referred to as neotropical echinococcosis.

5. *Echinococcus shiquicus*

The parasite was found in the Tibetan fox (*Vulpes ferrilata*) its definitive host and the plateau pika (*Ochotona curzoniae*), the intermediate host. In most species of *Echinococcus*, the gravid segment is connected to a mature segment; however, a strobila consisting of only two segments (a gravid segment directly attaching to a premature segment) is unique to this species (Xiao *et al.,* 2005). The adult stage is morphologically similar to *E. multilocularis* but differs by its smaller hooks, fewer segments, upper position of genital pore in the premature segment and fewer eggs in the gravid segment. It is easily distinguishable from *E. granulosus* by its shorter length, branchless gravid uterus and the anterior position of the genital pore in the gravid segment. The adult measures 1.3 to 1.7 mm.

The metacestode is found mainly in the lungs of pika and is essentially a unilocular minicyst containing fully developed brood capsules; however, oligovesicular forms have also been observed. It is differentiated from *E. granulosus* by the absence of daughter cysts within the fertile cyst (WHO/WOAH, 2001).

A detailed description of echinococcosis in humans and animals can be found in the WHO/WOAH Manual on echinococcosis (WHO/WOAH, 2001).

B. DIAGNOSTIC TECHNIQUES

Table 2. Test methods available for the diagnosis of echinococcosis and their purpose

Method	Purpose (metacestode cysts in intermediate hosts)					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases[(a)]	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Parasite Identification /meat inspection	++	–	++	++	++	–
Antigen detection	–	–	–	–	–	–
PCR	++	–	–	+++	++	–
Detection of immune response						
ELISA	–	–	–	–	+	+
Purpose (adult worms in carnivorous definitive hosts)						
Detection of the agent						
Parasite isolation/ microscopy	+	+	+++	+++	++	–
Antigen detection	+	++	+++	+++	++	–
PCR	–	++	+++	+++	+++	–
Detection of immune response						
ELISA	–	–	–	–	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[(a)]Meat inspection may only identify cysts seen and not include subsequent tests to confirm they are due to *Echinococcus granulosus* infection.

1. Detection of the agent

In the intermediate host, diagnosis depends on the meat inspection or post-mortem detection of the larval cyst form, which can occur in almost any organ, particularly in the liver and lungs. The diagnosis of echinococcosis in dogs or other carnivores requires the demonstration of the adult cestodes of *Echinococcus* spp. in the small intestine or the detection of specific coproantigens or coproDNA in faeces. Comprehensive reviews are available

relating to diagnostic procedures for *E. granulosus s.l.* (Craig *et al.,* 2015) and *E. multilocularis* (Conraths & Deplazes, 2015).

Investigators carrying out these procedures are exposed to the risk of infection and severe disease, which must be minimised by appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4). Infective (egg/adult) material can be decontaminated by freezing at –80°C (core temperature) for 5 days, or by heating to 70°C for 1 hour. Face masks, disposable gloves and an apron must be worn. Chemical disinfection is not reliable, although sodium hypochlorite (10% bleach) can be used to destroy eggs. Contaminated material must be destroyed by incineration or autoclaving.

1.1. Diagnosis of larval echinococcosis in intermediate hosts

1.1.1. Necropsy

Whereas surveillance for *E. granulosus s.l.* in domestic animals may take place in licensed slaughter houses, that for *Echinococcus* spp. in wildlife must be done by field surveys. When undertaking surveillance work with *E. granulosus s.l.* in intermediate hosts, it is vitally important that data are stratified and reported according to the age of animals slaughtered. Prevalence rates are strongly age dependent and reports from abattoirs that may slaughter only young animals will substantially under-represent the true situation. This is because older animals may be heavily infected even when animals have very few larvae.

CE cysts can be observed in many organs, but in large animals, such as sheep and cattle, palpation or incision should be done. Pigs, cattle, sheep and goats may also be infected with larval *Taenia hydatigena,* and it is sometimes difficult to differentiate between these two parasites when they occur in the liver. In wild animals, such as ruminants and rodents, several other larval cestodes should be considered for differential diagnosis. Please refer to Chapter 3.9.5 *Cysticercosis* for information on other cestodes found at meat inspection.

i) Suspect parasite material should be removed from the organ by cutting with a scalpel to include the immediate host tissue, and kept in a cool location. (**NB**: parasite material in intact cysts will remain viable for more than 24 hours after death even at ambient temperatures. However viability will be prolonged by storage at 4°C for up to 72 hours. If material cannot be examined within this time, it should be stored either in 10% formol saline for subsequent microscopic examination or in 70–90% ethanol for subsequent DNA analysis. Ideally a sample of parasite material should be preserved in both media. Parasite tissues that are frozen will not be viable but can be examined morphologically on thawing and subjected to DNA analyses.

ii) For morphological analysis of cyst contents, fluid should be removed and retained using a syringe. The material inside the cyst should then be washed with saline and the contents examined under the microscope (×4 objective) for the presence of protoscoleces. Note that some CE cysts may be sterile and not contain protoscoleces. If no protoscoleces are present, the germinal layer on the inside of the cyst cavity may be observed as a gelatinous structure that can easily be pulled away. Formalin-fixed material can be stained by conventional histological techniques. The presence of a periodic-acid-Schiff (PAS) positive acellular laminated layer, underlying a connective tissue layer, and with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of the metacestodes of *Echinococcus* spp.

iii) In all cases exact species/genotype identification can only be made through extraction of DNA from ethanol-fixed or frozen material and subsequent genotyping by polymerase chain reaction (PCR) and, where needed, sequencing. This requires either protoscoleces or pieces of germinal layer to be present. Cysts removed from animals should be cut open after the fluid has been removed and pieces of cyst wall removed to 70% ethanol. It is important to remember that identification of the parasite genotype can give significant information on transmission cycles and that an individual animal may contain mixed infections of more than one genotype. Specific primers based on mitochondrial genes (cox 1, NAD1) and ribosomal genes (12s) have been identified for all *Echinococcus* species and related taeniids and are summarised by Roelfsema *et al*. (2016). These also include primers listed for the detection of adult worms in Table 3, Section B.2.2.1.

1.2. Diagnosis of adult parasites in carnivores

1.2.1. Necropsy

Necropsy is invariably employed in studies of echinococcosis in wildlife and is useful if domestic carnivores are humanely culled. It should be emphasised that it is necessary to isolate and identify the adult *Echinococcus*, because under normal conditions of faecal examination, the eggs of *Echinococcus* cannot be differentiated from those of *Taenia* spp. The eggs of *E. granulosus* and *E. multilocularis* can now be identified and differentiated from other taeniid eggs by PCR. It should also be emphasised that any possible contact with eggs is potentially very hazardous and requires risk management. Tissues should be deep frozen at between –70°C and –80°C for 3–7 days before necropsy to kill any eggs.

The small intestine is removed as soon as possible after death, and tied at both ends. If the material is not frozen or formalin fixed (4–10%), it should be examined quickly, as the parasite can be digested within 24 hours. Formalin does not kill eggs. The fresh intestine is divided into several sections and immersed in 0.9% saline at 38±1°C for examination. Worms adhering to the intestinal wall may be observed and counted by means of a hand lens (for *E. granulosus* and *E. vogeli*). For accurate counts, the unfixed intestine is best divided into four or six sections, opened up and immersed in 0.9% saline at 38±1°C for 30 minutes to release the parasites. The contents are washed into another container for detailed examination, and the intestinal wall is scraped with a spatula. All material is boiled and washed by sieving to eliminate most of the particulate material and to make it non-infectious. The washed intestinal contents and scrapings are placed on a black tray, and the worms are counted with the aid of a hand lens or stereoscopic microscope. *E. granulosus* is usually found in the first third of the small intestine of dogs and *E. multilocularis* in the mid/posterior sections. This approach has a greater than 95% sensitivity, except under low worm burdens where false negative results may occur.

Necropsy is considered to be the most reliable form of diagnosis for *E. multilocularis* in definitive hosts. It is a useful method for determining the prevalence in a population and the best way to determine worm burden. Carcasses or intestines of definitive hosts for examination should be deep frozen at between –70°C and –80°C for 3–7 days before necropsy to kill any eggs. Eggs of *E. multilocularis* are resistant to freezing to –50°C. *Echinococcus multilocularis* can survive in liquid nitrogen (around –200°C) for 35 years and still be infective.

1.2.2. Sedimentation and counting technique (SCT) (Eckert, 2003)

This well established technique has been widely used, but is less sensitive than the coproDNA (PCR) test.

i) The small intestine is incised longitudinally and cut into 20 cm long segments or into five pieces of approximately the same length. These pieces are transferred to a glass bottle containing 1 litre physiological saline (0.9% NaCl) solution.

ii) The glass bottle is shaken vigorously for a few seconds and the pieces of intestine are removed. The superficial mucosal layer is stripped by exerting pressure between thumb and forefinger to dislodge attached helminths.

iii) The glass bottle is left for 15 minutes for sedimentation to occur; the supernatant is then decanted. The glass bottle is refilled with physiological saline solution. This procedure is repeated 2–6 times until the supernatant is cleared of coloured particles.

iv) The sediment fraction is examined in small portions of about 5–10 ml in rectangular plastic or Petri dishes with a counting grid (9× 9 cm) in transmission light under a stereomicroscope at a magnification of ×120.

v) If up to 100 worms are found, the entire sediment fraction is checked; if higher numbers are present, the total worm burden is calculated from the count of one subsample.

1.2.3. Preserving specimens

Intact worms are fragile and for morphological studies are best handled in normal saline with a Pasteur pipette. They are washed free of other material and left for approximately 30 minutes for all movement to cease. For all DNA characterisation, worms should be transferred to 70–90%

ethanol. For morphological studies, the worms should be fixed in 5–10% formalin. Persons involved in such examinations should receive serological screening for anti-*Echinococcus* serum-antibodies at least once a year (WHO/WOAH, 2001).

Methods have been developed aimed at simplifying and improving epidemiological investigations in final host populations and allowing diagnosis in living animals. These methods include the detection of coproantigens and PCR DNA detection (see below).

1.3. Arecoline surveys and surveillance

Purgation with arecoline has been used to perform surveys of tapeworm infections in dog populations. Its use as a control agent has been superseded by praziquantel. Arecoline can cause discomfort to dogs and its use for diagnostics is not recommended.

2. Coprological tests

Adult *Echinococcus* worms inhabiting the intestine will release both surface or secretory molecules (antigens) and DNA (usually contained within eggs). Both types of molecules can be detected by assaying faecal samples. The sensitivity of the tests is strongly influenced by the worm burden and stage of maturity.

2.1. Coproantigen tests

Coproantigen ELISA (enzyme-linked immunosorbent assay) or coproELISA provides an alternative method for diagnosing canine echinococcosis, and both polyclonal and monoclonal antibodies have been used, directed against either somatic or excretory/secretory (ES) antigens. To date, only a few commercial coproELISAs have been reported. Wang *et al.* (2021) evaluated these tests and found that they have good sensitivity and specificity. However, the kits are not easily available. Moreover, several tests developed within individual research laboratories, have been described even if, a certain amount of variability between tests from different laboratories regarding sensitivity and specificity are reported.

CoproELISAs are usually genus-specific for *Echinococcus* spp. (Allan & Craig, 2006). For canine echinococcosis due to *E. granulosus* most authors report reasonable sensitivity (78–100%) and good genus specificity from 85% to greater than 95% as well as a degree of pre-patent detection (Deplazes *et al*., 1992). Where cross-reactions occur these generally appear to be caused by infection with *Taenia hydatigena*, the most common taeniid of dogs, and attempts to improve specificity by using monoclonal antibodies in coproELISAs have not been able to eliminate this problem. CoproELISA sensitivity broadly correlates with worm burden of *E. granulosus*, however some low intensity infections (worm burdens <50–100) may give false negatives in coproELISA (Allan & Craig, 2006).

For detection of *E. multilocularis* infection of foxes, necropsy is time-consuming. Coproantigen testing by ELISA may offer a specific practical alternative. Fox faecal samples should be taken at post-mortem from the rectum rather than from the small intestine. *Echinococcus* coproantigens are also stable in fox or dog faeces left at 18–25°C for 1 week and in dog faeces frozen at –20°C. Coproantigen testing has also been successfully used to evaluate the efficacy of deworming wild foxes infected with *E. multilocularis* using praziquantel-laced bait, which proved to be a successful combination of eliminating the source of infection.

2.1.1. Typical coproantigen test procedure (*Echinococcus* genus specific) (Craig *et al.,* 1996)

i) The faecal sample (collected per rectum or from the ground) is mixed with an equal volume of phosphate buffered saline (PBS), pH 7.2, containing 0.3% Tween 20 (PBST), in a capped 5 ml disposable tube. This is shaken vigorously and centrifuged at 2000 ***g*** for 20 minutes at room temperature. Faecal supernatants can be tested immediately or stored at –20°C or lower. Supernatants that appear very dark or viscous are still acceptable for use.

ii) A 96-well ELISA microtitre plate is coated with optimal concentration (typically 5 µg per ml) of a protein A purified IgG fraction of rabbit anti-*E. granulosus s.l.* proglottid extract in 0.05 M bicarbonate/carbonate buffer, pH 9.6 (100 µl per well). The plate is covered and incubated overnight at 4°C.

iii) The wells are rinsed three times in PBST with 1 minute between washes; 100 µl of the same buffer is added to each well, and the plate is incubated for 1 hour at room temperature.

iv) The PBST is discarded and 50 µl of neat fetal calf serum is added to all wells. This is followed by the addition of 50 µl per well of faecal sample supernatants is added (in duplicate wells). The plate is incubated at room temperature for 1 hour with plastic film to seal the plate.

v) The wells are rinsed as in step iii, but the contents are discarded into a 10% bleach (hypochlorite) solution.

vi) An optimal dilution concentration of around 1 µg/ml of an IgG rabbit anti-*E.-granulosus* proglottid extract peroxidase conjugate in PBST is prepared and 100 µl per well is added to all wells. The plate is incubated for 1 hour at room temperature (22–24°C).

vii) The wells are rinsed as in step iii.

viii) Next, 100 µl per well of tetramethyl benzidene (TMB) or similar peroxidase substrate is added and the plate is left in the dark for 20 minutes at room temperature (22–24°C).

ix) Absorbance of wells is read at 650 nm. The enzyme-substrate reaction can be stopped by adding 100 µl of 1 M phosphoric acid (H_3PO_4) to each well. The colour turns from blue to yellow if positive and is read at 450 nm.

x) Laboratories should establish their own end-point criteria using standard positive and negative samples. Standards can also be obtained from the WOAH Reference Laboratory[1]. Usually, the positive to negative threshold is taken as 3 standard deviations above the mean absorbance value of control negatives, or against a reference standard control positive using absorbance units equivalence.

2.2. CoproDNA methods

2.2.1. Definitive hosts

While coproantigen ELISAs provide a better overall and practical alternative to arecoline purgation for pre-mortem detection of canine echinococcosis, their lack of species specificity is a disadvantage, especially for epidemiological studies. The amplification of small fragments of species-specific *Echinococcus* DNA in eggs or in faeces by PCR was first reported for *E. multilocularis* infections in foxes, with reduced inhibition and sensitivity subsequently increased by egg concentration through sieving and zinc chloride flotation of faecal samples (Mathis *et al*., 1996). Cabrera *et al.* (2002) applied this approach targeted to the mitochondrial cytochrome c oxidase subunit 1 (cox1) gene of *E. granulosus* as proof of principle for PCR identification of eggs of *E. granulosus* (with an analytic sensitivity of four eggs) isolated from adult tapeworms and faecal samples from necropsied dogs in Argentina. The ability to perform PCR with faecal samples or extracts directly without first isolating taeniid eggs is an advantage, especially when relatively large numbers of samples require testing. However faecal material preserved in formol saline is not suitable for DNA amplification and 70% ethanol or freezing should be used. Commercial extraction kits designed for faecal specimens can be used to extract total DNA from canid faecal samples (1–2 g). This approach has been used with at least two coproPCRs based on the EgG1 Hae III repeat (Abbasi *et al.,* 2003) and the NADH dehydrogenase subunit 1 gene (ND1) (Boufana *et al.,* 2013).

In recent years there have been a number of key developments attempting to simplify DNA amplification (e.g. loop-mediated isothermal amplification [LAMP]) (Ni *et al*., 2014; Salant *et al*., 2012) and improve sensitivity and specificity (e.g. real-time PCR) (Dinkel *et al.,* 2011; Knapp *et al.,* 2014; Øines *et al.,* 2014). This is important in relation to differential diagnosis between *E. granulosus* genotypes, *E. multilocularis* and other taeniids that occur in the same geographical area. Multiplex PCR in particular are a useful approach to multispecies detection. (Dinkel *et al.,* 2011; Trachsel *et al.,* 2007). Currently there are several published PCRs for the *E. granulosus* complex and *E. multilocularis* (Table 3) and their great value is an extremely high specificity to the extent that a result can be taken as an alternative to the finding of worms at necropsy or purgation. A practical and cost-effective way to undertake testing of dogs or foxes on a large-scale is to adopt a serial testing strategy based on primary screening of all samples using the coproELISA,

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

followed by testing of all positives using coproPCR ensuring that duplicate samples are taken from each animal and fixed appropriately for each technique.

Table 3. PCR primers used for coproDNA detection* (*modified from Craig* et al., *2015*). *Tissue indicates that the technique is also compatible with DNA extraction from metacestode tissues

Gene (all sequences 5' → 3')	Species	Copro-sample	Tissue	Reference
cox1 F: TCA-TAT-TTG-TTT-GAG-KAT-YAG-TKC R: GTA-AAT-AAM-ACT-ATA-AAA-GAA-AYM-AC	*E. granulosus s.l.*	Eggs	Yes	Cabrera *et al.,* 2002
EgG1HaeIII Eg1121a F: GAA-TGC-AAG-CAG-CAG-ATG Eg1122a R: GAG-ATG-AGT-GAG-AAG-GAG-TG	*E. granulosus* s.l	Faeces	Yes	Abbasi *et al.,* 2003
12sRNA Eg1f F: CAT-TAA-TGT-ATT-TTG-TAA-AGT-TG Eg1r, R: CAC-ATC-ATC-TTA-CAA-TAA-CAC-C	*E. granulosus* G1	Eggs/ Faeces	Yes	Stefanic *et al.,* 2004
12S rRNA G1: E.g.ss1 F: GTA-TTT-TGT-AAA-GTT-GTT-CTA E.g.ss1 R: CTA-AAT-CAC-ATC-ATC-TTA-CAA-T G5, G6, G7: E.g.cs1 F: (ATT-TTT-AAA-ATG-TTC-GTC-CTG) E.g.cs1 R: (CTA-AAT-AAT-ATC-ATA-TTA-CAA-C) To discriminate between *E. ortleppi* and *E. granulosus* G6/7, semi-nested PCRs specific for G6/7 (*g6/7 PCR*; e.g. camel. F: ATG-GTC-CAC-CTA-TTA-TTT-CA e.g.cs1 R) and for *E. ortleppi* (*g5 PCR*; e.g. cattle. F: ATG-GTC-CAC-CTA-TTA-TTT-TG E.g.cs1 R)	*E. granulosus* G1, G5, G6/7	No	Yes	Dinkel *et al.,* 2004
Cox1, NAD, rrnS Multiple sequences referred to	*E. multilocularis, E. granulosus*, *Taenia* spp.	Eggs	Yes	Trachsel *et al.,* 2007
Real-time multiplex-nested PCR Primer/probe sequence P60.short. F: TGG-TAC-AGG-ATT-AGA-TAC-CC P375.short. R: TGA-CGG-GCG-GTG-TGT-ACC CVF. F: TTA-ATG-ACC-AAC-ATT-CGA-AA CVF. R: AGG/T-ACA/G-TAG/C-CCC-ATA/G-AAA/T-GC Pnest. F: ACA-ATA-CCA-TAT-TAC-AAC-AAT-ATT-CCT-ATC Pnest. R: ATA-TTT-TGT-AAG-GTT-GTT-CTA CVF.light F: TCA/T-GCC/T-TGA-TGA/G-AAC-TTC-GGA/G-TCC CVF.light R: AC/TA/G-ATT-CCA-ATA/G-TTT-CAT-GTC/T-TCT emulti-fl: CTA-AAA-CTA-CAC-AAA-CTT-ACA-TTA-CTA--FL emulti-705; LC705-ACA-ATA-ATA-TCA-AAC-CAG-ACA-TAC-ACC-A-PH CaVuFe1-fl: ATA-CAC-TAT-ACA-TCT-GAC-AC-FL CaVuFe2-640: LC640-GCT-ACT-GCT-TTC-TCA-TCT-G-PH	*E. multilocularis, E. granulosus* (G1), *E. ortleppi, E. canadensis* (G6, G7), *other taeniids*	Faeces	Yes	Dinkel *et al.,* 2011
LAMP method Eg1121aGAA-TGC-AAG-CAG-CAG-ATGEg1122aGAG-ATG-AGT-GAG-AAG-GAG-TGFIP1echCTT-TTC-CGG-ATG-GGT-AGG-CAT-CTT-TTG-ATC-ACT-CCT-ATT-CTA-GCA-TGTBIP1echCGT-GCT-GTG-GAG-GTA-GTT-TCG-TTT-TCA-GTG-AGA-TGA-GTG-AGA-AGG	*E. granulosus* G1	Eggs	Yes	Salant *et al.,* 2012
ND1 Eg181, F: GTT-TTT-GGC-TGC-CGC-CAG-AAC Eg183, R: AAT-TAA-TGG-AAA-TAA-TAA-CAA-ACT-TAA-TCA-ACA-AT Em19/3, F: TAG-TTG-TTG-ATG-AAG-CTT-GTT-G Em6/1, R: ATC-AAC-CAT-GAA-AAC-ACA-TAT-ACA-AC	*E. granulosus* G1; *E. multilocularis; E shiquicus*	Faeces	Yes	Boufana *et al.,* 2013

Gene (all sequences 5' → 3')	Species	Copro-sample	Tissue	Reference
Many mitochondrial and nuclear primers	*E. granulosus* complex G1–(G10)	(Eggs)	Yes	Boubaker *et al.,* 2013
Nad5 gene primers inc. (LAMP method) Primer name and sequence FIP: TTA-ACC-AAC-CAA-TAA-CAA-CCC-AGT-gaattc-GTG-GTG-TTA-GTT-ATT-TGG-TTA-GG BIP: ATG-TGA-CGT-TTG-GTG-TGG-TAG-TTA-gaattc-AAG-AAC-CAC-CAA-AAT-AAT-GTC-T F3: GTG-TGT-TGC-TAT-ATT-GCT-TGT B3: AAC-TTT-AAC-AAC-ATA-CAC-CTA-GT	*E. granulosus s.s* (G1)	Faeces	Yes	Ni *et al.,* 2014
Magnetic capture – PCR mt 12S rRNA gene **EMrtCO1 F'** (TGG-TAT-AAA-GGT-GTT-TAC-TTG-G), **EMrtCO1 R'** (ACG-TAA-ACA-ACA-CTA-TAA-AAG-A), and Zen probe: 56-FAM/-TCT-AGT-GTA/Zen/-AAT-AAG-AGT-GAT-CCT-ATT-TTG-TGG-TGG-GT/3IABkFq/	*E. multilocularis*	Faeces	Yes	Isaksson *et al*., 2014
Real-time PCR large ribosomal subunit gene (rrnL) Em-rrn primer: F CTG-TGA-TCT-TGG-TGT-AGT-AGT-TGA-GAT-TT Em-rrn R GGC-TTA-CGC-CGG-TCT-TAA-CTC Em-probe with reporter 6-carboxyfluorescein (FAM) and quencher tetramethylrhodamine (TAMRA): TGG-TCT-GTT-CGA-CCT-TTT-TAG-CCT-CCA-T	*E. multilocularis*	Faeces	Yes	Knapp *et al.,* 2014
Real-time PCR using a hydrolysis probe targeting part of the mitochondrial "large ribosomal subunit" gene rrnL-Em F CTG-TGA-TCT-TGG-TGT-AGT-AGT-TGA-GAT-TT rrnL-Em R GGC-TTA-CGC-CGG-TCT-TAA-CTC	*E. multilocularis*	Faeces	Yes	Knapp *et al.,* 2014; Knapp *et al.,* 2016
Multiphase approach described in Santolamazza *et al.,* 2020 Phase1: DNA extraction; Phases 2: PCR Cox1; Phases 3: RFLP AluI specific digestion for distinction of G1, G3,G4, G5, G6/7 and G8/G10; Phases 4: multiple PCR showing the specific banding patterns for the G4-G10 genotypes	*E. granulosus sensu lato* G1, G3,G4, G5, G6/7 and G8/G10	Not available	Yes	Cox1: Bowles *et al.,* 1992 modified by Bart *et al.,* 2006 RFLP AluI: Kim *et al.,* 2017 Multiple PCR: Boubaker *et al.,* 2017
Real-time PCR for multiple sequences referred to Cox1, Cox3, NAD5	*E. granulosus sensu stricto* (G1, G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6–8, G10)	Faeces	Yes	Maksimov *et al.,* 2020

LAMP: loop-mediated isothermal amplification

3. Serological tests

3.1. Intermediate hosts

Serological diagnosis of ovine echinococcosis has long been considered a potentially important tool for epidemiological studies in endemic areas, as well as for surveillance of control programmes. It has been known for many years that sheep infected experimentally with *E. granulosus* can mount detectable specific IgG responses within weeks. However, serum antibody levels varied greatly in natural infections resulting in reduced sensitivity and cross-reactions with *Taenia hydatigena* or *T. ovis* infected animals. At present this approach cannot replace necropsy (Craig *et al.,* 2015; McManus, 2014).

3.2. Definitive hosts

Serodiagnostic tests for canine echinococcosis were considered to have good potential for practical testing of dogs for *E. granulosus* infection and, initially, as a potential substitute for arecoline purgation. Diagnostic specificity was good (>90%) but sensitivity was generally poor (35–40%) with natural infections, and was much lower when compared directly with coproantigen detection (Jenkins *et al.,* 1990). Further research to assess existing or develop better recombinant antigens may improve the sensitivity of serological tests for canine echinococcosis.

There is mounting evidence of cases where dogs developed alveolar echinococcosis (AE) caused by the larval stage. AE in dogs is fast-developing and life-threatening. Frey *et al.* (2017) evaluated the diagnostic performance of several antigens for serological detection of AE in dogs. Excellent performance of ELISA with recombinant EM95 antigen in combination with Western blot was demonstrated. The test can potentially detect AE in the early stages of development. Since AE in dogs may be concomitant with the intestinal adult stage, it was suggested that dogs from endemic areas should be tested for *E. multilocularis* using available methods, including the EM95 ELISA, before relocation into non-endemic regions.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Intermediate hosts

Application of an effective vaccine to reduce CE infection in livestock may have a substantial impact on the rate of transmission of the disease to humans (Lightowlers, 2006; Torgerson, 2003). A highly effective EG95 vaccine has been developed that can prevent infection with *E. granulosus s.s.* in the parasite's livestock intermediate hosts (Lightowlers *et al.,* 1996; Gauci *et al.,* 2011). Field trials of the vaccine have demonstrated that the vaccine reduces the level of cystic echinococcosis in sheep under natural conditions (Amarir *et al*., 2021; Larrieu *et al*., 2019). The ultimate aim of vaccination against *E. granulosus* is to reduce the parasite's transmission and reduce human exposure to cystic echinococcosis.

Two subcutaneous immunisations, approximately 1 month apart, induce protection against subsequent exposure to *E. granulosus* eggs. The vaccine is not believed to cure an infection existing prior to the animal being vaccinated. For that reason, young animals should receive their first vaccination around the time of weaning. The duration of immunity induced in young animals after a second vaccination is sufficient to protect them until 1 year of age, at which time a single booster vaccination elicits a strong protective response sufficient to induce long-lasting protection (Larrieu *et al*., 2019; Poggio *et al*., 2016). Hence, a vaccination programme involving two immunisations in recently weaned animals, followed by a single booster immunisation when animals are approximately 1 year of age, presents effective and practical programme.

The EG95 vaccine is manufactured in Argentina, China (People's Rep. of) and Morocco, and has gained regulatory approval in Morocco and a number of East and South Asian, and South American, countries.

1.2. Definitive hosts

Development of *E. granulosus* vaccines for dogs would ideally reduce worm fecundity and populations, and could be a valuable step towards the reduction (prevention) of the infection pressure on intermediate hosts, and thus reduce (prevent) infection in dogs. However, no clear evidence exists for immunologically-based protection against *Echinococcus* infection in definitive hosts. Attempts to actively immunise dogs against infection with *E. granulosus* have not produced consistent results.

2. Outline of production and minimum requirements for vaccines for intermediate hosts

The vaccine incorporates 50 µg of the EG95 antigen, expressed in *Escherichia coli*, together with adjuvant (Quil A or Montanide ISA 70 plus saponin) (Gauci *et al.,* 2011; Lightowlers *et al.,* 1996).

2.1. Characteristics of the seed

2.1.1. Quality criteria

Suitable *E. coli* strains (BB4 LE392.23 [F´ *lacI*^{q} ZΔM15 *proAB* Tn10 (Tet^{r})] or BL21 (DE3) F^{-}*omp*T *hsdS*$_{B}$ (r_{B}^{-}, m_{B}^{-}) *gal dcm* (DE3) for expression of recombinant vaccine antigen should be acquired from a source that has been established as sterile and pure (free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and those listed by the appropriate licensing authorities). The pGEX expression vector should be obtained from a source known to be free of extraneous agents. The EG95 DNA insert in the expression vector should be verified as having the sequence described (Gauci *et al.,* 2011; Lightowlers *et al.,* 1996).

2.2. Methods of manufacture

2.2.1. Procedure

Suitable *E. coli* strains are transformed with pGEX vector containing in-frame EG95 cDNA. Bacteria are cultured in a suitable medium such as Super Optimal Broth. Recombinant protein expression is induced by addition of isopropyl-ß-D-thiogalactosidase (IPTG) at a concentration of 0.2 mM with incubation for 3–5 hours, after which the culture supernatant is discarded and the bacterial pellets are resuspended in PBS, pH 7.4. After chilling on ice, *E. coli* are lysed by sonication, French Press or other suitable equipment. The extent of bacterial cell lysis is monitored by measuring an increase in the soluble proteins released from the ruptured cells by determination of protein concentration. Soluble and insoluble cellular proteins are separated by centrifugation. After a brief wash with PBS, insoluble proteins are solubilised in 8 M urea. Vaccine can be prepared from either the soluble fraction by glutathione agarose affinity purification as described by Lightowlers *et al.* (1996) or from the insoluble inclusion bodies as described by Gauci *et al.* (2011). Proteins are analysed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and quantified by scanning densitometry and determining protein concentration according to Bradford (1976).

2.2.2. Requirements for ingredients

All ingredients used for vaccine production should comply with the requirements referred to in Chapter 1.1.8 *Principles of veterinary vaccine production*.

2.2.3. Final product batch tests

i) Sterility

Must comply with chapter 1.1.8.

ii) Safety

Batch safety testing is performed unless consistent safety of the product is demonstrated and approved in the regulatory approval dossier and the production process is approved for consistency with the standard requirements referred to in chapter 1.1.8.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions. Local and general reactions must be examined. The tests must be performed by administering the vaccine to the sheep in the recommended dose and recommended route of administration. Objective and quantifiable criteria to detect and measure adverse reactions should be used; these would include temperature changes of vaccinated and control groups.

iii) Batch potency

Sheep are immunised according to the recommended protocol (two subcutaneous injections, 3–4 weeks apart). IgG antibody responses to the EG95 antigen are determined 2 weeks after the second immunisation as described by Heath & Koolaard (2012).

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NB: There is a WOAH Reference Laboratory for echinococcosis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for echinococcosis

NB: FIRST ADOPTED IN 1989 AS ECHINOCOCCOSIS/HYDATIDOSIS. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.1.7

EPIZOOTIC HAEMORRHAGIC DISEASE (INFECTION WITH EPIZOOTIC HEMORRHAGIC DISEASE VIRUS)

SUMMARY

Epizootic haemorrhagic disease (EHD) is a vector-borne infectious noncontagious viral disease of domestic and wild ruminants, primarily white-tailed deer (Odocoileus virginianus) *and cattle. Sheep, goats and camelids might also be susceptible, but usually do not develop overt disease.*

EHD virus (EHDV) is transmitted between ruminant hosts by species of Culicoides *biting midges, thus EHD infections are strongly seasonal. White-tailed deer is the most severely affected species, with the peracute form having a high mortality rate. In cattle, clinical signs occur rarely but fever, anorexia, dysphagia, emaciation, ulcerative stomatitis, lameness, respiratory distress and erythema of the udder have been reported.*

Detection of the agent: *EHDV belongs to the family* Reoviridae*, genus* Orbivirus*, and shares many morphological and structural characteristics with the other members of the genus, in particular bluetongue virus (BTV).*

EHDV particles are non-enveloped but have a double capsid with an icosahedral symmetry. Within the virus core, 10 double-stranded RNA genomic segments code for seven structural proteins (VP) and at least four nonstructural proteins (NS). The protein VP2 of the outer core is the major determinant of serotype specificity, while the VP7 of the inner core possesses the serogroup-specific antigens. At least seven distinct serotypes have been identified and two new putative serotypes; there is however, some uncertainty regarding the exact number of serotypes and a panel of reference strains of EHDV is not yet officially recognised.

Assays for detection of EHDV in field samples include virus isolation in cell culture and EHD serogroup-specific reverse-transcription polymerase chain reaction (RT-PCR) tests. Suspected isolates can be identified by competitive (antigen-capture) and sandwich enzyme-linked immunosorbent assays (ELISAs). Serotype-specific RT-PCR assays have been developed for serotype identification of cell culture isolates. Isolates may also be identified by high throughput sequencing or virus neutralisation tests.

Serological tests: *Antibodies to EHDV may be first detectable from 8 days post-exposure. Neutralising antibodies and the virus can co-exist in the infected animal, likely because of the strong association between the EHDV and the red blood cells.*

For the detection of anti-EHDV antibodies in the sera of exposed animals, a specific monoclonal antibody-based competitive ELISA (C-ELISA) is recommended. The C-ELISA is a rapid test, detecting antibodies against the VP7 protein. Virus neutralisation (VN) tests may also be performed. VN testing is usually performed to identify exposure to specific EHDV serotypes. The VN test is more time-consuming (3–5 days) and labour intensive, and cross reactions among serotypes may preclude optimal results. Tests such as agar gel immunodiffusion and the indirect ELISA can be used, but have the major drawback of being unable to distinguish between antibodies to EHDV and BTV.

Requirements for vaccines: *In the USA, an autogenous vaccine that can be used only in captive wild deer has been administered. In Japan, a vaccine has been developed for use in cattle. Apart from these two limited settings, there has been little interest from laboratories and vaccine companies elsewhere in developing vaccines to control the disease or EHDV circulation.*

A. INTRODUCTION

Epizootic haemorrhagic disease (EHD) is an infectious noncontagious viral disease transmitted by insects of the genus *Culicoides*. Available data suggest that the species of *Culicoides* that transmit EHD virus (EHDV) are likely to be similar though not necessarily the same as those that transmit bluetongue virus (BTV) (Carpenter *et al.,* 2008).The disease affects both wild and domestic ruminants, particularly North American cervids, and, to a lesser degree, cattle (Bréard *et al.,* 2004), although many countries describe only asymptomatic infection (Gard & Melville, 1992; St George *et al.,* 1983). Sheep, goats and camelids may be susceptible to EHDV infection but their role as hosts is uncertain.

In susceptible species, EHDV may cause a disease with clinical manifestations similar to BTV infection. White-tailed deer (*Odocoileus virginianus*) are the species most severely affected with the peracute form characterised by fever, anorexia, respiratory distress, and severe oedema of the head and neck. Swelling of the tongue and conjunctivae can also be present. In the acute (or classical) form, these clinical signs may be accompanied by haemorrhages in many tissues including skin and heart, and animals may develop ulcers or erosions of the tongue, dental pad, palate, rumen and abomasum (Savini *et al.,* 2011).

Histopathological lesions include widespread vasculitis with thrombosis, endothelial swelling, haemorrhages and necrosis in many organs especially the tongue, salivary glands, fore-stomachs, aorta and papillary muscle of the left ventricle of the myocardium. Scattered grey plaques on the surface of the gall bladder mucosa were also described (Noon *et al.,* 2002).

While the majority of infections in cattle are subclinical, the serotypes 1, 2, 6 and 7 have been described as potentially pathogenic (Bréard *et al.,* 2004; Cêtre-Sossah *et al.,* 2014; Temizel *et al.,* 2009). The disease is characterised by fever, anorexia, ulcerative stomatitis, swelling of eyelids, respiratory distress, nasal and ocular discharge, redness and scaling of muzzle and lips, lameness, erythema of the udder and difficulty swallowing (Temizel *et al.,* 2009). Ibaraki disease in cattle is caused by a strain of EHDV-2 (Anthony *et al.,* 2009). Animals become dehydrated and emaciated, and in some cases death occurs due to aspiration pneumonia. The lesions are histologically characterised by hyaline degeneration, necrosis and mineralisation of striated muscle accompanied by an infiltration of neutrophils, lymphocytes and histiocytes (Ohashi *et al.,* 1999; Savini *et al.,* 2011).

Taxonomically, EHDV is classified in the *Orbivirus* genus of the family *Reoviridae* (Maclachlan & Osburn, 2004). It is a double-stranded RNA virus with a genome of 10 segments. At least some of the genome segments are able to undergo reassortment during mixed infections in the field (Allison *et al.,* 2010; Anbalagan *et al.,* 2014). Seven serotypes (and two new putative serotypes) are currently recognised, but there is not yet a widely accepted consensus on the exact number of serotypes (Anthony *et al.,* 2010; Maan *et al.,* 2017; Shirafuji *et al.,* 2017; Wright, 2013). The virus is stable at –70°C and in blood, tissue suspension or washed blood cells held at 4°C. EHDV on laboratory surfaces is susceptible to 95% ethanol and 0.5% sodium hypochlorite solution.

EHDV particles are composed of three protein layers. The outer capsid consists of two proteins, VP2 and VP5. Like BTV, VP2 is the primary determinant of serotype specificity. VP5, the other external protein, might also elicit neutralising antibodies (Savini *et al.*, 2011; Schwartz-Cornill *et al.*, 2008). This outer capsid is dissociated readily from the core particle, and leaves a bi-layered icosahedral core particle composed of two major proteins, VP7 and VP3, surrounding the transcriptase complex (VP1, VP4, and VP6) and the genomic RNA segments. VP7 is the serogroup-specific immuno-dominant protein and the protein used in serogroup specific enzyme-linked immunosorbent assays (ELISAs) (Saif, 2011). The viral RNA also encodes five nonstructural proteins (NS1 to NS5) (Belhouchet *et al.,* 2011).

As a vector-borne viral disease, the distribution of EHD is limited to the distribution of competent *Culicoides* vectors (Mellor *et al.,* 2008).The EHDV has been isolated from wild and domestic ruminants and arthropods in North America, Ecuador (Verdezoto *et al.,* 2018), the Caribbean region (Brown-Joseph *et al.,* 2019), French Guiana (Viarouge *et al.,* 2014), Asia, Africa and Australia, and more recently in countries surrounding the Mediterranean Basin including Algeria, Israel, Jordan, Morocco, Tunisia, and Turkey. No cases of EHDV infection have been reported in Europe. Outbreaks generally coincide with the peak of vector population abundance, so most cases of EHD occur in the late summer and autumn (Mellor *et al.,* 2008; Stallknecht & Howerth, 2004).

As EHDV is a vector-borne infection it may be difficult to control or eradicate once established. Unpredicted and uncontrollable variables such as climatic and geographical factors, as well as abundance of suitable EHDV insect vectors are all important for the outcome and persistence (or reappearance) of EHDV in an area. Furthermore, to date, there are no detailed studies on the effect of control measures applied in the countries where the disease has affected cattle. Sera prepared from viraemic animals may represent some risk if introduced parenterally into naive

animals. The most significant threat from EHDV occurs when virus is inoculated parenterally into susceptible animals. If appropriate *Culicoides* are present, virus can be transmitted to other hosts. Therefore, EHDV-infected animals must be controlled for the period of viraemia and protected against *Culicoides* by physical means.

There is no known risk of human infection with EHDV. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.*

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of EHD and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Real-time RT-PCR	–	+++	+	++	++	–
RT-PCR	–	++	+	++	++	–
Isolation in cell culture	–	++	–	++	–	–
Detection of immune response						
C-ELISA (serogroup specific)	+++	+++	+++	–	+++	++
VN (serotype specific)	++	++	++	–	++	+++
AGID	+	–	+	–	+	+
CFT	+	–	+	–	+	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; C-ELISA = competitive enzyme-linked immunosorbent assay;
VN = virus neutralisation; AGID = agar gel immunodiffusion test; CFT = complement fixation test.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

Clinical signs of EHD in wild ruminants and cattle are similar to those of BT in sheep and cattle, and they may be similar to signs found in other cattle diseases like bovine viral diarrhoea/mucosal disease, infectious bovine rhinotracheitis, vesicular stomatitis, malignant catarrhal fever and bovine ephemeral fever. Definitive diagnosis of EHDV infection therefore requires the use of specific laboratory tests.

1. Detection of the EHDV agent

1.1. *In-vitro* culture

1.1.1. Isolation in cell culture

The same diagnostic procedures are used for domestic and wild ruminants. Virus isolation can be attempted from the blood of viraemic animals, tissue samples including spleen, lung and lymph

nodes of infected carcasses, and from *Culicoides* spp. EHDV can be isolated by inoculation of cell cultures such as those of cattle pulmonary artery endothelial, baby hamster kidney (BHK-21), and African green monkey kidney (Vero) (Aradaib *et al.*, 1994), the latter two being the most commonly used for growing the virus. *Aedes albopictus* (e.g. C6/36) and *Culicoides variipennis* (Kc) cell lines may also be used for virus isolation (Batten *et al.,* 2011; Eschbaumer *et al.,* 2012; Gard *et al.*, 1989); as can embryonated chicken eggs, but with less sensitivity (Eschembauer *et al.,* 2012). Cytopathic effect (CPE), which occurs only in mammalian cell lines, usually appears between 2 and 7 days post-inoculation, however a blind passage may be required.

Below is a general virus isolation procedure in cell culture that can be modified according to individual laboratory needs. Incubation of cell cultures for EHDV isolation is usually performed in a humid 5% CO_2 atmosphere.

i) For tissues from clinical cases, prepare a 10–30% tissue suspension in cell culture or other appropriate medium containing antibiotics. Centrifuge and save the supernatant for virus isolation.

ii) For uncoagulated whole blood, centrifuge the blood (0.2 ml) to separate the red blood cells (RBC) and plasma. Discard the plasma and replace with phosphate-buffered saline (PBS, pH 7.4). Centrifuge the blood again to separate the RBC. Perform three total washes with PBS. Add 0.4 ml of sterile distilled water to lyse the RBC. Cells may alternatively be lysed by sonication. Add 1.6 ml of PBS to the lysed RBC and save for virus isolation.

iii) Discard medium from the vessel containing fresh monolayer cells (1–3 days old).

iv) Inoculate the cells with a fraction of the clarified tissue or lysed RBC suspension, or previous passage cell culture.

v) Incubate at 34–37°C for 1 hour. Cell culture flask caps should be loosened or vented caps should be used to allow for gas transfer.

vi) Discard the inoculum and wash the monolayer with medium containing antibiotics once or twice. Add maintenance medium and return to the incubator.

vii) Observe the cells for CPE regularly. CPE is only observed in mammalian cell lines and usually appears between 2 and 7 days post-inoculation.

viii) If no CPE appears, a second and third passage should be attempted. Scrape the cells by using a scraper or freeze–thaw the cells once and inoculate fresh cultures.

ix) If CPE is present suggesting the presence of virus, the identity of the isolate may be confirmed by reverse-transcription polymerase chain reaction (RT-PCR), antigen capture ELISA, immunofluorescence, or virus neutralisation.

1.1.2. Serogroup identification of isolates

i) *Molecular methods*

See Section 1.2.1 for PCR methods.

ii) *Immunological methods*

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP7, that are conserved within each serogroup. The cross-reactivity between members of the EHD and BT serogroups raises the possibility that an isolate of BTV could be mistaken for EHDV on the basis of a weak immunofluorescence reaction with a polyclonal anti-EHDV antiserum. For this reason, an EHDV serogroup-specific monoclonal antibody (MAb) can be used. A number of laboratories have generated such serogroup-specific reagents (Luo & Sabara, 2005; Mecham & Jochim, 2000; Mecham & Wilson, 2004; White *et al.,* 1991). Commonly used methods for the identification of viruses to serogroup level are as follows.

a) Immunofluorescence

Monolayers of BHK-21 or Vero cells on chamber slides (glass cover-slips) are infected with either tissue culture-adapted virus or virus in insect cell lysates. After 24–48 hours at 37°C,

or after the appearance of a mild CPE, infected cells are fixed with agents such as paraformaldehyde, acetone or methanol, dried and viral antigen detected using anti-EHDV antiserum or EHDV-specific MAbs and standard immunofluorescent procedures.

b) Serogroup-specific sandwich ELISA

The serogroup-specific sandwich ELISA is able to detect EHDV in infected insects and tissue culture preparations (Thevasagayam *et al*., 1996). The assay is EHDV specific, with no cross-reactions with other orbiviruses such as BTV and African horse sickness virus (AHSV).

1.1.3. Serotype identification of EHDV isolates

i) *Molecular methods*

a) Polymerase chain reaction

Recent genome identification of EHDV isolates has enabled molecular identification of serotype and/or topotype by RT-PCR using serotype-specific primers followed by sequencing (Maan *et al.*, 2010; Sailleau *et al.,* 2012).

b) High throughput sequencing

High throughput sequencing may be performed on isolates with or without serotype-specific primers. Sequences may be compared with the GenBank library for serotype identity.

ii) *Immunological techniques*

a) Serotyping by virus neutralisation

There is a variety of tissue culture-based methods available to detect the presence of neutralising anti-EHDV antibodies. Cell lines commonly used are BHK-21 and Vero. Two methods to serotype EHDV are outlined briefly below. EHDV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included. The WOAH Reference Laboratory can supply control positive sera.

- Plaque reduction

  The virus to be serotyped is serially diluted and incubated with either no antiserum or with a constant dilution of individual standard antisera to a panel of EHDV serotypes. Virus/antiserum mixtures are added to monolayers of cells. After 1 hour adsorption at 37°C and 5% CO_2, and removal of inoculum, monolayers are overlaid with cell culture medium containing 0.8–0.9% agarose. Plates/flasks are incubated at 37°C and 5% CO_2. After 4 days' incubation, a second overlay containing 0.01% (1 part per 10,000) neutral red and 0.8–0.9% agarose in cell culture medium is applied and the plates/flasks are incubated at 37°C and 5% CO_2. Flasks are examined daily for visible plaques for up to 3 more days. The neutralising antibody titres are determined as the reciprocal of the serum dilution that causes a fixed reduction (e.g. 90%) in the number of plaque-forming units. The unidentified virus is considered serologically identical to a standard serotype if the latter is run in parallel with the untyped virus in the test, and is similarly neutralised.

- Microtitre neutralisation

  Approximately 100 $TCID_{50}$ (50% cell culture infective dose) of the standard or serial dilution of the untyped virus is added in 50 µl volumes to test wells of a flat-bottomed microtitre plate and mixed with an equal volume of a constant dilution of standard antiserum in tissue culture medium. After 1 hour incubation at 37°C and 5% CO_2 approximately 10^4 cells are added per well in a volume of 100 µl, and the plates incubated for 3–5 days at 37°C and 5% CO_2. The test is read using an inverted microscope. Wells are scored for the degree of CPE observed. Those wells that contain cells only or cells and antiserum, should show no CPE. In contrast, wells containing cells and virus should show 75–100% CPE. The unidentified virus is considered to be serologically identical to a standard EHDV serotype if both are

neutralised in the test to a similar extent, i.e. 75% and preferably 100% protection of the monolayer is observed.

1.2. Molecular methods – detection of nucleic acid

1.2.1. Conventional reverse-transcription polymerase chain reaction

In recent years, several efforts were directed towards the development of innovative molecular techniques such as the RT-PCR for the rapid detection of EHDV nucleic acid (Aradaib *et al.* 2003; Clavijo *et al.*, 2010; Wilson *et al.*, 2009; Viarouge *et al.,* 2014). RT-PCR allows the detection of EHDV RNA in blood samples and other tissues. Moreover, serotype-specific RT-PCRs targeting segment 2 of the viral RNA have been developed (Brodie *et al.*, 1998; Maan *et al.*, 2010; Sailleau *et al.,* 2012), as well as multiplex real-time RT-PCRs for the discrimination between EHDV and BTV (Wilson *et al.*, 2009; Yin *et al.*, 2010). Although RT-PCR has high sensitivity and specificity, RT-PCR-based diagnosis should be interpreted with caution: the RT-PCR technique detects viral RNA with a very high level of sensitivity, but this does not necessarily indicate the presence of infectious virus. The duration of EHDV positivity by RT-PCR in blood is not known, but there is evidence that it lasts longer than the period over which infectious virus can be isolated, as demonstrated for BTV.

The capacity of RT-PCR assays to detect very small numbers of nucleic acid molecules means that such tests are exquisitely sensitive to contamination by extraneous nucleic acids. The latter may include any primers in use in the laboratory or previously amplified polynucleotides. It is critical therefore to have a 'clean' area containing all equipment necessary for reagent and test preparation and a separate area with its own equipment for amplification. Impervious gloves should be worn and changed frequently at all stages of the procedure, particularly after working with sample RNA or amplified DNA. This will help protect reagents and samples from contamination by ubiquitous RNAses and other agents and from cross-contamination by DNA.

i) *RNA extraction from blood, insects and tissue samples*

A large variety of commercial kits are available; the RNA extraction step should be appropriate to the sample to be tested (e.g. blood vs tissue) and can be performed following directions suggested by the manufacturers.

ii) *Reverse-transcription polymerase chain reaction*

Several kits for one-step RT-PCR are currently available on the market. Alternatively, a double step procedure consisting of an initial RT step followed by cDNA amplification could be performed. Most of these methods still require a proper validation protocol. General recommendations that might be modified depending upon local/case-specific needs are indicated below. The method described by Aradaib *et al.* (2003) directed to S5 of EHDV has been taken as an example of conventional RT-PCR.

Primer sequences
For: 5'-TCG-AAG-AGG-TGA-TGA-ATC-GC-3';
Rev: 5'-TCA-TCT-ACT-GCA-TCT-GGC-TG-3'

a) Primer stock solutions are diluted to a final concentration of 10 pmol/µl.

b) One-step RT-PCR reaction tubes are labelled and 4.0 µl of primer mix is added to each tube. The tubes are held on ice.

c) Next, 4 µl of test, positive and negative control RNA samples are added to 4 µl of the primer mix in RT-PCR tubes.

d) Heat denaturation: 95°C for 5 minutes, then ice for further 3 minutes.

e) One-step RT-PCR mix is prepared, based on direction of the manufacturer, containing reagents in sufficient volume for the number of samples being tested.

f) One-step mix is added to the denatured mix to a final volume of 50 µl.

g) The tubes are placed in a thermal cycler programmed for reverse transcription and cDNA amplification as suggested by the manufacturer.

iii) *Electrophoretic analysis of RT-PCR product*

The presence of EHDV in the RT-PCR products could then be visualised by using standard electrophoretic analysis procedures.

1.2.2. Real-time reverse-transcription polymerase chain reaction

Assays based on RT-PCR have been used to detect EHDV RNA in clinical specimens. Serogroup-specific RT-PCRs are directed toward the highly conserved genes of EHDV such as S9 or S10 or less highly conserved genes including S3. However, none of these assays is able to discriminate simultaneously all EHDV serotypes. There is limited (or no) information regarding the detectability of published assays for all serotypes, a real-time RT-PCR (Clavijo *et al.*, 2010) is reported to recognise and quantify all EHDV serotypes. The targeted gene of this method is represented by the NS1 gene. More recently, new real-time RT-PCRs have been described for the detection and typing of seven serotypes of EHDV (Maan *et al.,* 2017; Viarouge *et al.,* 2015).

The real-time RT-PCR described below (Viarouge *et al.,* 2015) is a duplex pan-EHDV real-time RT-PCR assay that amplifies a portion of segment 9 of all EHDV strains coupled with a real-time RT-PCR that amplifies the beta-actin RNA of ruminant cells (housekeeping gene). Moreover, a set of seven duplex serotyping real-time RT-PCR assays that specifically amplify both the EHD segment 2 portion of each serotype and the beta-actin gene is described. The Ct (cycle threshold) value obtained with the real-time RT-PCR that amplifies the housekeeping gene is used to verify the integrity of the total RNA extracted and the absence of inhibitors that can interact during the RT-PCR steps for each biological sample tested. These assays were developed and validated to detect and serotype EHDV for application to biological samples from the field.

Assay type	Primers/Probe Name	Sequence (5'–3')
Pan EHDV	EHDV_Seg9_F_7-25	AAT-TGC-GCA-TGT-CAG-CTG-C
	EHDV_Seg9_R_76-55	TTT-AAT-TCC-TCG-GTC-GAA-CGT-T
	EHDV_Seg9_P_29-44	FAM-TTT-GCT-CGC-ACC-CGG-T-MGB
Type EHDV1	EHDV1_Seg 2_F_8-31	TGT-GTC-AGG-ATG-GAG-GAC-ATT-AAC
	EHDV1_Seg 2_R_ 231-255	CGA-ATT-ATT-CCR-GTR-CTT-AAC-GCT-T
	EHDV1_Seg2_P_ 128-143	FAM-CCG-CAT-CAA-ATG-TAT-G-MGB
	EHDV1_Seg2_P'_128-141	FAM-CCG-CAC-CAG-ATG-TAT-MGB
Type EHDV2	EHDV2_Seg2_F_1642-1665 (W)	CCT-TTA-AGA-TAA-GAC-GGG-TCG-AGA
	EHDV2_Seg2_F'_1640-1666 (E)	GTC-CTT-TAA-GGT-AAG-ACG-GGT-AGA-GAT
	EHDV2_Seg2_R_1798-1770	CTC-AAG-ATA-TTA-CCG-GTT-AAG-CAT-AGA-GT
	EHDV2_Seg2_R'_1788-1170	TGC-CGG-TCA-TAC-AGA-ACG-C
	EHDV2_Seg2_P_1728-1743	FAM-AAC-GAG-ATG-TGG-CTT-C-MGB
Type EHDV4	EHDV4_Seg2_F_2573-2591	TAT-CAA-GCG-ACC-CAG-TCG-C
	EHDV4_Seg2_R_2771-2743	CGT-ATG-ACA-TTC-TGC-AAG-TCA-GC
	EHDV4_Seg2_P_2635-2653	FAM-CAC-ATC-TAC-GGA-TAC-TGT-G-MGB
Type EHDV5	EHDV5_Seg2_F_403-424	ACG-AAT-CGG-AGG-ATA-CGG-ATC
	EHDV5_Seg2_R_522-501	TCG-CGT-ATG-ATC-ACA-CTG-GTC-T
	EHDV5_Seg2_P_471-489	FAM-ACT-ATC-GGT-AGT-GGT-GTT-C-MGB
Type EHDV6	EHDV6_Seg2_F_560-582	GGA-TCT-GGA-ACG-TGC-TAT-GAT-CT
	EHDV6_Seg2_F'_561-582	GTT-CCG-GGA-CAT-GCT-ATG-ATC-T
	EHDV6_Seg2_R_734-714	CAG-CCT-GAA-TCT-TCG-TTT-GCT
	EHDV6_Seg2_R'_734-715	CCG-CCT-GAA-TTT-TTG-TTT-GC
	EHDV6_Seg2_P_686-702	FAM-ATA-ACG-AAC-AGG-GAG-CC-MGB

Assay type	Primers/Probe Name	Sequence (5'–3')
Type EHDV7	EHDV7_Seg2_F_2490-2508	CGA-GAG-GAA-CCG-ACC-GAA-G
	EHDV7_Seg2_F'_2490-2512	CGA-GAA-GAG-CCG-ATT-GAA-GAA-G
	EHDV7_Seg2_R_2627-2605	GCT-TAA-ATG-CGT-ATT-CAT-GGG-AT
	EHDV7_Seg2_R'_2629-2605	GTG-CTT-AAA-TGC-GTA-TTC-ATA-GGG-T
	EHDV7_Seg2_P_2520-2537	FAM-ACT-GTA-TGG-CCG-TAT-CTA-MGB
Type EHDV8	EHDV8_Seg2_F_822-846	ACG-ATC-CTA-TAA-TAT-CAC-GCT-TGG-A
	EHDV8_Seg2_R_897-878	TCT-TCG-ATC-CGC-TCA-CTG-C
	EHDV8_Seg2_P_853-869	FAM-AGC-TGA-TGA-ATG-GAT-GC-MGB

Primer and probe sequences for endogenous gene (Toussaint *et al.,* 2007)

Primers:
ACT_F_1005–1029 (5'-CAG-CAC-AAT-GAA-GAT-CAA-GAT-CAT-C-3'), ACT_R_1135–1114 (5'-CGG-ACT-CAT-CGT-ACT-CCT-GCT-T-3').

Probe sequences
Probe: ACT_P_1081–1105 (VIC-TCG-CTG-TCC-ACC-TTC-CAG-CAG-ATG-T-TAMRA).

The PCR amplification is performed using a real-time one-step RT-PCR kit. The mixtures contain 1× buffer, 0.5 µM of each EHDV-primer, 0.25 µM EHDV-probe, 0.375 µM of each beta-actin-primers, 0.25µM beta-actin-probe, 1 µl of enzyme mix, and 5 µl of denaturated template in a total of 20 µl of reaction volume. After RNA denaturation by heating at 95°C for 3 minutes, 5 µl of denaturated RNA was added to the mix and the amplifications were carried out using the same cycling parameters for all of the assays: 45°C for 10 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fluorescence was measured during the 60°C annealing/extension step. Ct values were measured at the point at which the sample fluorescence signal crossed a threshold value (the background level). Negative results (for assays that did not exceed this signal level) are reported as 'No Ct'.

The Viarouge *et al.* (2015) method was validated according to the WOAH Validation Standard (except for the proficiency test, which was not undertaken). The real-time RT-PCR does not detect any of the 27 serotypes of BTV.

2. Serological methods

Following infection with EHDV, antibodies may be detectable from 8 days post-infection (Breard *et al.,* 2013; Eschbaumer *et al.,* 2012; Quist *et al.*, 1997). Like BTV, EHDV-infected animals may have neutralising antibodies and EHD viraemia at the same time; this is likely to be because of the strong association between the viruses and the RBCs. Duration of acquired immunity is still unknown but evidence from natural infections suggests it may last for life. A variety of serological methods, varying in sensitivity and specificity, are available to detect EHDV serogroup- and serotype-specific antibodies.

2.1. Competitive enzyme-linked immunosorbent assay (C-ELISA)

The EHD competitive ELISA (C-ELISA) was developed to measure EHDV-specific antibody without detecting cross-reacting antibody to other orbiviruses. These techniques making use of MAbs against EHDV VP7 are able to detect EHDV serogroup-specific antibodies (Luo & Sabara, 2005; Mecham & Jochim, 2000; Mecham & Wilson, 2004; White *et al.,* 1991), and currently are the preferred technique.

2.1.1. Test procedure

There are several test procedures described, and commercial kits are available. The following procedure gives one example of an EHD C-ELISA. The technique is similar to others except that in this technique the recombinant *Baculovirus*-expressed VP7 is captured by rabbit polyclonal anti-VP7-antibodies previously adsorbed to the wells (Mecham & Wilson, 2004).

i) 96-well microtitre plates are coated with 100 µl of the recombinant VP7 expressed in *Baculovirus* overnight at 4°C (Luo & Sabara, 2005), and diluted in 0.05 M carbonate buffer, pH 9.6.

ii) The plates are washed three times with PBST (0.01 M PBS containing 0.05% or 0.1% Tween 20, pH 7.4) and then blocked for 1 hour with 5% dry milk at room temperature.

iii) After being washed with PBST, 100 µl of test sera is added in duplicate at a single dilution, either 1/5, 1/10 or 1/20 in PBST containing 2.5% dry milk.

iv) Immediately, 100 µl of a predetermined dilution of MAb diluted in PBST is added to each well. MAb control wells contain diluent buffer in place of test serum.

v) Plates are incubated for 1 hour at 37°C and then further washed with PBST as described above

vi) After washing, wells are filled with 100 µl of an appropriate dilution of horseradish peroxidase-secondary antibody in PBST.

vii) Following incubation for 1 hour at 37°C, the conjugate solution is discarded and plates are washed three times using PBST. Wells are filled with 100 µl substrate solution and the plates are shaken at room temperature for 30 minutes.

viii) The reaction is stopped by addition of appropriate stopping reagent, and after blanking the ELISA reader on wells containing substrate and stopping reagent, the absorbance values are measured by using appropriate filters.

ix) Results are expressed as per cent inhibition and are derived from the mean absorbance values for each sample by the following formula:

% inhibition = 100 – [(mean absorbance test sample)/(mean absorbance MAb control) × 100].

NB: Some laboratories prefer to use a negative control serum that has previously been shown to have a percentage inhibition of zero as an alternative to the MAb control.

x) Percentage inhibition values >50% are considered to be positive. Inhibition between 40% and 50% is considered to be suspicious. The results of the test sera duplicates can vary as long as the results do not lie either side of the positive cut-off.

xi) Strong and weak positive sera and a negative serum should be included on each plate. The weak positive should give 60–80% inhibition and the negative should give less than 40% inhibition.

Commercially produced C-ELISAs based on recombinant VP7 and anti-VP7 MAb are now available. These commercial assays are routinely used in many laboratories across the world.

2.2. Virus neutralisation

The reference method for the identification and quantification of antibodies against EHDV serotypes present in test samples is the VN test. This technique detects and quantifies serotype-specific antibodies. The main disadvantage of the technique is that all suspected virus serotypes must be included in the assay, consequently, it can be a very time consuming and labour intensive test to perform. The VN test requires 3–5 days to be completed (Pearson *et al.,* 1992).

2.2.1. Test procedure

Cell lines commonly used are BHK-21 and Vero cells. A titre greater than or equal to 1/10 is usually considered specific for EHDV.

EHDV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included.

i) 50µl of serial sera dilutions, from 1/10 to 1/1280, are added to each test well of flat-bottomed microtitre plates and each mixed with an equal volume of known EHDV serotypes (100 CID_{50}). The plates are incubated at 37°C in 5% CO_2.

ii) After 1 hour of incubation, approximately 10^4 Vero cells are added per well in a volume of 100 µl, of minimal essential medium (MEM) containing antibiotics and, after incubation for 4–6 days, the test is read using an inverted microscope.

iii) Wells are scored for the degree of CPE observed. A sample is considered positive when it shows 75% to preferably 100% CPE inhibition at the lowest dilution (1/10). The serum titre represents the highest serum dilution capable of reducing more than 75% CPE in cell culture.

2.3. Complement fixation

The complement fixation test (CFT) is sensitive and specific for EHDV diagnosis and was used until 1980 for diagnosis and certification of animals for export. The test is serogroup-specific and inexpensive with a sensitivity similar to the virus neutralisation (VN) and agar gel immunodiffusion (AGID) tests. CFT allows detection and quantification of antibodies for 4–12 months after infection but is less reliable after this period (Pearson *et al.*, 1992). CFT is particularly useful for detecting recent EHDV infections but not for detecting older infections.

2.4. Agar gel immunodiffusion

The AGID was widely used to detect EHDV antibodies from sera of infected animals (Dubay *et al.*, 2004). In the past this test was used for animal trade. It is simple, economical and the antigen used in the assay is relatively easy to generate. However, the disadvantage of the AGID is its lack of specificity in that it cannot discriminate between BTV and EHDV. Thus AGID positive sera should be retested using a serogroup-specific test, at least in those areas where BTV and EHDV may be co-circulating. Furthermore, although semi-quantitative the result of AGID is generally reported out as positive or negative. The AGID can detect antibodies from 5–15 days after infection to 2 years or more (Pearson *et al.*, 1992).

C. REQUIREMENTS FOR VACCINES

As an effort to control the disease, vaccines were developed in USA for captive wildlife deer farmers and in Japan to be used in cattle. Apart from these two limited settings, there has only been minor interest from laboratories and biologicals companies elsewhere to develop vaccines to control the disease or the virus circulation. Autogenous inactivated vaccines have been prepared in North America from EHDV isolates originating from ill or dead animals in affected premises. Their use must be approved by government authorities. Prior to release, these vaccines are tested for purity and safety. To make the products available expediently, autogenous vaccines are not tested for efficacy. Most of the applications are by deer farmers. As the vaccines use inactivated virus, two doses given 2–4 weeks apart are generally necessary to initially immunise the animals and a yearly booster is recommended.

In Japan, both live modified and inactivated vaccines have been developed to control Ibaraki disease. The live attenuated vaccine derived from the Ibaraki-2 strain was used following the outbreaks in 1980s and has been demonstrated to be safe and effective. The vaccine has to be administered once subcutaneously during the low vector season. The inactivated vaccine includes bovine ephemeral fever and Ibaraki viruses grown in cell cultures and inactivated by formalin, as an aluminium-gel adjuvanted vaccine. In Japan both vaccines are used on a voluntary basis according to the epidemiological situation.

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NB: There is a WOAH Reference Laboratory for epizootic haemorrhagic disease
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for epizootic haemorrhagic disease

NB: FIRST ADOPTED IN 2014; MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.1.8.

FOOT AND MOUTH DISEASE (INFECTION WITH FOOT AND MOUTH DISEASE VIRUS)

SUMMARY

Foot and mouth disease (*FMD*) *is a highly contagious viral disease of mammals and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. There are seven serotypes of FMD virus* (*FMDV*), *namely, O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Infection with one serotype does not confer immunity against another. FMD cannot be differentiated clinically from other vesicular diseases, such as swine vesicular disease, vesicular stomatitis, vesicular exanthema and Seneca Valley virus infection. Laboratory diagnosis of any suspected FMD case is therefore a matter of urgency.*

Typical cases of FMD are characterised by a vesicular condition of the feet, buccal mucosa and, in females, the mammary glands. Clinical signs can vary from mild to severe, and fatalities may occur, especially in young animals. In some species the infection may be subclinical, e.g. African buffalo (Syncerus caffer). *The preferred tissue for diagnosis is epithelium from unruptured or freshly ruptured vesicles or vesicular fluid. Where collecting this is not possible, blood and/or oesophageal–pharyngeal fluid samples taken by probang cup in ruminants or throat swabs from pigs provide an alternative source of virus. Myocardial tissue or blood can be submitted from fatal cases, but vesicles are again preferable if present.*

It is vital that samples from suspected cases be transported under secure conditions and according to international regulations. They should only be dispatched to authorised laboratories.

Laboratory diagnosis of FMD is by virus isolation or by the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid. Detection of virus-specific antibody can also be used for diagnosis, and antibodies to viral nonstructural proteins (*NSPs*) *can be used as indicators of infection, irrespective of vaccination status. Confirmation of a case of FMD should take account of all relevant clinical, epidemiological and laboratory findings.*

Detection and identification of the agent: *The presence of FMD virus is confirmed by demonstration of specific antigen or nucleic acid, with or without prior amplification of the virus in cell culture* (*virus isolation*). *Due to the highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a laboratory with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4* Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.*

Enzyme-linked immunosorbent assays (*ELISA*) *can be used to detect FMD viral antigens and for serotyping. Lateral flow devices* (*LFD*) *are also becoming more readily available and can also be used to detect FMD viral antigens. The ELISA has replaced complement fixation* (*CF*) *in most laboratories as it is more specific and sensitive and it is not affected by pro- or anti-complement factors. If the sample is inadequate or the diagnosis remains uncertain, sample materials can be tested by reverse transcription polymerase chain reaction* (*RT-PCR*) *or virus isolation using susceptible cell to amplify any nucleic acid or live virus that may be present. The cultures should preferably be of primary bovine* (*calf*) *thyroid, but pig, lamb or calf kidney cells, or cell lines of comparable sensitivity may be used. Once a cytopathic effect* (*CPE*) *is complete in the cultures, harvested fluids can be tested for FMDV using ELISA, CF or RT-PCR.*

Serological tests: *The demonstration of specific antibodies to structural proteins in nonvaccinated animals is indicative of prior infection with FMDV. This is particularly useful in mild cases or where epithelial tissue cannot be collected. Tests for antibodies to some NSPs of FMDV are useful in*

providing evidence of previous or current viral replication in the host, irrespective of vaccination status. NSPs, unlike structural proteins, are highly conserved and therefore are not serotype specific and as a consequence, the detection of these antibodies is not serotype restricted.

Virus neutralisation tests (VNTs) and ELISAs for antibodies to structural proteins are used as serotype-specific serological tests. VNTs depend on tissue cultures and are therefore more prone to variability than ELISAs; they are also slower and subject to contamination. ELISAs for detection of antibodies have the advantage of being faster, and are not dependent on cell cultures. The ELISA can be performed with inactivated or recombinant antigens, thus requiring less restrictive biocontainment facilities.

Requirements for vaccines: *Inactivated virus vaccines of varying composition are available commercially. Typically, virus is used to infect a suspension or monolayer cell culture and the resulting preparation is clarified, inactivated with ethyleneimine and concentrated. The antigen is usually blended with oil or aqueous adjuvant for vaccine formulation. Many FMD vaccines are multivalent to provide protection against the different serotypes, or to accommodate antigenic diversity likely to be encountered in a given field situation.*

The finished vaccine must be shown to be free from residual live virus. This is most effectively done using in-vitro *tests on concentrated inactivated virus preparations prior to formulation of the vaccine and freedom from live virus is subsequently confirmed during* in-vivo *and/or* in-vitro *tests on the finished product. Challenge tests are also conducted in vaccinated cattle to establish a* PD_{50} *(50% protective dose) value or protection against generalised foot infection (PGP), although a serological test is considered to be satisfactory where a valid correlation between protection, and specific antibody response has been established.*

FMD vaccine production facilities should also have an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4.

Diagnostic and reference reagents are available from the WOAH Reference Laboratories for FMD or the FAO (Food and Agriculture Organization of the United Nations) World Reference Laboratory for FMD (The Pirbright Institute, UK).

A. INTRODUCTION

Foot and mouth disease (FMD) is caused by a virus of the genus *Aphthovirus*, family *Picornaviridae*. There are seven serotypes of FMD virus (FMDV), namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1, that infect cloven-hoofed animals. Infection with any one serotype does not confer immunity against another. Within serotypes, many strains can be identified by biochemical and immunological tests.

Of the domesticated species, cattle, pigs, sheep, goats and water buffalo (*Bubalus bubalis*) are susceptible to FMD (Food and Agricultural Organization of the United Nations [FAO]; 1984). Many species of cloven-hoofed wildlife may become infected, and the virus has occasionally been recovered from other species as well. Amongst the camelidae, Bactrian camels and new world camelids have been shown to be susceptible (Larska *et al*., 2009). In Africa, SAT serotypes of FMD viruses are often maintained by African buffalo (*Syncerus caffer*). There is periodic spillover of infection into livestock or sympatric cloven-hoofed wildlife. Elsewhere in the world, cattle are usually the main reservoir for FMD viruses, although in some instances the viruses involved appear to be specifically adapted to pigs (such as the pig-adapted Cathay strain of type O FMDV) and that requires cells of porcine origin for primary isolation. Small ruminants can play an important role in the spread of FMDV, but it is not clear whether the virus can be maintained in these species for long periods in the absence of infection of cattle. Strains of FMDV that infect cattle have been isolated from wild pigs, antelope and deer. The evidence indicates that, in the past, infection of deer was derived from contact, direct or indirect, with infected domestic animals, and that apart from African buffalo, wildlife has not, so far, been shown to be able to maintain FMD viruses independently for more than a few months.

Infection of susceptible animals with FMDV can lead to the appearance of vesicles on the feet, in and around the oral cavity, and on the mammary glands of females. The vesicles rupture and then heal whilst coronary band lesions may give rise to growth arrest lines that grow down the side of the hoof. The age of lesions can be estimated from these changes as they provide an indicator of the time since infection occurred (UK Ministry of Agriculture, Fisheries and Food; 1986). Mastitis is a common sequel of FMD in dairy cattle. Vesicles can also occur at other sites, such as

inside the nostrils and at pressure points on the limbs – especially in pigs. The severity of clinical signs varies with the strain of virus, the exposure dose, the age and breed of animal, the host species and the immunity of the animal. The signs can range from a mild or inapparent infection to one that is severe. Death may result in some cases. Mortality from a multifocal myocarditis is most commonly seen in young animals: myositis may also occur in other sites.

On premises with a history of sudden death in young cloven-hoofed livestock, close examination of adult animals may often reveal the presence of vesicular lesions if FMD is involved. The presence of vesicles in fatal cases is variable.

In animals with a history of vesicular disease, the detection of FMDV in samples of vesicular fluid, epithelial tissue, oesophageal–pharyngeal (OP) sample, milk, or blood is sufficient to establish a diagnosis. Diagnosis may also be established by the detection of FMDV in the blood, heart or other organs of fatal cases. A myocarditis may be seen macroscopically (the so-called "tiger heart") in a proportion of fatal cases.

FMD viruses may occur in all the secretions and excretions of acutely infected animals, including expired air. Transmission is generally effected by direct contact between infected and susceptible animals or, more rarely, indirect exposure of susceptible animals to the excretions and secretions of acutely infected animals or uncooked meat products. Following recovery from the acute stage of infection, infectious virus disappears with the exception of low levels that may persist in the oropharynx of some ruminants. Live virus or viral RNA may continue to be recovered from oropharyngeal fluids and cells collected with a probang cup. FMD virus has also been shown to persist in a nonreplicative form in lymph nodes (Juleff *et al*., 2008). Animals in which the virus persists in the oropharynx for more than 28 days after infection are referred to as carriers. Pigs do not become carriers. Circumstantial evidence indicates, particularly in the African buffalo, that carriers are able, on rare occasions, to transmit the infection to susceptible domestic animals with which they come in close contact: the mechanism involved is unknown. The carrier state in cattle usually does not persist for more than 6 months, although in a small proportion, it may last up to 3 years. In African buffalo, individual animals have been shown to harbour the virus for at least 5 years, but it is probably not a lifelong phenomenon. Within a herd of buffalo, the virus may be maintained for 24 years or longer. Sheep and goats do not usually carry FMD viruses for more than a few months, whilst there is little information on the duration of the carrier state in Asian buffalo species and subspecies.

FMD is considered a negligible zoonotic risk. However, because of its highly contagious nature for animals and the economic importance of FMD, all laboratory manipulations with live viral cultures or potentially infected/contaminated material such as tissue and blood samples must be performed at an appropriate containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Countries lacking access to appropriate containment facilities should send specimens to a WOAH FMD Reference Laboratory. Vaccine production facilities should also meet these containment requirements.

Diagnostic and standard reagents are available in kit form or as individual items from WOAH Reference Laboratories for FMD. The use of inactivated antigens in the enzyme-linked immunosorbent assay (ELISA), as controls in the antigen-detection test or to react with test sera in the liquid-phase blocking or solid-phase competitive ELISA, reduces the disease security risk involved compared with the use of live virus. Reagents are supplied freeze-dried or in glycerol or nonglycerinated but frozen and can remain stable at temperatures between +1°C and +8°C, –30°C and –5°C and –90°C and –50°C, respectively, for many years. There are a number of commercially available diagnostic test kits, for the detection of virus antigens or antibodies.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of FMD and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[a]						
Virus isolation	–	+	+++	+++	–	–
Antigen detection ELISA	–	–	+++	+++	–	–
CFT	–	–	+	+	–	–
LFD	–	–	+++	+++	–	–
Real-time RT-PCR	+	+	+++	+++	+	–
RT-PCR	+	+	+++	+++	+	–
Detection of immune response						
NSP Ab ELISA	+++	++	+++	+++	+++	–
SP Ab ELISA[b]	++	++	+++	+++	++	+++
VNT[b]	++	++	+++	+++	++	+++
AGID[b]	+	+	+	+	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
ELISA = enzyme linked immunosorbent assay; CFT = complement fixation test; LFD: lateral flow device; RT-PCR = reverse-transcriptase polymerase chain reaction; AGID = Agar gel immunodiffusion; NSP Ab ELISA = ELISA for antibodies against nonstructural proteins; SP Ab ELISA = ELISA for antibodies against structural proteins; VNT = Virus neutralisation test.
[a]It is essential to confirm the presence of FMDV following virus isolation by an antigen or nucleic acid detection test.
[b]The tests do not distinguish infected from vaccinated animals.

For laboratory diagnosis, the tissue of choice is epithelium or vesicular fluid. Ideally, at least 1 g of epithelial tissue should be collected from an unruptured or recently ruptured vesicle, usually from the tongue, buccal mucosa or feet. To avoid injury to personnel collecting the samples, as well as for animal welfare reasons, it is recommended that animals be sedated before any samples are obtained.

Epithelial samples should be placed in a transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer, pH 7.2–7.6, preferably with added antibiotics (penicillin [1000 International Units (IU)], neomycin sulphate [100 IU], polymyxin B sulphate [50 IU], mycostatin [100 IU]). If 0.04 M phosphate buffer is not available, tissue culture medium or phosphate-buffered saline (PBS) can be used instead, but it is important that the final pH of the glycerol/buffer mixture be in the range pH 7.2–7.6. FMDV is extremely labile in low pH and buffering of the transport media is critical for successful sample collection. Samples should be kept refrigerated or on ice until received by the laboratory.

Where epithelial tissue is not available from ruminant animals, for example in advanced or convalescent cases, or where infection is suspected in the absence of clinical signs, samples of OP fluid can be collected by means of a probang (sputum) cup (or in pigs by swabbing the throat) for submission to a laboratory for virus isolation or reverse-transcription polymerase chain reaction (RT-PCR). Viraemia may also be detected by examining serum samples by means of RT-PCR or virus isolation. For the collection of throat swabs from pigs, the animal should be held on its back in a wooden cradle with the neck extended. Holding a swab in a suitable instrument, such as an artery forceps, the swab is pushed to the back of the mouth and into the pharynx.

Before the collection of OP samples from cattle or large ruminants (e.g. buffalo), 2 ml transport fluid (composed of 0.08 M phosphate buffer containing 0.01% bovine serum albumin, 0.002% phenol red, antibiotics [1000 units/ml penicillin, 100 units/ml mycostatin, 100 units/ml neomycin, and 50 units/ml polymyxin], and adjusted to pH 7.2) should be added to a container of around 5 ml capacity capable of withstanding freezing above dry ice (solid carbon dioxide) or liquid nitrogen (Kitching & Donaldson, 1987).

An OP sample is collected by inserting a probang over the tongue into the oro-pharyngeal area and then passing it vigorously backwards and forwards 5–10 times between the first portion of the oesophagus and the back of the pharynx. The purpose is to collect oro-pharyngeal fluid and especially superficial epithelial cells from these areas, including the proximal part of the oesophagus, the walls of the pharynx, the tonsillar crypts and the surfaces of the soft palate. If the sample does not contain adequate cellular debris the actions may be repeated.

After collection of OP fluid by probang, the contents of the cup should be poured into a wide-necked transparent bottle of around 20 ml capacity. The fluid is examined, and should contain some visible cellular material. This fluid is then added to an approximately equal volume of transport fluid, ensuring that cellular material is transferred; the mixture is shaken gently and should have a final pH of around pH 7.6. Samples contaminated with ruminal contents may be unsuitable for culture. Samples seen to contain blood are not entirely satisfactory. Repeat sampling can be done after the mouth and throat of the animal have been rinsed with water or PBS. Where several animals are to be sampled, the probang must be cleaned and disinfected between each animal. This is done by washing the probang in tap water, immersing it in a suitable disinfectant (e.g. 0.5% [w/v] citric acid in tap water) and then rinsing off all the disinfectant with water before sampling the next animal.

OP samples from small ruminants are collected by putting 2 ml of transport fluid into a wide-necked bottle of about 20 ml capacity and, after collection, rinsing the probang cup in this transport fluid to discharge the OP sample. This is then transferred to a container of about 5 ml capacity for transport. The small container should be capable of withstanding freezing above dry ice or liquid nitrogen (Kitching & Donaldson, 1987).

Samples of OP fluid should be refrigerated or frozen immediately after collection. If they are to remain in transit for more than a few hours, they should preferably be frozen by being placed either above dry ice or liquid nitrogen. Before freezing, the containers should be carefully sealed using airtight screw caps or silicone. This is particularly important when using dry ice, as introduction of CO_2 into the OP sample will lower its pH, inactivating any FMDV that may be in the samples. Glass containers should not be used because there is a risk that they will explode on defrosting in the event of liquid nitrogen leaking into them. Samples should reach the laboratory in a frozen state or, if this is not feasible, maintained under reliable cold chain conditions during transit.

Special precautions are required when sending perishable suspect FMD material both within and between countries. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) has explicit requirements for packaging and shipment of diagnostic specimens by all commercial means of transport. These are summarised in Chapter 1.1.2 *Collection, submission and storage off diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*. Forms and guidance on sample submission and specifications for manufacture of probang cups can be found on the website of the Pirbright WOAH Reference Laboratory at http://www.wrlfmd.org/. Procedures for collection and shipment of field samples for the diagnosis of vesicular diseases and its differential diagnosis can be found at the Pan-American FMD WOAH Reference Laboratory at http://www.panaftosa.org.br

1. Detection and identification of the agent

A range of sample types, including epithelium, OP samples, milk, serum and heart muscle from cases with myocarditis, may be examined by virus isolation or RT-PCR. By contrast, ELISA, CF and the lateral flow device LFD are suited to the examination of epithelial suspensions, vesicular fluids or cell culture supernatants, but are insufficiently sensitive for the direct examination of OP samples or serum. It is essential to confirm the presence of FMDV following virus isolation by an FMDV-specific antigen or nucleic acid detection test. A virus isolate or an RT-PCR product can be further characterised by sequencing.

1.1. Virus isolation

The epithelium sample should be taken from the PBS/glycerol, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures, and weighed. A suspension should be prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added until a final volume of nine times that of the epithelial sample has been added, giving a 10% suspension. This is clarified on a bench centrifuge at 2000 ***g*** for 10 minutes. Once clarified, such suspensions of field samples suspected to contain FMDV are inoculated onto cell cultures. Sensitive cell culture systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IB-RS-2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity. The sensitivity of any cells used should be tested with standard preparations of FMDV. The use of IB-RS-2 cells aids the differentiation of swine vesicular disease virus (SVDV) from FMDV (as SVDV will only grow in cells of porcine origin) and is often essential for the isolation of porcinophilic strains, such as O Cathay. The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours. In the case of OP fluids, pretreatment with an equal volume of chloro-fluoro-carbons may improve the rate of virus detection by releasing virus from immune complexes.

1.2. Immunological methods

1.2.1. Enzyme-linked immunosorbent assay

The preferred procedure for the detection of FMD viral antigen and identification of viral serotype is the ELISA (Ferris & Donaldson, 1992; Roeder & Le Blanc Smith, 1987). This is an indirect sandwich test in which different rows in multiwell plates are coated with rabbit antisera to each of the seven serotypes of FMDV. These are the 'capture' sera. Test sample suspensions are added to each of the rows, and appropriate controls are also included. Guinea-pig antisera to each of the serotypes of FMDV are added next, followed by rabbit anti-guinea-pig serum conjugated to an enzyme. Extensive washing is carried out between each stage to remove unbound reagents. A colour reaction on the addition of enzyme substrate and chromogen indicates a positive reaction. With strong positive reactions, this will be evident to the naked eye, but results can also be read spectrophotometrically at an appropriate wavelength. In this case, an absorbance reading greater than 0.1 above background indicates a positive reaction; the serotype of FMDV can also be identified. Values close to 0.1 should be confirmed by retesting or by amplification of the antigen by tissue culture passage and testing the supernatant once a CPE has developed. A suitable protocol is given below. Other protocols are available with slightly different formats and interpretation criteria (Alonso *et al*., 1993).

Depending on the species affected and the geographical origin of samples, it may be appropriate to simultaneously test for SVDV or vesicular stomatitis virus (VSV). Ideally a complete differential diagnosis should be undertaken in all vesicular conditions.

Rabbit antiserum to the 146S antigen of each of the seven serotypes of FMDV (plus SVDV or VSV if required) is used as a trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

Control antigens are prepared from selected strains of each of the seven types of FMDV (plus SVDV or VSV if appropriate) grown on monolayer cultures of BHK-21 cells (IB-RS-2 cells for SVDV or VSV). The unpurified supernatants are used and pretitrated on ELISA plates. The final dilution chosen is that which gives an absorbance at the top of the linear region of the titration curve (optical density approximately 2.0), so that the five-fold dilutions of the control antigens used in the test give two additional lower optical density readings from which the titration curve can be derived. PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST).

Guinea-pig antisera prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes of FMDV (plus SVDV if required) and preblocked with normal bovine serum (NBS) is used as the detecting antibody. Predetermined optimal concentrations are prepared in PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM).

Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used at a predetermined optimum concentration in PBSTM. As an alternative to guinea-pig or rabbit antisera, suitable monoclonal antibodies (MAbs) can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody. A validated ready-to-use kit is available, based on pre-selected monoclonal antibodies, for detection and serotyping of six of the seven FMDV serotypes.

1.2.2. Test procedure

i) ELISA plates are coated with 50 µl/well rabbit antiviral sera in 0.05 M carbonate/bicarbonate buffer, pH 9.6. Rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (optional). In countries from regions where SAT and Asia 1 serotypes have never circulated those serotypes are not routinely included.

ii) Leave overnight at 4°C in a stationary position or place on an orbital shaker set at 100–120 revolutions per minute in a 37°C incubator for 1 hour.

iii) Prepare test sample suspension (10% original sample suspension or undiluted clarified cell culture supernatant fluid).

iv) The ELISA plates are washed five times in PBS.

v) On each plate, load wells of columns 4, 8 and 12 with 50 µl PBST. Additionally, add 50 µl of PBST to wells 1, 2 and 3 of rows A to H on plate 1. To well 1 of row A of plate 1 add 12.5 µl of control antigen type O, to well 1 of row B add 12.5 µl of control antigen type A; continue in this manner for control antigen of types C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (if appropriate) in order to well 1, rows C to H. Mix diluent in well 1 of rows A to H and transfer 12.5 µl from well 1 to 2 (rows A to H), mix and transfer 12.5 µl from well 2 to 3, mix and discard 12.5 µl from well 3 (rows A to H) (this gives a five-fold dilution series of each control antigen). It is only necessary to change pipette tips on the micropipette between antigens. The remainder of the plate can be loaded with the test sample(s). Add 50 µl of sample one to wells 5, 6 and 7 of rows A to H, the second sample is placed similarly in columns 9, 10 and 11, rows A to H.

   If more than two samples are to be tested at the same time, the other ELISA plates should be used as follows:

   Dispense 50 µl of the PBST to the wells (rows A to H) of columns 4, 8 and 12 (buffer control columns). Note that the control antigens are not required on these plates. These test samples may be added in 50 µl volumes in rows A to H to columns 1, 2, 3; 5, 6, 7; 9, 10, 11, respectively.

vi) Cover with lids and place on an orbital shaker at 37°C for 1 hour.

vii) Wash the plates by flooding with PBS – wash three times as before and empty residual wash fluid. Blot the plates dry.

viii) Transfer 50 µl volumes of each guinea-pig serum dilution to each plate well in the appropriate order, e.g. rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (optional).

ix) Cover the plates with lids and replace on the orbital shaker. Incubate at 37°C for 1 hour.

x) The plates are washed again three times, and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

xi) The plates are washed again three times, and 50 µl of substrate solution, containing 0.05% % H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

xii) The reaction is stopped after 15 minutes by the addition of 50 µl of 1.25 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

1.2.3. Lateral flow device test

There are commercially available lateral flow devices (LFD) for the detection of FMDV antigens (Ferris *et al*., 2009), but WOAH has not yet received a validation dossier for these tests. As soon as a dossier is received, the manufacturer could apply for inclusion on the WOAH test register.

1.2.4. Complement fixation test

In general, the ELISA is preferable to the complement fixation test (CFT) because it is more sensitive and it is not affected by pro- or anti-complementary factors (Ferris & Dawson, 1988). If ELISA reagents are not available, or if subtyping is pursued, the CFT may be performed as follows:

The CF50% protocol in tubes used widely in South America for typing, subtyping and for establishing serological relationships (r values) is performed as follows: 0.2 ml antiserum to each FMDV serotype diluted at a predetermined optimal dilution in veronal buffer diluent (VBD) or borate-saline solution (BSS) is placed in separate tubes. To these, 0.2 ml of test sample suspension is added, followed by 0.2 ml of a complement dilution containing 4 units of complement. The test system is incubated at 37°C for 30 minutes prior to the addition of 0.4 ml 2% standardised sheep red blood cells (SRBC) in VBD or BSS sensitised with rabbit anti-SRBC. The reagents are incubated at 37°C for further 30 minutes and the tubes are subsequently centrifuged and read. Samples with less than 50% haemolysis are considered positive.

Other protocols performed in microplates are available and are performed as follows: antisera to each of the seven types of FMDV are diluted in VBD in 1.5-fold dilution steps from an initial 1/16 dilution to leave 25 µl of successive antiserum dilutions in U-shaped wells across a microtitre plate. To these are added 50 µl of 3 units of complement, followed by 25 µl of test sample suspension(s). The test system is incubated at 37°C for 1 hour prior to the addition of 25 µl of 1.4% SRBC in VBD sensitised with 5 units of rabbit anti-SRBC. The reagents are incubated at 37°C for a further 30 minutes and the plates are subsequently centrifuged and read. Appropriate controls for the test suspension(s), antisera, cells and complement are included. CF titres are expressed as the reciprocal of the serum dilution producing 50% haemolysis. A CF titre ≥36 is considered to be a positive reaction. Titre values of 24 should be confirmed by retesting an antigen that has been amplified through tissue culture passage.

1.3. Nucleic acid recognition methods

RT-PCR can be used to amplify genome fragments of FMDV in diagnostic materials including epithelium, milk, serum and OP samples. Real-time RT-PCR has a sensitivity comparable to that of virus isolation and automated procedures enhance sample throughput (Reid *et al.,* 2003). Serotyping primers have also been developed (Vangrysperre & De Clercq, 1996). Simplified RT-PCR systems for potential field-use are under development (Callahan *et al.,* 2002).

1.3.1. Agarose gel-based RT-PCR assay

A gel-based RT-PCR procedure is described (Reid *et al.,* 2000). The RT-PCR assay consists of the three successive procedures of (i) extraction of template RNA from the test or control sample followed by (ii) RT of the extracted RNA, (iii) PCR amplification of the RT product and (iv) detection of the PCR products by agarose gel electrophoresis.

1.3.2. Test procedure

i) Add 200 µl of test sample to 1 ml of RNA extraction reagent in a sterile tube. Store at –70°C until required for RNA extraction.

ii) Transfer 1 ml of the solution from i) into a fresh, sterile tube containing 200 µl of chloroform. Vortex mix for about 10–15 seconds and leave at room temperature for 3 minutes.

iii) Centrifuge for 15 minutes at 20,000 ***g***.

iv) Transfer 500 µl of the aqueous phase into a fresh, sterile tube containing 1 µl of glycogen (20 mg/ml) and add 500 µl of iso-propyl-alcohol (propan-2-ol). Vortex mix for a few seconds.

v) Leave at room temperature for 10 minutes then centrifuge for 10 minutes at 20,000 ***g***.

vi) Discard the supernatant fluid from each tube and add 1 ml of 70% ethanol. Vortex mix for a few seconds.

vii) Centrifuge for 10 minutes at 20,000 ***g***.

viii) Carefully remove the supernatant fluid from each tube taking care not to dislodge or lose any pellet at the bottom of the tube.

ix) Air dry each tube at room temperature for 2–3 minutes.

x) Resuspend each pellet by adding 20 µl of nuclease-free water to the tube.

xi) Keep the extracted RNA samples on ice if the RT step is about to be performed. Otherwise store at –70°C.

NOTE: As an alternative to phenol/chloroform, RNA extraction can be performed using commercially available kits based on chaotropic salt lysis and silica RNA affinity.

xii) For each sample to be assayed, add 2 µl of random hexamers (20 µg/ml) and 5 µl of nuclease-free water into a sterile 0.5 ml microcentrifuge tube. It is recommended to prepare the dilution in bulk for the total number of samples to be assayed but allowing for one extra sample.

xiii) Add 5 µl of RNA from the extraction procedure described above to give a volume of 12 µl in each tube. Mix by gently pipetting up and down.

xiv) Incubate at 70°C for 5 minutes.

xv) Cool at room temperature for 10 minutes.

xvi) During the 10-minute incubation period, prepare the RT reaction mixture described below for each sample. Prepare the reaction mixture in bulk in a sterile 1.5 ml microcentrifuge tube for the number of samples to be assayed plus one extra sample.

First strand buffer, 5× conc. (4 µl); bovine serum albumin (acetylated), 1 mg/ml (2 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); DTT, 1 M (0.2 µl); Moloney Murine Reverse Transcriptase, 200 U/µl (1 µl).

xvii) Add 8 µl reaction mix to the 12 µl of random primer/RNA mix. Mix by gently pipetting.

xviii) Incubate at 37°C for 45 minutes.

xix) Keep the RT products on ice if the PCR amplification step is about to be performed, otherwise store at –20°C.

xx) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (35 µl); PCR reaction buffer, 10× conc (5 µl); $MgCl_2$, 50 mM (1.5 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); primer 1, 10 pmol/µl (1 µl); primer 2, 10 pmol/µl (1 µl); *Taq* Polymerase, 5 units/µl (0.5 µl).

xxi) Add 45 µl of PCR reaction mix to a well of a PCR plate or to a microcentifuge tube for each sample to be assayed followed by 5 µl of the RT product to give a final reaction volume of 50 µl.

xxii) Spin the plate or tubes for 1 minute in a suitable centrifuge to mix the contents of each well.

xxiii) Place the plate in a thermal cycler for PCR amplification and run the following programme:

94°C for 5 minutes: 1 cycle;
94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes: 30 cycles;
72°C for 7 minutes: 1 cycle.

Times and temperatures may need to be optimised to the particular enzymes, reagents and PCR equipment used in individual laboratories.

xxiv) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of staining solution and load onto a 1.5% agarose gel. After electrophoresis a positive result is indicated by the presence of a 328 bp band corresponding to FMDV sequence in the 5' untranslated region of the genome.

1.3.3. Stock solutions

i) Nuclease-free water, RNA extraction reagent, chloroform, glycogen, iso-propyl-alcohol (propan-2-ol), ethanol, random hexanucleotide primers, First strand buffer, BSA (acetylated), dNTPs, DTT, Moloney Murine Reverse Transcriptase, PCR reaction buffer (10×), $MgCl_2$ and *Taq* Polymerase are commercially available.

ii) Primers at a concentration of 10 pmol/µl: Primer 1 sequence 5'-GCCTG-GTCTT-TCCAG-GTCT-3' (positive strand); Primer 2 sequence 5'-CCAGT-CCCCT-TCTCA-GATC-3' (negative strand).

1.3.4. Real-time RT-PCR assay

The real-time RT-PCR assay can use the same procedures of extraction of total RNA from the test or control sample followed by RT of the extracted RNA as for the conventional agarose gel-based procedure. Automated extraction of total nucleic acid from samples followed by automated pipetting programmes for the RT and PCR steps (Reid *et al.,* 2003) can be used as an alternative to the manual procedures described above. PCR amplification of the RT product is performed by a different procedure. A more simple one-step method for combining the RT and PCR steps has also been described (Shaw *et al.,* 2007) and is widely used by laboratories. Detection of the PCR products in agarose gels is not required following real-time amplification.

i) Take the RT products from step xix (see above).

ii) Prepare the PCR mix described below for each sample. Again it is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample: nuclease-free water (6 µl); PCR reaction master mix, 2× conc. (12.5 µl); real-time PCR forward primer, 10 pmol/µl (2.25 µl); real-time PCR reverse primer, 10 pmol/µl (2.25 µl); labelled probe, 5 pmol/µl (1 µl).

iii) Add 24 µl PCR reaction mix to a well of a real-time PCR plate for each sample to be assayed followed by 1 µl of the RT product to give a final reaction volume of 25 µl.

iv) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.

v) Place the plate in a real-time PCR machine for PCR amplification and run the following programme:

50°C for 2 minutes: 1 cycle;
95°C for 10 minutes: 1 cycle;
95°C for 15 seconds, 60°C for 1 minute: 50 cycles.

Times and temperatures may need to be optimised to the particular enzymes, reagents and PCR equipment used in individual laboratories.

vi) *Reading the results:* Assign a threshold cycle (CT) value to each PCR reaction from the amplification plots (a plot of the fluorescence signal versus cycle number; different cut-off values may be appropriate for different sample types; Parida *et al.,* 2007). The CT values used to assign samples as either FMDV positive or negative should be defined by individual laboratories using appropriate reference material. For example at the WOAH Reference Laboratory at Pirbright, negative test samples and negative controls should have a CT value at >50.0. Positive test samples and positive control samples should have a CT value <40. Samples with CT values falling within the range 40–50 are designated "borderline" and can be retested. Strong positive FMD samples have a CT value below 20.0 (Reid *et al.,* 2001).

1.3.5. Stock solutions for real-time PCR assay

i) Nuclease-free water and real-time PCR reaction master mixes are available from commercial suppliers.

ii) Either of the two following primers and probe sets can be used for real-time PCR of FMDV:

5'UTR (Reid *et al.,* 2001) Forward primer: CACYT-YAAGR-TGACA-YTGRT-ACTGG-TAC; Reverse primer: CAGAT-YCCRA-GTGWC-ICITG-TTA and labelled probe: CCTCG-GGGTA-CCTGA-AGGGC-ATCC.

3D (Callahan *et al.,* 2002) Forward primer: ACTGG-GTTTT-ACAAA-CCTGT-GA; Reverse primer: GCGAG-TCCTG-CCACG-GA and labelled probe: TCCTT-TGCAC-GCCGT-GGGAC.

1.3.6. Molecular epidemiology

The molecular epidemiology of FMD is based on the comparison of genetic differences between viruses. Dendrograms showing the genomic relationship between vaccine and field strains for all seven serotypes based on sequences derived from the 1D gene (encoding the VP1 viral protein) have been published (Knowles & Samuel, 2003; see also http://www.wrlfmd.org/). Comparison of whole genome sequences can provide further discrimination between closely related viruses and help to recreate the transmission pathways between farms within outbreaks (Cottam *et al.,* 2008). RT-PCR amplification of FMDV RNA, followed by nucleotide sequencing, is the current preferred option for generating the sequence data to perform these comparisons. Many laboratories have developed techniques for performing these studies, and reference laboratories hold databases containing over 6000 partial sequences.

The recommended method for VP1 analysis is to:

i) Extract FMDV RNA directly from epithelial suspensions or from a low cell culture passage.

ii) Perform an RT-PCR of the complete 1D gene (or if only part of the 1D gene, then the 3' end of the gene is more useful).

iii) Determine the nucleotide sequence of the PCR product (or at least 170 nucleotides [preferably 420 for the SAT types] at the 3' end of the gene).

A protocol complete with primer sequences, is available (Knowles *et al*., 2016) or can be downloaded from the following World Wide Web URLs:

http://www.wrlfmd.org/

http://bvs.panaftosa.org.br/

2. Serological tests

Serological tests for FMD are performed in support of four main purposes namely: 1) to certify individual animals prior to import or export (i.e. for trade); 2) to confirm suspected cases of FMD; 3) to substantiate absence of infection; 4) to demonstrate the efficacy of vaccination. For substantiating freedom from infection, different approaches are required according to whether the population has been vaccinated or not and if vaccination has been used, whether this has been applied as an emergency application or as part of an ongoing programme of vaccination. Different tests and different interpretations of test results will be appropriate according to the above-mentioned purposes and the validation of the selected procedure must take account of the purpose. For example, test cut-offs may be set at a different threshold for herd-based serosurveillance than is appropriate for certifying freedom from infection for individual animals for the purposes of international trade.

Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs).

The SP tests are relatively serotype-specific and detect antibodies elicited by vaccination and infection; examples are the virus neutralisation test (VNT) (Golding *et al*., 1976), the solid-phase competition ELISA (SPCE; Brocchi *et al*., 1990; Chenard *et al*., 2003; Mackay *et al*., 2001; Paiba *et al*., 2004) and the liquid-phase blocking ELISA (LPBE; Hamblin *et al*., 1986; 1987). These tests are highly sensitive, providing that the virus or antigen used in the test is closely matched to the strain circulating in the field. They are the tests used to certify animals prior to movement, including for international trade purposes and are appropriate for confirming previous or ongoing infection in nonvaccinated animals as well as for monitoring the immunity conferred by vaccination in the field. The VNT requires cell culture facilities, the use of live virus and takes 2–3 days to provide results. The ELISAs are blocking- or competition-based assays that use serotype-specific polyclonal antibodies (PAbs) or MAbs, are quicker to perform and are not dependent on tissue culture systems and the use of live viruses. Low titre false-positive

reactions can be expected in a small proportion of the sera in either ELISA formats. An approach combining screening by ELISA and confirming the positives by the VNT minimises the occurrence of false-positive results. Reference sera to standardise FMD SP serological tests for some serotypes and subtypes are available from the WOAH Reference Laboratory at Pirbright.

The detection of antibody to the NSPs of FMDV can be used to identify past or present infection with any of the seven serotypes of the virus, whether or not the animal has also been vaccinated. Therefore the tests can be used to confirm suspected cases of FMD and to evaluate prevalence of infection or to substantiate freedom from infection on a population basis. For certifying animals for trade, the tests have the advantage over SP methods that the serotype of virus does not have to be known. However, there is experimental evidence that some cattle, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may not be detected in some anti-NSP tests, causing false-negative results (Brocchi *et al*., 2006). These assays measure antibody to NSPs using antigens produced by recombinant techniques in a variety of *in-vitro* expression systems. Antibody to the polyproteins 3AB or 3ABC are generally considered to be the most reliable indicators of infection (Mackay *et al*., 1997). In animals seropositive for antibody to 3AB or 3ABC, antibody to one or more of the other NSPs can aid in the final interpretation of the test (Bergmann *et al*., 2000; Mackay *et al*., 1997). However, lack of vaccine purity may affect diagnostic specificity as the presence of NSPs in some vaccine preparations may result in misclassification in animals that have been repeatedly vaccinated. Procedures for evaluating vaccine purity are covered in Section D of this chapter.

International standard sera for testing of cattle have been developed and are available from the WOAH Reference Laboratories in Brazil and UK (Campos *et al*., 2008). In the future, standard sera will also be made available for sheep and pigs. Bovine serum panels have also been established to compare the sensitivity of NSP tests (Parida *et al*., 2007).

2.1. Virus neutralisation test

The quantitative VN microtest for FMDV antibody is performed with IB-RS-2, BHK-21, lamb or pig kidney cells in flat-bottomed tissue-culture grade microtitre plates.

Stock virus is grown in cell monolayers and stored at –20°C after the addition of 50% glycerol. (Virus has been found to be stable under these conditions for at least 1 year.) The sera are inactivated at 56°C for 30 minutes before testing. The control standard serum is 21-day convalescent or post-vaccination serum. A suitable medium is Eagle's complete medium/LYH (Hank's balanced salt solution with yeast lactalbumin hydrolysate) with HEPES buffer and antibiotics.

The test is an equal volume test in 50 µl amounts.

2.1.1. Test procedure

i) Starting from a 1/4 dilution, sera are diluted in a twofold, dilution series across the plate, using at least two rows of wells per serum, preferably four rows, and a volume of 50 µl.

ii) Previously titrated virus is added; each 50 µl unit volume of virus suspension should contain about 100 $TCID_{50}$ (50% tissue culture infective dose) within an accepted range (e.g. 32–320 $TCID_{50}$).

iii) Controls include a standard antiserum of known titre, a cell control, a medium control, and a virus titration used to calculate the actual virus titre used in the test.

iv) Incubate at 37°C for 1 hour with the plates covered.

v) A cell suspension at 10^6 cells/ml is made up in medium containing 10% bovine serum (specific antibody negative) for cell growth. A volume of 50 µl of cell suspension is added to each well.

vi) Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 3–5% carbon dioxide at 37°C for 2–3 days.

vii) Microscope readings may be feasible after 48 hours. The plates are finally fixed and stained routinely on the third day. Fixation is effected with 10% formol/saline for 30 minutes. For staining, the plates are immersed in 0.05% methylene blue in 10% formalin for 30 minutes.

An alternative fixative/stain solution is naphthalene blue black solution (0.4% [w/v] naphthalene blue black, 8% [w/v] citric acid in saline). The plates are rinsed in tap water.

viii) Positive wells (where the virus has been neutralised and the cells remain intact) are seen to contain blue-stained cells sheets; the negative wells (where virus has not been neutralised) are empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected (Kärber, 1931). The test is considered to be valid when the amount of virus used per well is in the range $\log_{10}$ 1.5–2.5 $TCID_{50}$, and the positive standard serum is within twofold of its expected titre.

ix) Interpretation of tests can vary between laboratories in regard to the negative/positive cut-off threshold. Laboratories should establish their own criteria by reference to standard reagents that can be obtained from the WOAH Reference Laboratory at Pirbright. In general, a titre of 1/45 or more of the final serum dilution in the serum/virus mixture is regarded as positive. A titre of less than 1/16 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of 1/16 to 1/32 are considered to be doubtful, and further serum samples may be requested for testing; results are considered to be positive if the second sample has a titre of 1/16 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/45 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

2.2. Solid-phase competition enzyme-linked immunosorbent assay

The method described (Paiba *et al.,* 2004) can be used for the detection of antibodies against each of the seven serotypes of FMDV. As an alternative to guinea-pig or rabbit antisera, suitable MAbs can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody (Brocchi *et al.,* 1990). Commercial kits based on monoclonal antibodies are available for serotype O (Chenard *et al.,* 2003), serotype A, serotype Asia 1 and serotype SAT 2 with a different format but similar performance characteristics.

Rabbit antiserum to the 146S antigen of one of the seven types of FMDV is used as the trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

Antigens are prepared by inactivating viruses propagated in cell culture with ethyleneimine using the procedures described for vaccine manufacture. The final dilution chosen is that which, after addition of an equal volume of diluent, gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20, 10% NBS and 5% normal rabbit serum and phenol red indicator is used as a diluent (blocking buffer).

Guinea-pig antisera, prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes and preblocking with NBS, is used as the detecting antibody. Predetermined optimal concentrations are prepared in blocking buffer PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM).

Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used as conjugate at a predetermined optimum concentration in PBSTM blocking buffer.

Test sera are diluted in PBST blocking buffer.

The solid-phase competitive ELISA is more specific but as sensitive as the liquid-phase blocking ELISA (Mackay *et al.,* 2001; Paiba *et al.,* 2004). Methods have been described for the development of secondary and working standard sera (Goris & De Clercq, 2005a) and for charting assay performance (Goris & De Clercq, 2005b).

2.2.1. Test procedure

i) ELISA plates are coated with 50 µl/well rabbit antiserum homologous to the antigen being used, diluted in carbonate/bicarbonate buffer, pH 9.6, and left overnight in a humid chamber at 4°C.

ii) The ELISA plates are washed three times with PBS.

iii) Then 50 µl of the FMDV antigen diluted in blocking buffer is added to each well of the ELISA plates. (Blocking buffer: 0.05% [w/v] Tween 20, 10% [v/v] NBS, 5% [v/v] normal rabbit serum.) The plates are covered and placed on an orbital shaker at 37°C for 1 hour, with continuous shaking.

iv) After washing three times with PBS, 40 µl of blocking buffer is added to each well, followed by 10 µl of test sera (or control sera), giving an initial serum dilution of 1/5.

v) Immediately 50 µl of guinea-pig anti-FMDV antiserum diluted in blocking buffer is added, giving a final serum dilution of 1/10.

vi) The plates are covered and incubated on an orbital shaker at 37°C for 1 hour.

vii) After washing three times with PBS, 50 µl of anti-guinea-pig Immunoglobulin conjugate (preblocked by incubation for 1 hour at room temperature with an equal volume of NBS) diluted in blocking buffer is added. The plates are covered and incubated for 1 hour at 37°C on an orbital shaker.

viii) After washing three times with PBS, 50 µl of substrate solution, containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

ix) The reaction is stopped after 10 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

x) *Controls:* On each plate two wells are used for conjugate control (no guinea-pig serum), four wells each for strong and weak positive sera, two wells for negative sera, and four wells for 0% competition (no test sera).

xi) *Interpretation of the results:* A percentage of inhibition is calculated for each well, either manually or using a suitable computer programme (100 – [optical density of each test or control value/mean optical density of the 0% competition] × 100%), representing the competition between the test sera and the guinea-pig anti-FMDV antisera for the FMDV antigen on the ELISA plate. Laboratories should validate the assay in terms of the cut-off value above which sera should be considered positive in relation to (i) the particular serotypes and strains of virus under investigation (ii) the purpose of testing (iii) the population under test, using the methods described in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*. At the WOAH Reference Laboratory at Pirbright, for serotype O, for all species, for the purposes of demonstrating freedom from infection in a naïve population, greater than 60% inhibition is considered positive (Paiba *et al.,* 2004). For maximum sensitivity, for example when certifying individual animals for international trade, an inconclusive range may be set between 40 and 60%.

2.3. Liquid-phase blocking enzyme-linked immunosorbent assay

Antigens are prepared from selected strains of FMDV grown on monolayers of BHK-21 cells. The unpurified supernatants are used and pretitrated in a twofold dilution series but without serum. The final dilution chosen is that which, after addition of an equal volume of diluent (see below), gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST). The other reagents used in the test are the same as those in the solid-phase blocking ELISA. An example of the test procedure is described below. Temperature and incubation times can vary depending on the protocol.

2.3.1. Test procedure

i) ELISA plates are coated with 50 µl/well rabbit antiserum homologous to the antigen being used and left overnight in a humid chamber at room temperature.

ii) The ELISA plates are washed three times with PBS.

iii) In U-bottomed multiwell plates (carrier plates) 50 µl of a duplicate, twofold series of each test serum is prepared, starting at 1/8. To each well, 50 µl of a constant dose of viral antigen that is homologous to the rabbit antisera used to coat the plates is added and the mixtures are left overnight at 4°C, or incubated at 37°C for 1 hour. The addition of the antigen increases the final serum dilution to 1/16.

iv) Then 50 µl of serum/antigen mixtures is transferred from the carrier plates to the rabbit-serum coated ELISA plates and the plates are incubated at 37°C for 1 hour on a rotary shaker.

v) After washing, 50 µl of guinea-pig antiserum homologous to the viral antigen used in the previous step (iv) (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are then incubated at 37°C for 1 hour on a rotary shaker.

vi) The plates are washed and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

vii) The plates are washed again three times and 50 µl of substrate solution, containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

viii) The reaction is stopped after 15 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

ix) Controls: A minimum of four wells each of strong positive, weak positive and negative bovine reference sera at a final dilution of 1/32 should be included on each plate together with an equivalent number of reaction (antigen) control wells containing antigen in diluent alone without serum. For end-point titration tests, duplicate twofold dilution series of positive and negative homologous bovine reference sera should be included on at least one plate of every run.

x) *Interpretation of the results:* Antibody titres are expressed as the 50% end-point titre, i.e. the dilution at which the reaction of the test sera results in an optical density equal to 50% inhibition of the median optical density of the reaction (antigen) control wells (Kärber, 1931). The median is calculated as the mean of two mid-values of the reaction control wells, eliminating from the calculation the highest and lowest values (alternatively, the mean value can be used after setting suitable tolerance limits to control for inter-well variation). In general sera with titres greater than or equal to 1/90 are considered to be positive. A titre of less than 1/40 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of greater than 1/40, but less than 1/90 are considered to be doubtful, and further serum samples may be requested for testing; results are considered to be positive if the second sample has a titre of 1/40 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/90 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

2.4. Nonstructural protein (NSP) antibody tests

Antibody to expressed recombinant FMDV NSPs (e.g. 3A, 3B, 2B, 2C, 3ABC) can be measured by different ELISA formats or immunoblotting. These ELISAs either use purified antigens absorbed directly to microplates or use PAbs or MAbs to trap specific antigens from semi-purified preparations (Bergmann *et al*., 2000; De Diego *et al*., 1997; Mackay *et al*., 1997; Sorensen *et al*., 1998). The screening method used in Panaftosa is described in detail below. Other indirect and competitive ELISAs detecting bovine antibodies to 3ABC have been shown to have equivalent diagnostic performance characteristics (Brocchi *et al*., 2006). This same study corroborates preliminary data from Panaftosa that suggests that the diagnostic performance characteristics of these tests are similar in cattle, sheep and pigs. Commercial kits validated for identification of antibodies against FMDV NSPs in cattle and other species are also available.

2.4.1. Indirect enzyme-linked immunosorbent assay

2.4.1.1. Preparation of recombinant antigens

See Section B.2.4.2. *Enzyme-linked immunoelectrotransfer blot assay*.

2.1.1.2. Test procedure

i) Microplates are coated overnight at 4°C with 1 µg/ml of the fusion antigen 3ABC in carbonate/ bicarbonate buffer, pH 9.6 (100 µl per well). Antigen 3ABC was expressed and purified as indicated for the EITB (enzyme-linked immunoelectrotransfer blot) tests (Neizert *et al.,* 1991).

ii) The plates are washed six times with PBS, pH 7.2, supplemented with 0.05% Tween 20 (PBST).

iii) Test sera (100 µl per well) are added in a 1/20 dilution in blocking buffer consisting of PBS, 0.05% Tween 20, 5% nonfat dry milk, 10% equine sera and 0.1% *Escherichia coli* lysate. Each plate includes a set of strong and weak positive and negative controls calibrated against the International Standard Sera described below.

iv) The plates are incubated for 30 minutes at 37°C and washed six times in PBST.

v) Horseradish-peroxidase-conjugated rabbit anti-species IgG is diluted optimally in the blocking buffer, added at 100 µl per well and the plates are incubated for 30 minutes at 37°C.

vi) After six washings, each well is filled with 100 µl of 3'3', 5'5'-tetramethylbenzidine plus 0.004% (w/v) H_2O_2 in phosphate/citrate buffer, pH 5.5.

vii) The reaction is stopped after 15 minutes of incubation at room temperature by adding 100 µl of 0.5 M H_2SO_4. Absorbance is read at 450 nm and at 620 nm for background correction.

viii) *Interpreting the results:* Test results are expressed as per cent positivity relative to the strong positive control [(optical density of test or control wells/optical density of strong positive control) × 100] or alternatively as a test to control (T/C) index relative to a cut-off (i.e. threshold positive) control. Profiling the NSP antibody reactivity levels in herds along with age/vaccination stratification aids interpretation of herd infection status in vaccinated populations (Bergmann *et al.,* 2003). Test cut-off values, with or without suspicious zones, need to be determined with consideration to the purpose of testing and the intended target population. Inconclusive results may be followed up using confirmatory tests, retesting with EITB or a second NSP ELISA (taking account of the conditional dependence of the two tests). The overall test system sensitivity and specificity must be taken into account when designing the serosurveillance programme. Although not a suitable test for certifying animals prior to movement, NSP ELISAs may be a valuable adjunct in circumstances where the serotype or subtype of virus in the originating country is not known.

2.4.2. Enzyme-linked immunoelectrotransfer blot assay (EITB)

The EITB assay has been widely applied in South America as a confirmatory test for the above-described screening method. Further information is available from the WOAH Reference Laboratory, Panaftosa, PAHO/WHO.

2.4.2.1. Preparation of test strips containing the recombinant antigens

i) The five bioengineered FMDV NSPs 3A, 3B, 2C, 3D and 3ABC are expressed in *E. coli* C600 by thermo-induction. The 3D polypeptide is expressed in its complete form (McCullough *et al.,* 1992) whereas the rest of the proteins are obtained as fusions to the N-terminal part of the MS-2 polymerase gene (Strebel *et al.,* 1986).

ii) The expressed polymerase is purified over phosphocellulose, followed by poly(U) Sepharose columns. The fused proteins 3A, 3B, 2C and 3ABC are purified by sequential extraction of the bacterial extracts with increasing concentrations of urea. The 7M fraction containing the fusion proteins is further purified on a preparative 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The fusion protein band is excised from the gel and electroeluted (McCullough *et al.,* 1992).

iii) A mixture containing 20 ng/ml of each one of the purified recombinant polypeptides is separated on 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose (McCullough *et al.,* 1992).

2.4.2.2. Test procedure

i) The required amount of test strips should be assessed, taking into account that for each nitrocellulose sheet, which defines one transferred gel, a positive, a weakly positive, a cut-off and a negative control serum should be assayed. In general, 24 nitrocellulose strips, each 3 mm wide, should result from a gel.

ii) A volume of 0.8 ml of saturation buffer (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; 0.2% Tween 20; 5% nonfat dry milk; and 0.05% bacterial *E. coli* lysate) is added to each well. The antigen-coated strips are blocked by placing the trays on a rocker and agitating for 30 minutes at room temperature (20–22°C).

iii) A dilution of 1/200 of test sera and of each of the controls is added to the appropriate trough. The strips must be completely submerged and facing upwards, and maintained in that position during the whole process.

iv) Strips are incubated for 60 minutes on a rocker at room temperature.

v) Liquid is removed from the trays, and each test strip is washed three times with washing solution (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; and 0.2% Tween 20) by agitation for 5 minutes.

vi) The alkaline-phosphatase-conjugated rabbit anti-bovine solution is added to each test well, and the strips are incubated with shaking for 60 minutes at room temperature.

vii) The liquid is removed from the trays and each test strip is washed three times with washing solution as above.

viii) Substrate solution (0.015% bromochloroindolylphosphate/0.03% nitroblue tetrazolium) is prepared in substrate buffer (100 mM NaCl; 5 mM $MgCl_2$; and 100 mM Tris/HCl, pH 9.3), and is added to each test well.

ix) Strips are incubated by placing the test tray on the orbital mixer and agitating until the cut-off control shows five distinct, discernible bands. Strips are washed with running deionised water and air-dried.

x) *Interpreting the results:* The EITB may be scanned with a densitometer but visual reading, although more subjective, is considered suitable as well. Individual control sera are tested that exhibit minimal but consistent staining for each of the five antigens. A test sample is considered positive if antigens 3ABC, 3A, 3B and 3D (±2C) demonstrate staining densities equal to or higher than that of their appropriate controls. A sample is considered negative if two or more antigens demonstrate densities below their control sera. Test samples not fitting either profile are considered indeterminate.

C. REQUIREMENTS FOR VACCINES

The control of FMD is a national and regional responsibility and, in many countries, the vaccine may be used only under the control of the Veterinary Authority.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements. Varying requirements relating to quality, safety and efficacy apply in particular countries or regions in order for manufacturers to obtain an authorisation or licence for a veterinary vaccine. Where possible, manufacturers should seek to obtain such a license or authorisation for their FMD vaccines as independent verification of the quality of their product.

FMD vaccine production facilities should operate under appropriate biosecurity and containment procedures and practices as outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.*

Routine vaccination against FMD is used in many countries or zones recognised as free from foot and mouth disease with vaccination and in countries where the disease is endemic. In contrast, a number of disease-free countries have never vaccinated their livestock but have preferred the use of strict movement controls and culling of infected and contact animals when outbreaks have occurred. Nevertheless, many disease-free countries

maintain the option to vaccinate and have their own strategic reserves of highly concentrated inactivated virus preparations. Such antigen reserves offer the potential of supplying formulated vaccine in an 'emergency' at short notice. (See also Chapter 1.1.10 *Vaccine banks*.)

Traditional FMD vaccines may be defined as a fixed formulation containing defined amounts (limits) of one or more chemically inactivated cell-culture-derived preparations of a seed virus strain blended with a suitable adjuvant/s and excipients. See chapter 1.1.8 for biotechnology-derived vaccines such as recombinant or peptide vaccines.

Antigen banks may be defined as stockpiles of antigen components, registered or licensed according to the finished vaccine, and which can be stored under ultra-low temperatures for a long time for subsequent formulation into vaccine as and when required.

The vaccines are formulated for their specific purpose and in the case of vaccines destined for use in cattle, both aluminium hydroxide saponin adjuvanted and oil adjuvanted vaccines may be used. For use in swine, double oil emulsions are preferred due to their efficacy.

FMD vaccines may be classified as either 'standard' or 'higher' potency vaccines. Standard potency vaccines are formulated to contain sufficient antigen and appropriate adjuvant to ensure that they meet the minimum potency level required (recommended 3 PD_{50} [50% protective dose]; 75% EPP (expected percentage of protection) or 12 protected out of 16 vaccinated and challenged in PGP [protection against generalised foot infection] test) for the duration of the shelf life claimed by the manufacturer. This kind of vaccine is usually suitable for use in routine vaccination campaigns. For vaccination in naïve populations to control FMD outbreaks, higher potency vaccines (e.g.> 6 PD_{50} for the duration of the shelf life claimed by the manufacturer) are recommended for their wider spectrum of immunity as well as their rapid onset of protection.

Because of the presence of multiple serotypes of the virus, it is common practice to prepare vaccines from two or more different virus serotypes. In certain areas, it may be advisable to include more than one virus strain per serotype to ensure broad antigenic coverage against prevailing viruses.

1. Seed virus management

1.1. Characteristics of the seed virus

Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture, virus yield, antigenic stability at industrial level and broad antigenic spectrum. Isolates to prepare MSVs should be characterised and distributed, preferably by WOAH FMD Reference laboratories; they should be selected in accordance with the regional epidemiological importance of each strain.

The exact source of the isolate should be recorded and should include details such as the location, species and the type of material from which the virus was derived. Unique nomenclature should be used to identify the FMDV strain. The *in-vitro* passage history of the virus and details of the ingredients should be recorded in accordance with chapter 1.1.8. The passage level of the seed virus should be kept to a minimum to avoid antigenic or genetic changes.

1.2. Method of culture

Methods of culture shall comply with the chapter 1.1.8. Where no suitable established vaccine strain exists, new vaccine strains are derived through the establishment of MSVs from local field isolates by adapting them to growth in suspension or monolayer cells by serial passages. In order to remove the risk of contaminating lipid-enveloped viruses, it is recommended that putative MSVs undergo a validated organic solvent treatment prior to, or during, adaptation.

1.3. Validation as a vaccine strain

MSVs must be, antigenic and genetically well characterised and proven to be pure and free from extraneous agents in accordance with Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and those listed by the appropriate licensing authorities. Homology should be established with the original candidate isolates and effectiveness against the circulating strains from which they were developed should be proven. This often

encompasses a number of methods, the most reliable being *in-vivo* protection assays. Alternatively, *in-vitro* tests (preferably virus neutralisation) can also be used, which require the availability of post-vaccination sera against these master seeds (see Section D of this chapter).

Seed viruses may be stored at low temperature (e.g. –70°C) or freeze-dried. Working seed viruses may be expanded in one or a few more passages from the master seed stock and used to infect the final cell culture.

Consideration should also be given to minimising the risk of transmission of transmissible spongiform encephalopathy agents (TSEs) by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

1.4. Emergency procedures for provisional acceptance of new MSV, and subsequent release of formulated vaccines

In the case of incursion in a region of a new strain to which protection is not elicited by existing vaccine strains, it may be necessary to develop a new vaccine strain from a representative field isolate. Before the new MSV can be accepted, full compliance should be demonstrated with the relevant guidelines to demonstrate freedom from all extraneous agents listed by the appropriate licensing authorities using both general and specific tests, and to establish homology to the original candidate isolates. The time taken to raise the specific antisera necessary to neutralise the new strain for use in the general tests for detection of extraneous agents and to conduct other specific tests that require specialised techniques may be lengthy. Therefore, in emergency situations where there is insufficient time to complete full testing of the MSV, provisional acceptance of the new strain should be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account that the virus is inactivated using a chemical inactivant with first order kinetics. Further assurance is provided by the requirement for the kinetics of inactivation to be monitored and recorded for each production batch.

2. Method of manufacture

The recommended method of virus propagation for antigen production is the growth of FMDV in large-scale suspension cultures or monolayers using cell lines under sterile conditions.

A suitable strain of the virus is used to infect a suspension or monolayers of an established cell line, such as BHK-21. Such cell cultures should be proven to be free from contaminating microorganisms.

When the virus is expected to have reached its maximum yield, the culture is clarified, often by centrifugation and/or filtration. The virus is subsequently inactivated by addition of an inactivant of first order, usually ethyleneimine (EI) in the form of binary ethyleneimine (BEI) (Bahnemann, 1990). It is important that the necessary safety precautions for working with BEI/EI are fully observed. The BEI is added to a virus suspension, to give a predetermined final concentration. Inactivation must be duly validated and documented to show the inactivation kinetic and the results of the inactivation controls. The time period for BEI treatment and temperature used for inactivation must be validated for the actual conditions and equipment used. To decrease the likelihood of live virus failing to contact the inactivant, e.g. BEI/EI, it is essential to transfer the vessel contents immediately to a second sterile vessel where inactivation is allowed to go to completion according to the validated inactivation kinetic and taking into account possible regulatory requirements for additional waiting times.

During inactivation, the virus titre is monitored by a sensitive and reproducible technique. After inactivation any residual BEI/EI in the harvest should be neutralised, for example by adding excess sodium thiosulphate solution to a final concentration of 2%.

The inactivated virus is concentrated by ultrafiltration. Concentrated inactivated virus may be purified further by procedures such as chromatography. These concentrated and purified antigens can be formulated into vaccines or stored at low temperatures for many years, and made into vaccine when required by dilution in a suitable buffer and addition of adjuvants (Doel & Pullen, 1990).

Conventional FMD vaccines are usually formulated as oil adjuvanted or aqueous. Oil-adjuvanted vaccines are usually formulated as water-in-oil emulsion using mineral oils. The mineral oil is usually premixed with an emulsifying agent before the addition of a proportion, or all, of the aqueous phase of the vaccine, and emulsified by

use of a colloid mill or continuous mechanical or flow ultrasonic emulsifier. More complex double emulsions (water/oil/water) may be produced by emulsifying once more in an aqueous phase containing a small amount of detergent such as Tween 80. Ready-to-use oil adjuvants are now available commercially for different types of emulsion.

Aqueous vaccine is prepared by adsorbing the virus on to aluminium hydroxide gel, one of the adjuvant constituents of the final vaccine blend. The final blend of the vaccine may include other components, such as antifoam, lactalbumin hydrolysate, tryptose phosphate broth, amino acids, vitamins, buffer salts and other substances. An adjuvant such as saponin is also usually incorporated, as well as preservatives.

When using novel components, including adjuvants or preservatives, in any vaccine it is important to take into account that its status, with regard to residues in products derived from food-producing species, must be assessed to ensure that adequate assurance can be giving to licensing authorities in relation to safety for consumers. This requirement limits considerably the choice of adjuvants and preservatives for use in food-producing species.

3. In-process control

In general, virus titres reach optimum levels between 18 and 24 hours of the cell culture being infected, depending on the serotype. The time chosen to harvest the culture may be based on a number of assays; for instance cell death. Virus concentration may be assessed by an infectivity test, sucrose or CsCl density gradient or serological techniques. It is preferable to use more than one method as they may complement one another.

3.1. Inactivation kinetics

During inactivation of the virus, timed samples should be taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of cell cultures proven to be highly susceptible to FMDV, e.g. BHK. Such cultures permit the testing of statistically meaningful samples under reproducible conditions. The $\log_{10}$ infectivity of the timed samples are plotted against time, and the inactivation procedure is not considered to be satisfactory unless at least the latter part of the slope of the line is linear and extrapolation indicates that there would be less than one infectious particle per 10^4 litres of liquid preparation at the end of the inactivation period.

3.2. Inactivation control

The test for innocuity is an in-process test that should be carried out for every batch of antigen. Cells used to test for absence of residual live virus should undergo a sensitivity test to prove that they are suitable for virus replication. Following inactivation, a sample of each batch of inactivated antigen representing at least 200 doses of vaccine antigen should be tested for freedom from infectious virus by inoculation of sensitive monolayer cell cultures, preferably of the same origin as those used for the production of antigen. It may be necessary to concentrate the antigen to do this, in which case it must be shown that the concentrated material does not interfere with the sensitivity or reading of the assay. The cell sheets are examined daily over a period of 2–3 days, after which the spent medium is transferred to fresh monolayers and the original monolayers are replenished with fresh medium. Using this method, traces of live virus can be amplified by the passage procedure and detected on the basis of CPE observed. Three passages of the original virus preparation are commonly used. A variant on this method is to freeze–thaw the old monolayers to release intracellular virus, which can be detected by further passage.

4. Final product batch tests

4.1. Innocuity testing

The bulk inactivated antigen and the final formulated product should undergo innocuity test to prove absence of infectious virus. In the final product, antigen must be extracted from adjuvant following an appropriate validated method. A sample representing at least 200 doses of vaccine (including all product presentations) must be used for testing for freedom of infectious virus by inoculation of sensitive cell culture monolayers. After elution, antigen may be concentrated for inoculation in cell monolayers. Test procedure is as described in Section C.3.2 *Inactivation control*.

4.2. Sterility testing

The bulk inactivated antigen, concentrated antigen and the final formulated product should undergo sterility testing. Guidelines on techniques and culture media, which allow the detection of a wide range of organisms, are described in Chapter 1.1.9.

4.3. Identity testing

The bulk inactivated antigen, concentrated antigen and the final formulated product should undergo identity testing to demonstrate that the relevant strains are present. No other FMD virus serotype registered on the manufacturing site should be present in the vaccine, to be assured by appropriate tests such as serotype-specific RT-PCR.

4.4. Purity testing

Purity relates to the level of FMD NSPs in the final product, which should not induce antibodies that would interfere with serological tests used for sero-surveillance of virus circulation in vaccinated populations. Products claiming to be purified from NSPs have to demonstrate their level of purification. Lack of reactivity has to be demonstrated in the final product (see Section C.5. *Requirements for registration of vaccine*). In cases where consistency of purification is demonstrated and approved in the registration dossier, and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8, the Veterinary Authority may agree to omit the test in the final product.

Confirmation of vaccine purity may be shown by testing sera from animals vaccinated at least twice with the batch for absence of antibodies to NSPs.

4.5. Safety testing

The safety of the final product should be proven batch to batch. The safety testing is conducted to detect any abnormal local or systemic adverse reactions. In cases where consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8, Veterinary Authority may agree to omit the test in the final product.

Safety could be checked in animals used for the potency test. Animals are inoculated by the recommended route of administration with the recommended dose of vaccine. When potency is assessed by PGP or EPP, all animals are observed for local and systemic reactions to vaccination for the 30 days duration of the potency test. When PD_{50} test is used, at least two healthy sero-negative target animals inoculated as above are observed for local and systemic reactions to vaccination for no fewer than 14 days. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch.

4.6. Potency testing

Potency is examined on the final formulated product, or alternatively for antigen banks on a representative batch of vaccine prepared from the same bulk inactivated antigen.

The potency testing standard is the vaccination challenge test. However, indirect tests can also be used for practicability and animal welfare considerations, as long as correlation has been validated to expectancy of protection in the target animal. Frequently indirect potency tests include antibody titration after vaccination of target species. Alternative methods could be used if suitably validated.

Ideally, indirect tests are carried out for each strain for one species and each formulation of vaccine to establish correlation between the indirect test results and the vaccine efficacy.

4.6.1. Expected percentage of protection (EPP) (Maradei *et al.*, 2008; Periolo *et al.,* 1993)

The EPP estimates the likelihood that cattle would be protected against a challenge of 10,000 bovine infective doses after a single vaccination.

i) Individual sera collected 30–60 days post-vaccination using a full dose of the vaccine are required from a group of either 16 or 30 18–24 month-old cattle.

ii) This panel of sera and sera of two control cattle are tested for antibody titres to the homologous FMD vaccine strain in a LPB-ELISA or VN test (see Sections B.2.1 *Virus neutralisation* and B.2.3 *Liquid-phase blocking enzyme-linked immunosorbent assay*).

iii) The antigens used in the ELISA may be inactivated using BEI.

iv) The EPP is determined by reference to predetermined tables of correlation between serological titres and clinical protection[1] (Maradei *et al.,* 2008; Periolo *et al.,* 1993).

v) Batches with at least 75% EPP (with 16 vaccinated cattle) or at least 70% EPP (with 30 vaccinated cattle) are satisfactory for potency.

The presence of more than one serotype in a vaccine does not diminish the induction of antibodies against another serotype or the correlation of antibody titre with protection.

4.6.2. Other methods for evaluating protection

Other tests were published using different ELISA methods and VNT methods to indirectly evaluate the protection given by vaccines. Their results could be accepted only if a valid correlation with protection in relation to the vaccine strain being tested and the serological method being used has been scientifically demonstrated.

5. Requirements for registration of vaccine

5.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.1–4) should be submitted to the National Veterinary Authority. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the minimum allowed industrial batch volume from the country of origin.

5.2. Safety

For the purposes of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration in an *in-vivo* test in at least eight animals of each target species. Double dose (e.g. two injections) and repeat single dose (after 14 days) tests using vaccines formulated to contain the maximum permitted payload and number of antigens are recommended to be conducted. In total animals receive three injections. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days after each injection. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine by the National Veterinary Authority.

5.3. Efficacy

Vaccine efficacy is estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge or indirectly through *in-vitro* testing using well established correlations. The uncertainty of measurement in tests should be taken into account when interpreting its significance (Goris *et al.,* 2008). Vaccine efficacy should be established for every strain to be authorised for use in the vaccine.

Live reference FMD viruses corresponding to the main vaccine virus strains used in the region are available under certain conditions from WOAH Reference Laboratories for FMD in the region and from the National Veterinary Authority. These reference viruses are stored at ultralow temperatures and are sent, in strict accordance with shipping regulations.

The stock of challenge virus to be aliquoted is prepared from lesions collected from at least two cattle above 6 months of age that have been recognised as free from FMDV antibodies. These animals are

1 The WOAH Reference Laboratory in Brazil can provide tables upon request

tranquillised, then inoculated intradermally in the tongue with the suspension in about 20 sites of 0.1 ml each. The vesiculated tongue tissue or other body parts and vesicular fluid is harvested at the peak of the lesions, approximately 2 days later.

Harvested tissue is macerated and a 2% suspension is prepared, filtered through a 0.2 μm filter, aliquoted and quickly frozen in the gas phase of liquid nitrogen; this constitutes the challenge virus stock. The infective titres of this stock are determined both in cell culture ($TCID_{50}$) and in two cattle (BID_{50}) (50% bovine infectious dose). Two tranquilised cattle are injected intradermally in the tongue with tenfold dilutions (1/10 through 1/10,000), using four sites per dilution. The cattle titrations are read 2 days later. Titres are usually above 10^6 $TCID_{50}$ for 0.1 ml and above 10^5 BID_{50} for 0.1 ml, calculated using the Spearman–Kärber method. The dilution for use in the cattle challenge test is 10 000 BID_{50} in a total volume of 2 × 0.1 ml and is injected intradermally in the upper surface of the tongue both for the PD_{50} test and PGP test.

5.3.1. PD_{50} test

The number of protective doses in a vaccine is estimated from the resistance to infectious virus challenge of animal groups receiving different amounts of vaccine. Cattle of at least 6 months of age, obtained from areas free from FMD that have not previously been vaccinated against FMD and are free from antibodies to FMDV should be used. Three groups of no fewer than five cattle per group should be vaccinated by the route recommended by the manufacturer. The vaccine should be administered at different doses per group by injecting different volumes of the vaccine. For example, if the label states that the injection of 2 ml corresponds to the administration of 1 dose of vaccine, a 1/4 dose of vaccine would be obtained by injecting 0.5 ml, and a 1/10 dose would be obtained by injecting 0.2 ml. These animals and a control group of two unvaccinated animals are challenged either 3 weeks (aqueous) or up to 4 weeks (oil) after vaccination with a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine. The challenge test is done by inoculating the equivalent of a total of 10,000 BID_{50} intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Animals are observed for at least 8 days. Unprotected animals will show lesions at sites other than the tongue. Control animals must develop lesions on at least three feet. From the number of animals protected in each group, the PD_{50} content of the vaccine is calculated. There are a variety of methods for calculating PD_{50} but procedures based on the Kärber (1931) method are generally preferred when interpreting PD_{50} estimates calculated in this way. The vaccine should contain at least 3 PD_{50} per dose for cattle.

5.3.2. PGP test (protection against generalised foot infection)

For this method, a group of 16 FMD-seronegative cattle of 18–24 months of age, with the same characteristics described for the PD_{50} test, are vaccinated with a full bovine dose by the route and in the volume recommended by the manufacturer. These animals and a control group of two unvaccinated animals are challenged 4 weeks or more after vaccination with a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine. The challenge test is performed by inoculating the equivalent of a total of 10,000 BID_{50} intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Animals are observed for 7–8 days. Unprotected animals will show lesions on the feet within 7 days after inoculation. Control animals must develop lesions on at least three feet. For routine prophylactic use, the vaccine should protect at least 12 animals out of 16 vaccinated. This test does not provide an estimate of how many protective doses are in a single vaccine dose but comparison between tests suggested that 12 protected out of 16 vaccinated and challenged animals correlates with 3 PD_{50} (Vianna Filho *et al.,* 1993).

5.3.3. Efficacy estimated by indirect tests

When direct challenge test are not available to estimates efficacy, National Veterinary Authority may decide to use indirect tests (such as VN or LPB-ELISA), provided there is a correlation determined between antibody level and protection against challenge with 10,000 BID_{50}.

5.3.4. Efficacy in other species

Efficacy tests in other target species, such as sheep, goats, pigs or buffalo are either different or not yet standardised. In general, a successful test in cattle is considered to be sufficient evidence

of the quality of a vaccine to endorse its use in other species. Under circumstances where a vaccine is produced for use primarily in a species other than cattle, it may be more appropriate to potency test the vaccine in that same species. With respect to sheep, goats and African (*Syncerus caffer*) or Asiatic buffalo (*Bubalus bubalis*), due to the often inapparent nature of the disease in these species, potency results from a cattle test may be a more reliable indicator of vaccine quality than attempting a potency test reliant on the detection of clinical signs in these other species.

5.4. Purity: testing for antibody against NSP

Viral circulation within a defined population can be assessed by testing for presence of antibodies against NSPs. Furthermore the WOAH *Terrestrial Animal Health Code* stipulates that a criterion for regaining FMD free status following an outbreak, if vaccine is used, is to test the vaccinated animals for antibodies against NSP. Likewise, countries wishing to be recognised as FMD free with vaccination must demonstrate the absence of virus circulation by showing that vaccinated animals are free from antibody to NSPs arising as a result of infection. Consequently, FMD antigens used to formulate vaccines that may be used in these circumstances should be purified to reduce the NSP content. In addition to providing supporting documentation on the processes involved in such purification, manufacturers should demonstrate lack of immunogenicity against NSPs as part of the licensing procedure in order to make such a claim on their product literature. When purity tests include the use of full vaccine doses, at least 8 cattle should be booster vaccinated at 28–30 days after first vaccination and tested for purity 28–30 days later. Up to one reactive animal at 28–30 days post-booster vaccination could be accepted. If more than two animals are reactive at 28–30 days post-booster vaccination, the batch should be rejected. If two animals are reactive, the manufacturers have the option to ask for a batch retest. A recommended test method that can be used is to vaccinate not less than 8 naïve cattle with a full dose of the vaccine containing the maximum number of strains and amounts of antigen permitted on the authorisation. Cattle should be vaccinated at least twice at 21- to 30-day intervals and then tested before each revaccination and 30–60 days after the last vaccination for the presence of antibody to NSPs using the tests described in Section B.2.4 *Nonstructural protein* (*NSP*) *antibody tests*. Negative results in NSP assays may support claims that the vaccine does not induce antibody to NSPs for the number of injections tested. These cattle may be the same as those used for the safety test described in Section C.5.2 *Safety*.

5.5. Duration of immunity

The duration of immunity (D.O.I) of an FMD vaccine will depend on the efficacy (formulation and antigen payload). As part of the authorisation procedure, the manufacturer should be required to demonstrate the D.O.I. of a given vaccine by either challenge or the use of a validated alternative test, such as serology using the same test and animals described in Section C.5.3 *Efficacy* tested at the end of the claimed period of protection, in compliance with Section C.5.3. D.O.I. studies should be conducted in each species for which the vaccine is indicated or the manufacturer should indicate that the D.O.I. for that species is not known. Likewise, the manufacturer should demonstrate the effectiveness of the recommended booster regime in line with these guidelines, usually by measuring the magnitude and kinetics of the serological response observed.

5.6. Stability

The stability of all vaccines, including oil emulsion vaccines, should be demonstrated as part of the shelf-life determination studies for authorisation.

The shelf life of conventional FMD vaccines is usually 1–2 years at 2–8°C. Vaccines should never be frozen or stored above the target temperature.

Stability should be tested using the same methods described in Section C.5.3 *Efficacy*, but vaccinating the animals at the end of the shelf life of the product.

5.7. Precautions (hazards)

Current FMD vaccines are innocuous and present no toxic hazard to the vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of a vaccine.

6. Storage and monitoring of concentrated antigens

Chapter 1.1.10 provides international standards for vaccine banks.

The process of storing concentrated antigens at ultra-low temperature for later formulation into FMD vaccine as described in Section C.2, is a well established procedure for building stocks of immunogenic material ready to be formulated into vaccines in case of need. It not only forms the basis for the storage of antigens in a strategic reserve for emergency purposes, but allows the manufacturer immediate access to many different antigen strains that can be rapidly formulated and dispatched to the customer (Lombard & Fussel, 2007). Such stockpiling minimises delays subsequent to an order, particularly where a multivalent vaccine is requested. Another advantage of this procedure is that much of the quality testing can be completed well in advance of shipment. It is necessary to state that the concentrated antigens have to be controlled using standards indicated in Sections C.1–4.

6.1. Storage conditions

6.1.1. Facilities

It is important that all aspects of the storage of concentrated antigens conform fully to internationally accepted requirements such as those referred to in chapter 1.1.8. Housing, facilities and procedures should ensure the security of the stored antigen and prevent tampering, contamination or damage.

6.1.2. Containment of stored antigens

The dose numbers or volumes stored are an important consideration, particularly where a reserve is shared between WOAH Members and there is variation in number of doses perceived to be needed by each Member in an emergency. Where the requirement is for a large stockpile of a particular vaccine strain that can only be produced from several separate production runs, vaccine bank managers must consider the need to either formulate each lot into a representative final blend for testing purposes or mixing the individual batches, at some convenient point, for ease of formulating and/or testing.

The type of container used to hold antigen concentrate is important. Under ultra-low temperature conditions it is important to use containers made from materials that do not become brittle or fragile at a temperature range allowing for heat sterilisation and cold storage.

6.1.3. Labelling of stored antigens

The concentrated antigens do not need to be labelled according to final or finished vaccine requirements and may be labelled as “in process” materials. Under ultra-low temperature conditions, the method of labelling must be of a durable nature. From experience, wire tagging bottles is the most preferred option using a metal/plastic tag sizeable enough to allow the necessary detail. Such detail should include the antigen/vaccine strain, batch number, date received and should also include an individual container or stock number. This information should be clear to read and marked on the tag using an indelible marker pen. Storage records and positions of containers should be carefully maintained.

6.2. Monitoring of stored concentrated antigens

It is vitally important that antigen concentrates are optimally maintained and routinely monitored in order to have some assurance that they will be efficacious when needed. Therefore arrangements should be made to monitor these antigen concentrates on a routine basis and to include where necessary, and at appropriate time intervals, a testing regime to ensure integrity of the antigen component or acceptable potency of the final product.

146S quantification, vaccination serology or vaccination challenge studies can be used for monitoring FMD antigen banks. It is recommended to carry out these tests on receipt (year 0) and every 5 years thereafter.

To support these testing requirements for depositories of antigen, concentrates should include a number of small samples that are representative of the larger stock. Small aliquots/stocks of FMD

antigen have usually consisted of a volume representing approximately one milligram of antigen. These aliquots should be stored side by side with the bulk antigen,

7. Emergency release of vaccines prepared from concentrated antigens

In situations of extreme urgency and subject to agreement by the National Veterinary Authority, a batch of vaccine may be released before completion of the tests and the determination of potency if a test for sterility has been carried out on the bulk inactivated antigen and all other components of the vaccine and if the test for safety and the determination of potency have been carried out on a representative batch of vaccine prepared from the same bulk inactivated antigen. In this context, a batch is not considered to be representative unless it has been prepared with not more than the amount of antigen or antigens and with the same formulation as the batch to be released.

D. VACCINE MATCHING TESTS

1. Introduction

Appropriate vaccine strain selection is an important element in the control of FMD and is necessary for the application of vaccination programmes in FMD-affected regions as well as for the establishment and maintenance of vaccine antigen reserves to be used in the event of new FMD incursions. The decision to change or include new strains in vaccine formulations is a multifaceted process and, among other issues, experimental, epidemiological and field observations should be considered.

Vaccination against one serotype of FMDV does not cross-protect against other serotypes and may also fail to protect fully or at all against other strains of the same serotype. The most direct and reliable method to measure cross-protection is to vaccinate relevant target species and then to challenge them by exposure to the virus isolate against which protection is required. This will take account of both potency and cross-reactivity.

However, such an approach requires the use of live FMDV and appropriate biosecurity procedures and practices must be used. The facility should meet the appropriate level of biocontainment, determined by risk analysis as outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.* In addition to the safety concerns, this procedure is slow and expensive and requires specific expertise that is best available in WOAH Reference laboratories. The use of animals for such studies should be avoided where possible by the use of *in-vitro* alternatives.

A variety of *in vitro* serological methods can be used to quantify antigenic differences between FMDV strains and thereby estimate the likely cross-protection between a vaccine strain and a field isolate. Genetic characterisation and antigenic profiling together with epidemiological observations can also reveal the emergence of new strains for which vaccine matching may be required and, conversely, may indicate that an isolate is similar to one for which vaccine matching information is already available. Such tests should be carried out in laboratories that work according to the standard specified in chapter 1.1.4 and Chapter 1.1.5 *Quality management in veterinary testing laboratories*, preferably WOAH Reference Laboratories for FMD.

Shipping of samples should be in accordance with Chapter 1.1.3 *Transport of biological materials*.

Vaccine potency and booster doses may contribute to the range of antigenic cover provided by a vaccine. A highly potent vaccine that stimulates a strong immune response may give protection to heterologous viruses. Furthermore, booster doses of vaccine can increase efficacy and the subsequent breadth of antigenic cover provided by a given vaccine, although the onset of full protection may be delayed (Pay, 1984).

2. Selection of field viruses for vaccine matching

For vaccine matching, preferably, more than one representative isolate should be evaluated from any outbreak.

Viruses should be selected based on epidemiological information, for instance isolation at different stages of an outbreak, from different geographical locations, or from different hosts (Alonso *et al.,* 1987). Field evidence for a suspected lack of vaccine quality as shown by reduced apparent protection, is an important criterion for vaccine matching, but other reasons for apparent vaccine failure (inadequate coverage, inadequate cold chain, insufficient boosting, lack of complementary control measures, etc.) should be also thoroughly explored.

The serotype of the field isolate is usually determined by ELISA or CFT using type-specific serological reagents, although methods based on MAbs or genetic typing may also be used. If the number of viruses exceeds the capacity of the laboratory to carry out methods described in Section D.4 *Vaccine matching tests*, a pre-selection of isolates should be done.

In order to minimise the risk of missing a relevant sample, the pre-selection should be carried out using all the isolates received by the laboratory. The recommended approach is to engage in serological validated antigenic profiling methods using MAb ELISA or CFT. VP1 sequencing should be used to verify the genetic homogeneity of the virus isolate population and its genetic distance with respect to the available vaccine strains.

The emergence of a new virus strain may be characterised by rapid dissemination with many outbreaks, and isolates from such epidemics are a priority for vaccine matching. Furthermore, isolates showing prevalence within the outbreak are the best candidates for vaccine matching tests. Isolates showing important differences with vaccine strains and not prevalent within the outbreak should be monitored closely through active surveillance in the field.

3. Selection of vaccine strains to be matched

The serotype of the virus, the region of origin and any information on the characteristics of the field isolate and, as appropriate, the vaccine strain used in the region of origin, may give indications as to the vaccine strains to be selected for vaccine matching tests. The availability of reagents for matching to particular vaccine strains may limit the extent of testing that is possible. To avoid this problem it is expected that vaccine manufactures should provide, upon request of the vaccine purchaser and WOAH Reference Laboratories, post-vaccination sera produced during final product batch potency testing. It is also recommended that WOAH Reference Laboratories guarantee the availability of reference post-vaccination sera produced with relevant vaccine strains. Vaccine matching approaches have two purposes: first, to guide the selection of the most effective vaccine strain for use in a particular field circumstance, either routine prophylactic vaccination or emergency use, for which matching requirements are not necessarily the same; and second, to monitor, on an ongoing basis, the suitability of vaccine strains maintained in strategic antigen reserves.

4. Vaccine matching tests

The serological relationship between a field isolate and a vaccine virus ('r' value) can be determined by VNT, ELISA or CFT. One way testing is recommended (r_1) with a vaccine antiserum, rather than two way testing (r_2), which also requires an antiserum against the field isolate to be matched. *In-vitro* neutralisation may be more relevant to predict *in-vivo* protection by the vaccine than other measures of virus-antibody interaction. VNT using the chequer-board titration method or other layout can be undertaken based in the laboratory experience. ELISA depends on the availability of trapping and detector antibodies suitable to the field strains, but it is more reproducible than VNT and can be carried out with inactivated virus. CFT can be used as a screening method to select strains to be tested by VNT or ELISA. The reproducibility of VNT results can be improved by incorporating multiple virus dilutions into the test so that the virus titre can be determined accurately by logistical regression.

For either VNT or ELISA, post-vaccination sera should be derived from at least five cattle 21–30 days after primary vaccination and/or 21–30 days after booster vaccination. The titre of antibody to the vaccine strain is established for each serum. Sera are used individually or pooled, after excluding low responders (Mattion *et al.,* 2009).

4.1. Relationship between the field isolate and the vaccine strain

The recommended standard test is the VNT. The ELISA can also be used if suitable reagents are available or as a screening test. The CFT is suitable as a screening test to select strains to be analysed by VNT or ELISA.

4.1.1. Vaccine matching by two-dimensional (chequerboard) neutralisation test

This test uses antiserum raised against a vaccine strain. The titres of this serum against 100 $TCID_{50\%}$ of the homologous vaccine strain and the same dose of a field isolate are compared to estimate the immunological coverage of the vaccine strain in relation to the field virus. For the test each 100 µl of virus/serum mixture contains 100 $TCID_{50}$ of test virus.

4.1.1.1. Test procedure

The procedure is similar to that of the VNT (see Section B.2.1 *Virus neutralisation*).

Additional biological reagents are: monovalent 21–30 day post-vaccination bovine sera (inactivated at 56°C for 45–60 minutes), the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain.

i) Field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, determine the virus titre (log_{10} $TCID_{50}$/ml) by end-point titration.

ii) For each test and vaccine virus a chequerboard titration is performed of virus against vaccine serum along with a back-titration of virus. Cells are added and incubated at 37°C for 48–72 hours after which time CPE is assessed.

iii) Antibody titres of the vaccine serum against the vaccine strain and field isolate for each virus dose used are calculated using the Spearman–Kärber method. The titre of the vaccine serum against 100 $TCID_{50}$ of each virus can then be estimated by regression. The relationship between the field isolate and the vaccine strain is then expressed as an 'r' value as:

$$r_1 = \frac{\text{reciprocal arithmetic titre of reference serum against field virus}}{\text{reciprocal arithmetic titre of reference serum against vaccine virus}}$$

4.1.1.2. Interpretation

Interpretation of the results of cross-reactivity tests: It has been generally accepted that in the case of neutralisation, r_1 values greater than 0.3 indicate that the field isolate is sufficiently similar to the vaccine strain so that use of a vaccine based on this strain is likely to confer protection against challenge with the field isolate. However, protection depends on both the cross-reactivity of antibodies elicited by the vaccine and the strength of the antibody response. The latter will be influenced by the potency of the vaccine and the number of doses given.

When deciding whether or not to use vaccines for which r_1 values lower than 0.3 are observed, factors to be considered include the availability of better matching vaccine strains, the potency of the vaccine and the potential for this to be increased to provide heterologous responses, the possibility of using additional booster doses, and the extent to which control of disease will be complemented by other zoosanitary measures or will be dependent on vaccination. The combined impact of potency and match can be estimated from the serological titre of the vaccine antiserum against the field virus, although correlating this precisely to protection requires a cross-protection test.

Alternatively, a suitable field isolate could be adapted to become a new vaccine strain.

Tests should always be repeated more than once. The confidence with which 'r' values can be taken to indicate differences between strains is related to the number of times that the examination is repeated. In practice, a minimum of at least three repetitions is advised.

4.1.2. Vaccine matching by expectancy of protection (EPP) determined by one-dimensional neutralisation test

Vaccine matching based on EPP values is widely used in South America where correlation tables are available for the vaccine strains used in the Region. This test uses antiserum raised against a vaccine strain (primo and booster vaccination). The titres of sera against 100 $TCID_{50}$/100 µl of sera/virus mixtures of the homologous vaccine strain and the same dose of a field isolate are compared to estimate the immunological coverage of the vaccine strain in relation to the field virus.

4.1.2.1. Test procedure

The procedure is similar to that of the VNT (see Section B.2.1 *Virus neutralisation*).

Additional biological reagents are: monovalent 21–30 day post-vaccination, and/or 21–30 day post-booster vaccination bovine sera (inactivated at 56°C for 45–60 minutes); the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain. For regions where multivalent vaccines are used, it is advisable to use sera panels raised against the vaccine commonly used in the vaccination programme.

i) Field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, determine the virus titre (log_{10} $TCID_{50}$/ml) by end-point titration.

ii) For each test and vaccine virus, a titration of antibodies is performed against a fixed amount of virus (100 $TCID_{50}$ of virus/100 µl of mixture sera/virus), along with a back-titration of working virus. Sera/virus mixtures and virus back titration are incubated at 37°C for 60 minutes and then inoculated on to microplates with preformed cell monolayers. Each serum dilution is inoculated in four wells and at least six sera dilutions are used. Microplates are incubated at 37°C for 48 hours under CO_2 atmosphere after which time CPE is assessed.

iii) Antibody titres of the vaccine serum against the vaccine strain and field isolate are calculated using the Spearman–Kärber method. The titre of the vaccine serum against 100 $TCID_{50}$ of each virus can then be estimated and expressed per ml. Individual EPP values are determined for each individual neutralisation titre using predefined correlation tables, and then the mean of the EPP is calculated for each group of sera (vaccinated and booster vaccinated). The immunological coverage of the vaccine strain is expressed by the EPP value.

b) Interpretation

i) Interpretation of the results

To interpret the results it is necessary to have a correlation defined between *in-vitro* titres and *in-vivo* challenge protection against 10,000 BID_{50} of vaccine virus. In the PANAFTOSA experience with FMDV control and eradication programmes in South America, a mean EPP value of 75% in booster vaccinated animals indicates that the vaccine strain is suitable to be used together with appropriate field measures to control outbreaks with the field strain under test (correlation tables for O1, A24 and C3 are available upon request to PANAFTOSA).

4.1.3. Vaccine matching by ELISA

Similarly, the liquid-phase blocking ELISA described in Section B.2.3 is recommended for vaccine matching calculating the r_1 or EPP value.

4.1.3.1. Test procedure

The procedure is similar to that of the LPBE (see Section B.2.3 *Liquid-phase blocking enzyme-linked immunosorbent assay*).

Additional biological reagents are: monovalent 21–30 day post-vaccination and/or 21–30 day post-booster vaccination bovine sera (inactivated at 56°C for 45–60 minutes); the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain. For regions where multivalent vaccines are used, it is advisable to use sera panels raised against the vaccine commonly used in the vaccination programme.

4.1.3.2. Interpretation

i) Interpretation of r1 results

It has been proposed that in the case of LPBE, r_1 values greater than 0.4 indicate that the field isolate is sufficiently similar to the vaccine strain so that use of a vaccine based on this strain is likely to confer protection against challenge with the field isolate. However, protection depends on both the cross-reactivity of antibodies elicited by the vaccine and the strength of the antibody response. The latter will be influenced by the potency of the vaccine and the number of doses given. When deciding whether or not to use vaccines for which r_1 values lower than 0.4 are observed, factors to be

considered include the availability of better matching vaccine strains, the potency of the vaccine and the potential for heterologous responses to be increased, the possibility of using additional booster doses, and the extent to which control of disease will be complemented by other zoo sanitary measures or will be dependent on vaccination.

ii) Interpretation of EPP results

To interpret the results it is necessary to have a correlation defined between *in-vitro* titres and *in-vivo* challenge protection against 10,000 BID_{50} of vaccine virus. In the PANAFTOSA experience with FMDV control and eradication programmes in South America, a mean EPP value of 75% in booster vaccinated animals indicates that the vaccine strain is suitable to be used together with appropriate field measures to control outbreaks with the field strain under test (correlation tables for O1, A24 and C3 are available upon request to PANAFTOSA).

4.1.4. Vaccine matching by CFT

The CFT preferably performed in a tube, can be used as a screening test to select those strains to be tested by VN or ELISA. The test is performed as described in Section B.1.2.4. Additional biological reagents are guinea-pig hyper-immune sera against vaccine strains. Sera antibody titre is determined against homologous virus and field strains. The r_1 value is calculated as previously described:

$$r_1 = \frac{\text{reciprocal arithmetic titre of reference serum against field virus}}{\text{reciprocal arithmetic titre of reference serum against vaccine virus}}$$

Interpretation of the results: it is generally accepted that r_1 values equal or greater to 0.25 indicate that the field isolate is sufficiently similar to the vaccine strain. Studies by VNT or ELISA need to be performed with selected strains to confirm suggested CFT classification.

4.2. Testing the fitness for purpose of a vaccine

The "r" value should not be used in isolation to select the most appropriate vaccine strain to be used in the field. Particularly when it suggests an insufficient match of a certain vaccine strain, the suitability of a vaccine based on such a vaccine strain could be demonstrated by a heterologous cross-protection challenge test carried out as described in Section C.4.3 *Identity testing* in animals vaccinated with a known vaccine and challenged with the (heterologous) field virus. Vaccinate at least seven cattle without FMD antibodies, with a commercial dose of the current vaccine to be used in the region. Between 28 and 30 days later, boost all these animals with a second commercial dose in the same conditions and vaccinate a second group of at least seven animals with the same vaccine dosage and same route. Challenge the two groups and two control animals (not vaccinated) 30 days later with the equivalent of a total of 10,000 BID 50% of the new field strain duly titrated. The results are valid if each of the two control animal shows podal lesions on at least three feet. Final results are reported either as the number of protected animals (without podal lesion) over the total number of animal per group, or by percentage of protection where 100% is the total number of animals used per group. If results in the group of once vaccinated cattle indicate a protection level under 50%, and in the group of twice vaccinated cattle, protection under 75%, the change for a more appropriate vaccine strain is recommended.

The use of the EPP method is possible when correlation studies have been performed for the vaccine strain. The EPP method proved to be useful in some regions of the world applied together with epidemiological observations and active surveillance in the field. This method measures the reactivity of a panel of post-vaccination antisera using either VNT or ELISA and relates the serological titres to the probability of protection, established through correlation tables associating antibody titres with protection against challenge of 10000 BID_{50} of the homologous vaccine strain.

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NB: There are WOAH Reference Laboratories for foot and mouth disease
(please consult the WOAH web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
FMD diagnostic tests, reagents and vaccines.

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.1.9.

HEARTWATER

SUMMARY

Description and importance of the disease: *Heartwater (also known as cowdriosis) is an acute, fatal, non-contagious, infectious, tick-borne rickettsial disease of ruminants caused by* Ehrlichia ruminantium *(formerly* Cowdria ruminantium*) and transmitted by* Amblyomma *ticks. It occurs in nearly all sub-Saharan countries of Africa, in its neighbouring islands, and also in the Caribbean. The disease can cause high mortality (up to 90%) in susceptible domestic ruminants. Goats and sheep are more susceptible than cattle, and European breeds are generally more susceptible than indigenous African breeds.*

Clinically, the disease most commonly presents as an acute form characterised by a sudden high fever, depressed demeanour, nervous signs and a high mortality. Hydropericardium and hydrothorax and lung oedema are commonly associated post-mortem lesions. Acute and peracute clinical forms of the disease occur: in the former, there are high death rates without many clinical manifestations and, in the latter, there is a higher recovery rate.

Recovered animals become carriers of infection. Certain wild animals can play a role as reservoir; Rusa deer, white-tailed deer, and springbok are susceptible to this infection and can experience high mortality.

Identification of the agent: *The specific diagnosis of heartwater is based on the observation of colonies of* E. ruminantium *in capillary endothelial cells of the brain. In the absence of molecular diagnostic tools, a piece of cerebellum can easily be removed with a curette through the foramen magnum after cutting off the head, while a sample of cerebral cortex can be obtained through a hole made in the skull with a hammer and a large nail. Brain smears are prepared by crushing to a paste and spreading thinly a small piece of cerebral or cerebellar cortex between two microscope slides. The capillaries are spread out in a single cell layer by drawing one slide across the other. The smears are air-dried, fixed with methanol and stained with Giemsa. The colonies (clusters) of* E. ruminantium *are reddish-purple to blue, and very often close to the nucleus of the infected endothelial cell. They can be scanty and difficult to find, particularly in peracute cases, but they are always present in the brain of a ruminant that died from heartwater, if not treated with drugs. Colonies are not likely to be detected in animals that were treated with antibiotics. The colonies are still visible 2 days after death in a brain that has been stored at room temperature (20–25°C) and up to 34 days in a brain that has been stored in a refrigerator at 4°C.*

Ehrlichia ruminantium *can be isolated from the blood of an infected host using cultivation on ruminant endothelial cells. When a cytopathic effect consisting of plaques of cell lysis appears, the presence of characteristic morulae is confirmed by staining the cell monolayer with Giemsa or RAL555 or by immunofluorescence.*

Molecular tools such as nested polymerase chain reaction (PCR) and real-time PCR targeting E. ruminantium*-specific genes are currently available for the detection of the presence of* E. ruminantium *in the blood of animals with clinical signs, in organs from dead animals (confirming clinical cases of heartwater), and also in the tick vectors. They could not however, detect* E. ruminantium *in asymptomatic carriers. Two multi-pathogen methods, including* E. ruminantium *detection, have been developed allowing differential diagnosis for tick-borne diseases to be undertaken. Apart from diagnosis, molecular methods are widely used for research on the* E. ruminantium *genome and for epidemiological studies, including* E. ruminantium *tick prevalence.* No commercial molecular kits targeting *E. ruminantium* are currently available.

Serological tests: *Two enzyme-linked immunosorbent assays (ELISAs) have been evaluated: an indirect ELISA and a competitive ELISA targeting major antigenic protein 1 (MAP1) antibodies.*

The current indirect ELISA uses a recombinant antigen expressed as a partial fragment of the MAP1 antigens – the MAP1-B ELISA – which gives improved specificity over earlier methods. However the assay still detects cross-reacting antibodies to other Ehrlichial organisms including Ehrlichia chaffeensis*,* E. canis *and Panola Mountain* Ehrlichia*. Hence, definitive proof of heartwater must rely on epidemiological evidence and additional molecular testing indicating the presence of the organism. This ELISA can help to monitor experimental infections and to measure the immune response of immunised animals, whose pre-immunisation serological history is known. Serology has very limited diagnostic use as clinically infected animals remain sero-negative during the febrile reaction and sero-convert after recovery.*

Serology is also not an effective import test. Prior to importation of animals from a heartwater endemic region, it is important to study the epidemiological data to try to establish that the herd and the resident ticks are not infected. Repeated serology on the herds and PCR on tick samples in targeted herds can be carried out to demonstrate that they are free of E. ruminantium*.*

Requirements for vaccines: *Immunisation against heartwater by the 'infection and treatment' method using infected blood is still in use in some countries. A first-generation vaccine consisting of inactivated purified elementary bodies of* E. ruminantium *emulsified in an oil adjuvant has given promising results in experimentally controlled conditions and has demonstrated significant protection in the field. Further improvement of the method to produce the inactivated vaccine using bioreactors, antigen storage conditions, and different adjuvant has demonstrated good efficiency in controlled conditions. An additional isolate, Welgevonden, has been attenuated and shown to confer good protection in controlled conditions, and significant protection has also been obtained using DNA vaccination. However, none of these experimental vaccines has been fully validated under field conditions. Field trials and studies on genetic characterisation of strains have revealed the presence of a high number of* E. ruminantium *strains in restricted areas. Thus, antigenic diversity is important in formulating effective vaccines, and further investigations are critical for the delivery of any vaccine in the field.*

A. INTRODUCTION

Heartwater (cowdriosis) is a rickettsial disease of domestic and wild ruminants caused by *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) and transmitted by *Amblyomma* ticks (Marcelino *et al.,* 2016). *Ehrlichia ruminantium* is classified in the order Rickettsiales and in the family Anaplasmataceae, together with the genus *Anaplasma*. Although ruminants remain the primary target of the pathogen, in South Africa a possible canine *E. ruminantium* infection has been reported, and *E. ruminantium* has been strongly suspected in several cases of rapidly fatal encephalitis in humans. However, in all cases, evidence of *E. ruminantium* infection was based on molecular detection. Isolation and characterisation of the infectious agent is necessary before *E. ruminantium* can be considered an emerging pathogen in species other than ruminants and especially in humans. Since then, no other clinical human case associated with heartwater has been observed.

Heartwater is an important tick-borne disease of livestock in Africa occurring in nearly all the sub-Saharan countries where *Amblyomma* ticks are present and in the surrounding islands: Madagascar, Reunion, Mauritius, Zanzibar, the Comoros Islands and Sao Tomé. The disease is also reported in three Caribbean islands (Guadeloupe, Marie-Galante and Antigua) (Marcelino *et al.,* 2016). All domestic and wild ruminants can be infected, but the former appear to be the most susceptible. Indigenous domestic ruminants are usually more resistant to the disease. Wild animals could play a role as reservoir, but Rusa deer, white-tailed deer, springbok, chital, timor deer, which are used in wildlife farming, seem to be the main wild ruminant species in which heartwater can have a significant economic impact.

The average natural incubation period is 2–3 weeks, but can vary from 10 days to 1 month. In most cases, heartwater is an acute febrile disease, with a sudden rise in body temperature, which may exceed 41°C within 1–2 days after the onset of fever. It remains high for 4–5 weeks with small fluctuations and drops shortly before death.

Fever is followed by inappetence, sometimes listlessness, diarrhoea, particularly in cattle, and dyspnoea indicative of lung oedema. Nervous signs develop gradually. The animal is restless, walks in circles, makes sucking

movements and stands rigidly with tremors of the superficial muscles. Cattle may push their heads against a wall or present aggressive or anxious behaviour. Finally, the animal falls to the ground, pedalling and exhibiting opisthotonos, nystagmus and chewing movements. The animal usually dies during or following such an attack.

Subacute heartwater with less pronounced signs, and peracute heartwater with sudden death, can also occur, according to the breed of ruminant and the strain of *E. ruminantium* involved.

The most common macroscopic lesions are hydropericardium, hydrothorax, pulmonary oedema, intestinal congestion, oedema of the mediastinal and bronchial lymph nodes, petechiae on the epicardium and endocardium, congestion of the brain, and moderate splenomegaly (Marcelino *et al.,* 2016).

A tentative diagnosis of heartwater is based on the presence of *Amblyomma* vectors, nervous signs, and presence of transudates in the pericardium and thorax on post-mortem examination. When making a diagnosis based on clinical signs, the following other diseases should be considered: bovine cerebral babesiosis and theileriosis, anaplasmosis, botulism, haemonchosis in small ruminants, rabies and poisoning.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of heartwater and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
In-vitro bacterium isolation	–	–	–	+	–	–
Real-time PCR	–*	–	–	+++	–	–
Nested PCR	–*	–	–	+++	–	–
Multi-pathogen real-time PCR	–*	–	–	+++	–	–
Detection of immune response						
ELISA	++	+	–	–	+++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
*can be used to screen tick populations, in parallel with serology on hosts.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

During the febrile reaction, *E. ruminantium* can be readily isolated in culture from blood or plasma; however, it is difficult to detect these organisms in a blood smear. Typical colonies of *E. ruminantium* can be observed in brain smears made after death and this represents a definitive diagnosis for heartwater.

Opening the cranium is not necessary. An alternative method is to cut off the head in front of the first cervical vertebra. Then, introduce a curette through the foramen magnum, between the medulla and the meninges. The curette is turned over towards the brain and removed with a piece of cerebellum. Another method consists of making a hole in the skull with a hammer and a large nail and aspirating a sample of brain cortex with a needle

attached to a syringe. These methods also lessen the danger to the operator in cases where the nervous signs have been caused by rabies.

In the live animal, a brain biopsy may be obtained aseptically after local anaesthesia, although with difficulty; appropriate restraint must be used especially with large and horned animals. Colonies of *Ehrlichia* are observed during the febrile period. This method is useful for experimental studies, but not suitable for routine diagnosis.

Colonies of *E. ruminantium* are still present 48 hours after death in a brain that has been stored at room temperature (20–25°C) and for up to 34 days in a brain that has been stored in a refrigerator at 4°C.

A small fragment of grey matter (approximately the size of a match head) is placed on a microscope slide, crushed to a paste consistency by another slide and, while maintaining pressure, the slides are drawn over each other lengthwise to produce a single layer of cells. The slides are air-dried, fixed in methanol, and stained with eosin and methylene blue or Giemsa. They are then examined under a microscope at a low magnification (×10 objective) to locate the cerebral capillaries and with a magnification of at least ×50 to identify the colonies of rickettsiae. *Ehrlichia ruminantium* are reddish-purple to blue coccoid organisms in the cytoplasm close to the cell nucleus which is coloured in pink (Marcelino *et al.,* 2016). Experience is required as *E. ruminantium* colonies must be differentiated from other haemoparasites (*Babesia bovis*), certain blood cells (thrombocytes, granulocytes), normal subcellular structures (mitochondria, mast cell granules), or stain artefacts (stain precipitates).

Ehrlichia ruminantium colonies are formed from clusters of granules (0.2–0.5 μm), sometimes arranged in the shape of a ring or a horseshoe (1–3 μm), that are placed close to the nucleus inside the endothelial cell. The granules can be scanty, particularly in peracute cases, but they are always present in the brain of an animal that died from heartwater. However, if the animal has been treated with doxycyclin or oxytetracyclin 48 hours before, the granules of *Ehrlichia* tend to fuse, making the diagnosis very difficult, and sometimes impossible. Transmission electron microscopy has been used to demonstrate that the *E. ruminantium* organisms develop inside a vacuole-like structure, which is surrounded by a membrane in the endothelial cell's cytoplasm. Each organism is enclosed by a double membrane. Within the vacuole-like structure, *E. ruminantium* electron-dense forms (elementary bodies), as well as intermediate reticulate forms, are identified.

1.1. Isolation of *Ehrlichia ruminantium* using *in-vitro* culture

Although numerous cell lines have been shown to support the growth of *E. ruminantium,* isolation is not the test of first choice for rapid diagnosis of heartwater, as it is labour intensive and time-consuming. For a rapid diagnosis, molecular methods are preferred. However, *E. ruminantium* isolation should be encouraged for typing the strains present in a region for the purpose of vaccination programmes. *Ehrlichia ruminantium* can be isolated from the blood of reacting animals by cultivation on ruminant endothelial cells. Endothelial cells from umbilical cord, aorta, or the pulmonary artery of different ruminant species (cattle, goat, sheep) are used most often for isolation, although other endothelial cell types (brain capillaries, circulating endothelial cells, etc.) have been described for the routine culture of the microorganism. Endothelial cell lines from sable, eland, buffalo, kudo and bush pig can also be used to grow *E. ruminantium*. No standard cell line has yet been designated for isolation.

1.1.1. Isolation procedure

i) The blood of the clinically affected animal (optimally on the second or third day of febrile reaction) is collected in anticoagulant (heparin or sodium citrate, not ethylene diamine tetra-acetic acid) and diluted 1/2 in the culture medium consisting of Glasgow minimal essential medium (MEM) supplemented with 10% inactivated fetal bovine serum, 200 mM L-glutamine, and antibiotics if necessary (penicillin 100 U/ml, streptomycin 100 μg/ml).

ii) The culture medium is poured off the endothelial cell monolayer, and sampled blood (approximately 2 ml for a 25 cm^2 flask) is added. The flask is incubated at 37°C (if possible with 5% CO_2) on a rocking platform for 2 hours.

iii) After incubation, the blood is poured off and the monolayer is gently washed three times with culture medium prewarmed at 37°C. Fresh culture medium (5 ml per 25 cm^2 flask) is added and the flask is incubated at 37°C with 5% CO_2. The medium is changed every 2 days.

(The *use of plasma instead of blood is more efficient* when taken from an animal with a febrile reaction >41°C. In this case, steps ii and iii above may be replaced with the following:

a) Seed 4 ml of plasma (smaller inoculum can be used if there is a limited amount of plasma available) on to a susceptible endothelial cell culture and incubate for 1 hour at 37°C on a rocking platform.

b) Wash plasma three times with culture medium prewarmed at 37°C. and then add 5 ml of culture medium (per 25 cm^2 flask) and observe for development of cytopathic effect.

iv) The monolayer is inspected regularly for the appearance of small plaques of cell lysis. The first plaques generally appear after about 2 weeks. Passaging on uninfected cell monolayers is performed when the lysis reaches 80% of the cell layer. The remaining cells are stained with eosine and methylene blue or Giemsa and examined microscopically for the presence of *E. ruminantium* morulae (Marcelino *et al.,* 2016).

2. Molecular methods

2.1. Detection of *Ehrlichia ruminantium* using nested polymerase chain reaction

Two nested polymerase chain reaction (PCR) assays have been developed to enhance detection of low levels of rickettsemia (Martinez *et al.,* 2004; Semu *et al.,* 2001). Both use the *pCS20* region as the target sequence. The Semu *et al.* assay uses two external primers: U24 (5'-TTT-CCC-TAT-GAT-ACA-GAA-GGT-AAC-3') and L24 (5'-AAA-GCA-AGG-ATT-GTG-ATC-TGG-ACC-3'), and then the AB 128 (5'-ACT-AGT-AGA-AAT-TGC-ACA-ATC-TAT-3') and AB 129 (5'-TGA-TAA-CTT-GGT-GCG-GGA-AAT-CCT-T-3') for the nested reaction. The analytical sensitivity of detection of this assay is one organism per reaction. The other nested PCR assay (Martinez *et al.*, 2004) uses a pair of external primers that comprise AB128/AB130 (5'-ACT-AGC-AGC-TTT-CTG-TTC-AGC-TAG 3') followed by a second amplification based on AB128/AB129 primers. The final PCR product is 278 pb long. The nested PCR shows a hundred-fold improvement in sensitivity compared with the simple AB128/AB129 PCR, and an average detection limit of 6 organisms per reaction.

Lack of amplification with conventional *pCS20* nested PCR was observed due to single nucleotide polymorphisms (SNPs) on primer hybridisation areas of the *pCS20* fragment DNA. Thus, AB128/129 and 130 primers modified using universal nucleotides allowed the detection of a wider range of *E. ruminantium* strains: AB128' (5'-ACT-AGT-AGA-AAT-TGC-ACA-ATC-YAT-3'), AB130' (5'-RCT-DGC-WGC-TTT-YTG-TTC-AGC-TAK-3') and AB129' (5'-TGA-TAA-CTT-GGW-GCR-RGD-ART-CCT-T-3'). This *pCS20* nested PCR is used routinely at the WOAH Reference Laboratory for diagnostic purposes on blood samples from clinical cases and for tick screening (Adakal *et al.,* 2009; 2010a; Cangi *et al*., 2016). The *pCS20* nested PCRs allow detection in organs (lung and brain) from infected dead animals, blood from infected animals during hyperthermia, and ticks fresh, frozen or preserved in 70% ethanol. The detection of *E. ruminantium* by nested PCR is possible in the blood of animals 1 or 2 days before hyperthermia and during the hyperthermia period but not on asymptomatic animals.

A nested PCR targeting the entire *map*1 polymorphic gene has been developed in parallel in order to type the strains by restriction fragment length polymorphism (RFLP) or sequencing of the amplification fragment directly from the pathological samples testing positive in the *pCS20* nested PCR (Martinez *et al.*, 2004). The *map*1 nested PCR performs well although with a slightly lower sensitivity than the *pCS20* nested PCR with 60 copies per sample. This tool is useful for genetic characterisation of *E. ruminantium* but not for diagnosis due to the polymorphic property of *map1* targeted genes.

The genetic characterisation and structure of the *E. ruminantium* population at the regional level is essential for the selection of potential vaccinal strains. The genetic typing of strains was previously done using RFLP on the *map1* polymorphic gene after PCR amplification (Adakal *et al.,* 2010b; Faburay *et al.,* 2007). However, multi-locus methods adapted to *E. ruminantium* have been validated such as multi-locus sequence typing (Adakal *et al.,* 2009) and multi-locus variable number of tandem repeat sequence analysis (Pilet *et al.,* 2012). These tools are being used on field tick samples for molecular epidemiological studies to better characterise the genetic structure of *E. ruminantium* strains (Adakal *et al.,* 2010a; Cangi *et al.,* 2016). However, these genetic characterisations are not associated with clusters of protection.

2.2. Detection of *Ehrlichia ruminantium* using real-time PCR

Several real-time PCR tests targeting *map1*, *map1-1* and *pCS20* region genes have been developed for the detection of *E. ruminantium* organisms. SYBR Green real-time PCRs targeting *map1* and *map1-1* were used to detect and quantify *E. ruminantium in vitro* during mass antigen production in a bioreactor and in experimentally infected sheep during the hyperthermia period (Marcelino *et al.,* 2005; 2007; Peixoto *et al.,* 2005; Postigo *et al.,* 2002). They were tested on a limited number of strains (up to six strains) and therefore they are not recommended for diagnostic purposes due to the polymorphic characteristics of *map1* multigenic family.

A real-time PCR assay targeting the *pCS20* region using a fluorescent-labelled probe has been developed to detect *E. ruminantium* in livestock blood and ticks from the field, and has a sensitivity similar to the nested PCR with seven copies per sample. The sequences of primers and probes are: CowF (5'-CAA-AAC-TAG-TAG-AAA-TTG-CAC-A-3'), CowR (5'-TGC-ATC-TTG-TGG-TGG-TAC-3') and Cow probe (5'-FAM-TCC-TCC-ATC-AAG-ATA-TAT-AGC-ACC-TAT-TA-XT-PH-3'). Unfortunately, this assay displayed cross reaction with *E. canis* and *E. chaffeensis.* It successfully detected 15 different *E. ruminantium* strains (Steyn *et al.,* 2008). As shown previously for the *pCS20* nested PCR, the presence of SNPs on hybridisation regions could inhibit strain detection. Testing more strains is necessary to further validate the method.

More recently a new real-time PCR targeting another *pCS20* region has been developed and demonstrated a good reproducibility, sensitivity and specificity with a limit of detection of 6 copies per sample. It can be used with appropriate fluorescent probes. Primers and probes are: Sol1F (ACA-AAT-CTG-GYC-CAG-ATC-AC), Sol1R (CAG-CTT-TCT-GTT-CAG-CTA-GT) and Sol1^{TqM} (6-FAM-ATC-AAT-TCA-CAT-GAA-ACA-TTA-CAT-GCA-ACT-GG-BHQ1). It detects 16 *E. ruminantium* strains from different geographical areas and there is no cross protection with *Anaplasma marginale, A. phagocytophilum, A. platys, Babesia bovis*, *B. bigemina, E. canis*. *E. muris* and *Rickettsia felis* and *parkeri* (Cangi *et al*., 2017). It has been tested on 700 tick field samples from Mozambique and will be used routinely in the WOAH Reference Laboratory for diagnostic use and tick screening.

Although nested and real-time PCR methods have proved highly effective in detecting infection in ticks or in animal samples during the clinical phase of the disease or after death, they could not allow detection of *E. ruminantium* in healthy carrier ruminants. A useful technique for confirming the status of a suspected carrier animal, whose blood is PCR negative, is to feed batches of naive ticks on the animal and then test the ticks by *pCS20* nested or real-time PCR. It is not known whether ticks act simply by concentrating circulating organisms, or by amplifying their number or even by inducing release of micro-organisms in the circulation during feeding.

2.3. Detection of *E. ruminantium* using multi-pathogen real-time PCR

A single FRET-real-time PCR has been developed to differentiate eight species in four distinct groups in a single reaction: *E. canis, E. chaffeensis, E. ewingii, E. muris, E. ovina, Ehrlichia* sp. *BOV2010,* Panola Mountain *Ehrlichia* and *E. ruminantium* (Zhang *et al.,* 2015). It is based on 16S recombinant RNA amplification using two fluorescent probes; different dissociation curves are detected depending on the species. *Ehrlichia ruminantium* could be clearly differentiated from other species with the lowest melting temperature. The limit of detection is five copies per sample and simultaneous detection is possible with a mix of 300 copies of each species. However, only four *E. ruminantium* strains were tested. Even if the lack of amplification is limited due to the targeted conserved gene 16S recombinant RNA, validation on more *E. ruminantium* strains is necessary.

On the other hand, Sayler *et al.* developed a dual-plex Taqman real-time PCR targeting the groEL gene of Panola Mountain *Ehrlichia* and *E. ruminantium* (Sayler *et al.,* 2016). This assay allows Panola Mountain *Ehrlichia,* which is endemic in the USA, to be differentiated from *E. ruminantium,* which is currently not present. The limit of detection is 10 copies per sample, and 27 *E. ruminantium* strains from 11 countries were detected. It seems therefore to be a promising method for differential diagnosis between the two species.

2.4. Detection of *Ehrlichia ruminantium* using the reverse line blot technique

The reverse line blot technique (RLB) has been used for the simultaneous detection and identification of *Anaplasma* and *Ehrlichia* species known to occur in ruminants, on the basis of differences in the small subunit rRNA gene (Bekker *et al.,* 2002). Primers 16S8FE and B-GA1B-new were designed from conserved domains and used to amplify a 492–498 bp fragment of the 16S rRNA gene spanning the variable V1 region. Species-specific oligonucleotide probes were designed in this V1 loop to allow species-specific detection of *E. ruminantium*, *E. ovina*, *Ehrlichia* sp. strain Omatjenne, *Anaplasma marginale*, *A. centrale, A. bovis*, *A. ovis* and *A. phagocytophilum*. One oligonucleotide probe cross-reactive with all species (catch-all probe) was also designed to serve as a control in case a PCR product does not hybridise to any of the species-specific probes. In the method, the species-specific probes are covalently linked to the hybridisation membrane, which is hybridised with the PCR product obtained using primers 16S8FE and B-GA1B-new. PCR products obtained from all above-mentioned microorganisms were shown to bind with specific oligonucleotide probes only. No PCR product was detected and no hybridisation occurred when the PCR-RLB was applied to *Theileria annulata*, *Babesia bigemina* or mammalian DNA. Similarly, negative control ticks were always negative in the RLB assay whereas it was possible to detect *Ehrlichia ruminantium* infection in 15–70% of ticks fed on experimentally infected or long-term carrier sheep. In Mozambique, *E. ruminantium* could also be detected in the blood of 12 sentinel small ruminants placed in the field with the infected animals; mixed infection was detected in five of the infected sentinel animals, thus demonstrating the usefulness of the method for detecting multiple infections. The RLB has been used recently in several studies in Western Kenya and in Nigeria to evaluate the prevalence of tick-borne diseases in cattle (Lorusso *et al.,* 2016; Njiiri *et al.,* 2015) however, they obtained very low prevalence of *E. ruminantium*. It was suggested by the authors that sequences of the primers and probes of RLB were not adapted to Kenya *E. ruminantium* strains.

2.5. Reading the results

As *E. ruminantium* is an obligate intracellular bacterium that cannot be cultivated in acellular media and its isolation is complex and takes several weeks, molecular tools are the best methods for the diagnosis of heartwater. Nested and real-time PCRs prove to be easier to perform and more sensitive than RLB. With all PCRs, however, care must be taken to ensure that no cross-contamination occurs between samples. Negative and positive controls must be included in each test. For each PCR assay, nested or real-time, positive and negative extraction controls (from experimentally infected and uninfected blood or organs, infected or uninfected ticks) should be included allowing detection of a default in the extraction procedures. The absence of inhibitor products in each sample should be proven by targeting a housekeeping gene from the vector or the host such as 16S or 18S ribosomal DNA (for tick screening, Cangi *et al*., 2017). As heartwater serology has several limitations (see Section B.3), the PCR could be used to help confirm if seronegative animals, originating from an endemic area, are not infected, prior to translocating them to a heartwater-free area that has the risk of becoming infected, because of the presence of potential vectors. Screening of ticks by PCR, along with serology on targeted herds over time could be used to establish the herd status before any movement of animals from this endemic area to a free area. However, *E. ruminantium* cannot be detected in asymptomatic carriers by molecular methods. The results obtained with nested PCRs, the RLB assay and real-time PCR, show that the direct detection of *E. ruminantium* in the blood is only reliable during and around the febrile phase of the disease. PCR-based methods appear to be more reliable in detecting infection in ticks, and this could have epidemiological value in determining the geographical distribution of *E. ruminantium*. In addition, when necessary in endemic areas, the inclusion of testing (originally naive) ticks fed on a suspect animal would greatly improve the sensitivity of carrier detection when serology and PCR on blood have failed. The procedure is nevertheless not suitable for routine diagnostic laboratories as it requires the maintenance of tick colonies and the capacity to experimentally infect animals.

3. Serological tests

To minimise the problem of cross-reactions with *Ehrlichia* spp., two enzyme-linked immunosorbent assays (ELISAs) based on a recombinant MAP1 antigen have been developed. The first is an indirect ELISA that uses an immunogenic region of the MAP1 protein (called MAP1-B) and gives far fewer cross-reactions with *Ehrlichia* spp. (MAP1-B ELISA) (Semu *et al.,* 2001). The second is a competitive ELISA that uses the *map*1 gene cloned in a baculovirus and monoclonal antibodies (MAbs) raised against the MAP1 protein (MAP1 C-ELISA) (Mondry *et al.,* 1998). Both tests have dramatically improved specificity, but they still show some reactivity with high titre sera

against *E. canis*, *E. chaffeensis* and Panola Mountain *Ehrlichia*. Cross reaction of serum from Panola Mountain *Ehrlichia*-infected goats has been observed with *E. ruminantium* MAP1-B antigen and, conversely, serum from heartwater-infected sheep with MAP1-B of Panola Mountain *Ehrlichia,* thus preventing their use in the detection of *E. ruminantium* introduction on the American mainland (Sayler *et al.,* 2016). The MAP1-B ELISA has been the most extensively used and will be described in detail. Serology as a diagnostic tool for detecting individual animals exposed specifically to *E. ruminantium* is therefore unreliable. Serology should be considered at the herd level taking into consideration the epidemiological environment and, if necessary, be complemented by molecular techniques.

3.1. MAP1-B enzyme-linked immunosorbent assay (Semu *et al.*, 2001)

Using the vector pQE9, the PCR fragment MAP1-F2R2, which encodes the amino acids 47–152 of the MAP1 protein including the immunogenic region MAP1-B, is expressed in *Escherichia coli* M15[pREP4] as a fusion protein containing six additional histidine residues. The recombinant MAP1-B is purified using Ni^{2+}-NTA agarose (nitrilotriacetic acid agarose) under denaturing conditions as described by the manufacturer. The antigen is preserved at 4°C and each batch is titrated.

The antigen is diluted at 0.5 μg/ml in 0.05 M sodium carbonate buffer, pH 9.6, and immobilised onto polystyrene plates by incubation for 1 hour at 37°C, and stored at 4°C until use. However, in initial trials, an antigen concentration of 2 μg/ml reduced background noise and improved specificity (data not shown: Semu *et al.*, 2001).

3.1.1. Test procedure

i) Plates are blocked for 30 minutes by adding 100 μl per well of 0.1 M phosphate buffered saline (PBS), pH 7.2, supplemented with 0.1% Tween 20 (PBST) and 3% non-fat dry milk (PBSTM).

ii) The plates are washed three times with PBS supplemented with 0.1% Tween 20 (PBST) and twice with distilled water.

iii) 100 μl of test serum diluted 1/100 in PBSTM is added in duplicate to wells, which are then incubated for 1 hour at 37°C.

iv) Plates are washed three times in PBST and twice in distilled water.

v) Horseradish-peroxidase-conjugated anti-species IgG optimally diluted in PBSTM is added at 100 μl per well and the plate is incubated for 1 hour at 37°C.

vi) After washing as in step iv, each well is filled with 100 μl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml orthophenylene-diamine and 3 μl/ml of 9% H_2O_2.

vii) The reaction is stopped after 30 minutes of incubation at room temperature (20–25°C) by adding 50 μl of 2 N H_2SO_4. Absorbance is read at 495 nm. Positive and negative controls are included in each plate.

3.2. MAP1 competitive enzyme-linked immunosorbent assay (Mondry *et al.*, 1998)

Recombinant MAP1 antigen is prepared as follows: 8-day-old *Trichoplusia ni* insect larvae are infected by a baculovirus expressing the *map1* gene and moribund larvae are homogenised (10% [w/v]) in PBS supplemented with 0.001% (v/v) Triton X-100.

Anti-MAP1 MAb is prepared as follows: spleen cells of BALB/C mice previously inoculated with larval homogenate are fused to SP2/0 cells. Supernatant fluids from hybridoma cell cultures are screened for reactivity with MAP1 by immunoblotting and immunoperoxidase methods. A reactive cell culture is subcloned, isotyped and subsequently used for MAb production.

After a further 1/800 (v/v) dilution in PBS, the antigen is immobilised on to polystyrene plates (Nunc-Immuno Plates PolySorp) by incubation overnight at 4°C, and stored at –70°C.

3.2.1. Test procedure

i) Prior to use, the plates are blocked for 30 minutes by adding 100 µl per well of PBS, pH 7.2, supplemented with 0.05% Tween 20 and 5% nonfat dry milk.

ii) Plates are washed three times with PBS/Tween, 50 µl/well of test serum diluted 1/50 in PBS supplemented with 0.05% Tween 20 and 1% nonfat dry milk (PBSTM) is added in duplicate and the plates are incubated for 30 minutes at 37°C.

iii) Without an intervening washing step, 75 µl/well of the MAb diluted 1/4000 (v/v) in PBSTM is added and the plates are incubated for another 30 minutes at 37°C.

iv) Plates are washed three times in PBS/Tween and horseradish-peroxidase-conjugated anti-mouse IgG optimally diluted in PBSTM is added at 50 µl per well. The plate is incubated for 1 hour at 37°C.

v) After three washings as before, 100 µl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml O-phenylene diamine and 3 µl/ml of 9% H_2O_2 are added to each well. After 30 minutes of incubation at room temperature in the dark, the reaction is stopped by adding 50 µl of 2 N H_2SO_4 and the absorbance is read at 495 nm. Positive and negative controls are included in each plate.

3.3. Reading the results

Both the MAP1-B ELISA and the MAP1 C-ELISA have shown a high specificity after evaluation in 3000 ruminant sera (goat, sheep and cattle) collected from 14 *A.-variegatum*-infested islands of the Lesser Antilles, among which only three are known to be infected by *E. ruminantium* (Mondry *et al.*, 1998). Overall specificity calculated from the 11 heartwater-free islands was 98.5% and 99.4% for the MAP1 C-ELISA and the MAP1-B ELISA, respectively. In another study undertaken in the Caribbean, ELISA MAP1-B positive samples were found in four of six islands free of heartwater (Kelly *et al.,* 2011). Moreover, high seroprevalence in vector-free areas of Zimbabwe or South Africa has also been reported although not explained (it may be caused by a cross-reacting agent not transmitted by *Amblyomma*) and should be kept in mind when interpreting the results (Kakono *et al.,* 2003).

Evaluating the sensitivity of the tests is more problematic as it would require knowledge of the exact status of a high number of animals sampled in the field. As mentioned before there is currently no simple technique available to confirm if an animal is infected. Experimentally, the sensitivity of the C-ELISA in goats was reported to be 91.6–95.4% for the MAP1-B ELISA, and 96.3–96.9% for the MAP1 C-ELISA (Mondry *et al.,* 1998). However, in another study the sensitivity averaged 95% for cut-off values set at 31% and 26.6% of the positive control serum for sheep and goat sera, respectively (Mboloi *et al.,* 1999). Indeed, calculations are based on a limited number of experimentally inoculated animals in a period of time soon after inoculation, when almost all the animals are still positive. Sensitivity in cattle is even lower and several reports show that after infection most of the animals become seronegative again in less than 6 months and some animals never seroconvert (Mahan *et al.,* 1998b; Semu *et al.,* 2001). This observation is in line with the difference in antibody prevalence observed between small ruminants and cattle in epidemiological surveys that cannot be explained by a lower risk of infection of the latter. For example, in Zimbabwean farms situated in endemic areas, more than 90% of goats presented antibodies in their serum compared with only 33% of cattle maintained in the same conditions (Mahan *et al.,* 1998b). Similar observations were made in the Caribbean.

Serological tests are useful for the assessment of heartwater antibody response in vaccinated animals. The tests should not be used to screen animals for importation into heartwater-free areas. Antibodies are maintained at detectable levels in naturally infected domestic ruminants for a few months only and circulating antibodies disappear more rapidly in cattle than in small ruminants. It is thus possible that serologically negative animals may be carriers of infection. Serology should therefore only be considered as a diagnostic method to be applied at the herd level and not at the individual animal level (Peter *et al.,* 2001). When interpreting diagnostic serology results, other epidemiological parameters must be considered.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No commercial vaccines are available at present. The only method of immunisation against heartwater remains the "infection and treatment" method using infected blood followed by treatment of reacting animals with tetracycline. This method is still in use in several areas, but it is likely to be replaced by preparations using attenuated or inactivated organisms, which have given promising research results.

1. Inactivated vaccine preparations

Inactivated vaccine based on *E. ruminantium* elementary bodies chemically inactivated or lysed, emulsified in oil adjuvant, conferred good protection against homologous and field challenges (Adakal *et al.,* 2010b; Mahan *et al.,* 1998a; Marcelino *et al.,* 2015a; Martinez *et al.,* 1996). However, it does not prevent vaccinated animals from developing clinical signs, and morbidity is observed after virulent challenge.

The development of a large-scale production process and optimisation of storage conditions for the inactivated vaccine has led to a decrease in the cost of a vaccinal dose to 0.11 euros (Marcelino *et al.,* 2007; Peixoto *et al.,* 2005). In 2015, Marcelino *et al.* developed a ready-to-use inactivated vaccine that could be easily used in the field. It was shown that even after breaking the cold-chain of 3 days at 37°C, mimicking field conditions, the vaccine was still efficient (Marcelino *et al.,* 2015a). This study showed the robustness of the vaccine under field conditions.

In Zimbabwe, field trials of the inactivated vaccine emulsified in oil adjuvant have also demonstrated protection of sheep against natural tick challenge (Mahan *et al.,* 1998a). In larger field trials conducted in eastern and southern Africa, a significant reduction in mortality has been achieved in cattle, goats and sheep using either a prototype strain from Zimbabwe (Mbizi strain) or a local strain from the experimental sites (Mahan *et al.,* 2001). However, in three out of four sites, the vaccine prepared from the local isolate was less effective than the prototype Mbizi vaccine, strongly suggesting an inadequate coverage of the antigenic repertoire of isolates present in each site. Vaccination trials in Burkina Faso showed a significant increase in the protective effect of inactivated vaccine when a local strain was added to the Gardel vaccinal strain (Adakal *et al.,* 2010b).

Lack of cross-protection between *E. ruminantium* isolates due to genetic or antigenic diversity is well established, but the complexity of the *E. ruminantium* population structure in the field has been underestimated. A large *E. ruminantium* genetic diversity has been observed throughout Africa, the Caribbean islands and the Indian Ocean, which raises the problem of the protective effect of the vaccinal strain against field strains (Adakal *et al.,* 2010a; Cangi *et al.,* 2016; Raliniaina *et al.,* 2010). Even if genetic characterisation is defined, there is a lack of a genetic marker associated with protection; moreover, it is essential to isolate *in-vitro* field strains to know their capacity for protection against heterologous strains and be able to mix several strains in the inactivated vaccine in order to cover widely the genetic diversity of field strains.

Inactivated vaccine is being developed commercially in South Africa. These inactivated vaccines do not prevent infection but do prevent or reduce death of vaccinated animals when exposed to live virulent challenge. The advantage however is that several field strains can be incorporated to make the vaccine more widely cross-protective.

A major challenge remains the identification of *E. ruminantium* genetic markers associated with protection in order to identify the vaccinal strains to include in the inactivated vaccine adapted to a region.

2. Attenuated vaccine preparations

Infection of ruminants with live *E. ruminantium* strains induces a strong long-lasting protection against an homologous isolate. This is the basis for the "infection-and-treatment" method using virulent isolates. Isolates of attenuated virulence that do not require the treatment of animals would be ideal, but a limited number of such attenuated isolates are available. An attenuated Senegal isolate has been obtained and shown to confer 100% protection against an homologous lethal challenge, but very poor protection against a heterologous challenge. The Gardel isolate, which gives a significant level of cross-protection with several isolates (although far from complete), has also been attenuated (Marcelino *et al.,* 2015b). A third isolate named Welgevonden from South Africa has been attenuated and shown to confer complete protection against four heterologous isolates under experimental conditions (Zweygarth *et al.*, 2005). However, it has not been tested in field conditions. The main drawback of attenuated vaccines is their extreme lability, which necessitates their storage in liquid nitrogen and their distribution in frozen conditions. In addition, they have to be administered intravenously. Moreover, there is

also a possible reversion to virulence and, as it is a live vaccine, it could not be used in heartwater free areas. Despite the recent efforts to understand the mechanism of virulence and attenuation (Marcelino *et al.,* 2015a), these are still largely unknown independently from the strain.

3. Recombinant vaccine preparations

Several reports show partial protection of mice using *map1* DNA vaccination and an improvement of protection by vaccination following a prime (plasmid) – boost (recombinant MAP1) protocol (Nyika *et al.,* 2002). However, protection of ruminants has never been demonstrated using this strategy. In opposition, significant protection of sheep was reported against homologous and heterologous experimental challenge following plasmid vaccination using a cocktail of four ORFs (open reading frames) from the 1H12 locus in the *E. ruminantium* genome (Collins *et al.,* 2003). No further results have been described since then. Recombinant vaccines will probably not be available in the near future. A prime DNA/boost recombinant protein vaccine has been developed (Pretorius *et al.,* 2008). An efficient protective effect was obtained using a cocktail of four open reading frames (ORFs) against homologous challenge, but the vaccine did not give satisfactory results during field tick challenge. Moreover, simple intramuscular immunisation is not sufficient to induce protection. The use of a gene gun is necessary for prime DNA injection, which is not suitable for a large vaccination campaign. A polymorphic gene was identified as an efficient component of a recombinant vaccine against heartwater using the prime/boost method (Pretorius *et al*., 2010). However, as this gene is polymorphic, a recombinant vaccine should include at least three different genotypes.

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NB: There is a WOAH Reference Laboratory for heartwater (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for heartwater

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.1.10.

JAPANESE ENCEPHALITIS

SUMMARY

Description of the disease: *Japanese encephalitis virus* (*JEV*) *is a member of the genus* Flavivirus *in the family* Flaviviridae *and causes encephalitis, principally in horses and humans. JEV also infects pigs where it causes abortions and stillbirths. JEV is maintained in nature between mosquitoes, pigs and water birds. The major vector of JEV throughout most of Asia is* Culex tritaeniorhynchus, *however other species may be locally important. Pigs act as important amplifiers of the virus, and birds can also be involved in its amplification and spread in the environment. The disease has been observed in large parts of Asia and recently in the western Pacific region. In horses, the infection is usually inapparent. Affected horses show clinical signs that include pyrexia, depression, muscle tremors, and ataxia. In pigs, abortions and stillbirths can occur when pregnant sows are infected with JEV for the first time. Infected pregnant sows usually show no clinical signs.*

Detection of the agent: *For virus isolation, brain material is collected from sick or dead horses that have demonstrated the clinical signs of encephalitis. The virus can be isolated in primary cell cultures made from chicken embryos, porcine or hamster kidney cells and established cell lines such as African green monkey kidney* (*Vero*)*, baby hamster kidney* (*BHK-21*)*, or mosquito* (*C6/36*) *cells. Identification of the virus isolated in tissue cultures is confirmed by serological or nucleic acid detection methods such as reverse-transcription polymerase chain reaction assay.*

Serological tests: *Antibody assay is a useful technique for determining the prevalence of infection in a horse population, and also for diagnosing Japanese encephalitis in diseased individuals. The assay methods include virus neutralisation* (*VN*)*, haemagglutination inhibition* (*HI*)*, complement fixation* (*CF*)*, and enzyme-linked immunosorbent assay* (*ELISA*)*. There is serological cross reactivity with other flaviviruses, such as West Nile virus, which can confuse the diagnosis. The plaque reduction VN test is the most specific and can be used to differentiate JEV infection from other flavivirus infections. Because of the cross-neutralisation within the Japanese encephalitis serocomplex, serological investigations should include related co-circulating flaviviruses tested in parallel.*

Requirements for vaccines: *Two types of vaccines are commercially available in several Asian countries for humans and animals. For horses, inactivated vaccines prepared in cell cultures have been used. For pigs, inactivated and live-attenuated vaccines are available.*

A. INTRODUCTION

Japanese encephalitis (JE) is a disease caused by a mosquito-borne flavivirus that elicits clinical signs of encephalitis in infected humans and horses and can be fatal (Fenner *et al*., 1992; Hoke Jr & Gingrich, 1994; Mansfield *et al.,* 2017). However infections in humans and horses usually result in subclinical infection. JE virus (JEV) also causes reproductive failure in sows, leading to abortion, stillbirths or fetal mummification, though infected pregnant sows usually demonstrate no clinical signs and the infection does not affect the future pregnancies (Williams *et al.,* 2012).

JEV is maintained in nature among mosquitoes, wild birds and pigs (Oliveira *et al.,* 2018). Pigs act as important amplifiers of the virus, and birds can also be involved in its amplification and spread. In pigs, the possibility of vector-free transmission has been described (Ricklin *et al.,* 2016). The principal vector of JEV is *Culex tritaeniorhynchus* in most parts of Asia. Other culicine mosquitoes also play a role as vectors. Because of low titres and short duration of viraemia, humans and horses do not transmit viruses to biting mosquitoes and are considered as dead-end hosts. JEV is widespread in eastern, south-eastern and southern Asian countries and has recently spread to western India

and to the western Pacific region including the eastern Indonesian archipelago, Papua New Guinea and Northern Australia (Mackenzie *et al.*, 2007).

JEV belongs to the genus *Flavivirus* in the family *Flaviviridae*. JEV is the type member of the Japanese encephalitis serocomplex, along with several important zoonotic viruses including West Nile virus (see chapter 3.1.24), St Louis encephalitis virus and Murray Valley encephalitis virus. Only a single serotype of JEV has been identified, although antigenic and genetic differences among JEV strains have been demonstrated by several techniques including complement fixation, haemagglutination inhibition, neutralisation tests using polyclonal or monoclonal antibodies (Ali & Igarashi 1997; Banerjee, 1986; Hale & Lee, 1954; Hasegawa *et al*., 1994; Kimura-Kuroda & Yasui, 1986) and oligonucleotide fingerprints of viral RNA (Banerjee & Ranadive, 1989; Hori *et al*., 1986). Envelope (E) gene analysis was shown to be a good representative of the phylogenetic analysis of JEV. To date, five genotypes of JEV have been described based on phylogenetic analysis of the viral E gene (Schuh *et al.,* 2014; Solomon *et al*., 2003; Uchil & Sachidanandam, 2001; Williams *et al*., 2000).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of Japanese encephalitis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Virus isolation	–	–	–	+++	–	–
Antigen detection	+	+	+	+	+	–
Real-time RT-PCR	++	++	++	+++	++	–
Detection of immune response						
HI	++	+++	++	+++	+++	+++
CFT	+	+	+	+	+	+
ELISA	++	++	++	++	++	++
VN (PRNT)	+	++	+	+++	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; HI = haemagglutination inhibition; CFT = complement fixation test; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation; PRNT: plaque reduction neutralisation test.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

The definitive diagnosis of JE in horses depends on the isolation or detection of the causal virus in neurological specimens. The isolation rate of virus from diseased or dead horses is usually very low, which may be due to the instability of the virus under certain environmental conditions, and also to the presence of antibody in infected animals. Clinical, serological and pathological findings are of assistance in diagnosis. Diagnosis is also possible by the detection of specific IgM and IgG antibodies in cerebrospinal fluid by enzyme-linked immunosorbent assay (ELISA) methods (Burke *et al*., 1982). Viral nucleic acid has been detected in the brain of infected horses by reverse-transcription polymerase chain reaction (RT-PCR) (Lian *et al*., 2002; Lam *et al.,* 2005).

In horses, the specimens collected for virus isolation or detection (nucleic acid or antigen) are portions of the corpus striatum, cortex or thalamus of the brain. Blood and spinal cord samples can also be used. In fetuses, stillborns or neonates of sows, virus may be isolated or detected from brain, tonsils, spleen, liver or placental tissues. All materials should be refrigerated immediately after collection and frozen to –80°C if specimens are to be stored for more than 48 hours. All laboratory manipulations with live cultures or potentially infected/contaminated material

must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*), to prevent the risk of human infection. Humans may be infected by direct contact of infectious material with broken skin or mucous membranes, accidental parenteral inoculation or aerosol. Diagnosticians collecting samples should also take the appropriate precautions. A human vaccine is available and at risk field veterinarians and laboratory workers should be vaccinated.

1. Detection of the agent

Tissue samples are homogenised in a 10% suspension in buffered saline, pH 7.4, containing calf serum (2%) or bovine serum albumin (0.75%), streptomycin (100 μg/ml) and penicillin (100 units/ml). The calf serum should be free from antibodies to JEV. The suspension is centrifuged at 1500 ***g*** for 15 minutes, and the supernatant fluid is removed for testing. Virus isolation in cell culture can use primary cultures of chicken embryo, African green monkey kidney (Vero), baby hamster kidney (BHK) cells, or the C6/36 mosquito cell line (a cloned cell line from *Aedes albopictus*). Homogenates of specimens, such as brain and blood taken from animals suspected of being infected are inoculated onto the cell cultures. Unlike vertebrate cells, JEV does not normally cause cytopathic effect (CPE) in C6/36 cells. Therefore, confirmation may require further culture in vertebrate cells and/or detection of viral antigen or RNA. Monoclonal antibodies specific to flavivirus and JEV can also be used to identify the virus in fixed infected cell monolayers using the indirect fluorescent antibody test (Lian *et al.,* 2002).

To detect JE virus RNA from clinical specimens, from vertebrate cells showing CPE or from mouse brains of infected mice, RT-PCR can also be employed using appropriate primers specific for JEV (Jan *et al*., 2000; Lian *et al*., 2002; Tanaka, 1993; Williams *et al*., 2001). Recently a new nucleic acid detection method, reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of JEV RNA was reported (Parida *et al*., 2006). Other RT-PCR methods have been described for human diagnosis, although there is little published data on nucleic acid detection methods in veterinary applications.

2. Serological tests

Serological tests are useful to determine the prevalence of infection in an animal population, the geographical distribution of the virus, and the degree of antibody production in vaccinated horses and pigs. If serology is to be used for the diagnosis of infection or disease in domestic animals or wildlife, it should be remembered that in an endemic area prior infection with the virus may have occurred. When testing horses and pigs, consideration should also be given to vaccination status when interpreting positive serology results. Maternal antibody can also persist in pigs for up to 8 months. Antibody assay is a useful technique for determining the prevalence of infection in an animal population, and also for diagnosing JE in diseased horses or pigs. The assay methods include virus neutralisation (VN), haemagglutination inhibition (HI), complement fixation (CF), and enzyme-linked immunosorbent assay (ELISA). Diagnosis requires a significant rise in antibody titre in paired sera collected during the acute and convalescent phases (for example a four-fold rise in VN titre). The specificity of each serological test should also be considered. A latex agglutination test to detect swine antibodies to Japanese encephalitis has recently been described (Xinglin et al., 2002). An ELISA for antibodies to a nonstructural protein (NS1) of JEV can be used to differentiate antibodies following natural infection from those induced by inactivated vaccines.

In some regions of the world, there is a need to carry out additional tests for related viruses before an unequivocal diagnosis of Japanese encephalitis can be made. For example, in Australia Murray Valley encephalitis and West Nile-Kunjin virus occur; these viruses are members of the JEV serocomplex and are antigenically closely related to JEV. Recent expansion of the distribution of West Nile virus in North America, where St Louis encephalitis virus was known to be endemic, further illustrates the flexibility of flaviviruses to adapt to new environments. The presence of antibody to these other flaviviruses can make serological diagnosis of Japanese encephalitis difficult. There is some cross reactivity with other flaviviruses on all the tests; the plaque reduction VN test is the most specific, especially if a 90% neutralisation threshold is used.

2.1. Virus neutralisation (plaque reduction neutralisation test)

2.1.1. Cell culture

African green monkey-derived Vero cells (ATCC No. CCL-81) are recommended to propagate viruses and for use in the plaque reduction neutralisation test (PRNT).

The Vero cells are cultured in a complete alpha minimal essential medium (α-MEM) supplemented with fetal bovine serum (FBS) and antibiotics. To prepare the cells for PRNT in a 24-well format, use the following protocol:

i) Verify that the Vero cells are in the log phase (approximately 2×10^7 cells in 175 cm^2 flask) with greater than 95% viability.

ii) Add 1.0×10^4 cells ~ 5×10^4 cells to each well of the plate; the cells are maintained at 37°C in a 5% CO_2 incubator for 2 or 3 days.

A confluent monolayer of cells should be prepared 2–3 days before the course of the assay, because cell monolayer is critical for plaque forming and to evaluate accurate results.

2.1.2. Virus strain and propagation

The JEV strain (Nakayama, JaGar-01 or appropriate JEV strain) is used for PRNT.

The conditions for virus preparation should be standardised with the use of an appropriate multiplicity of infection (MOI: 10^{-2} to 10^{-3}).

2.1.3. Reagents

i) α-MEM supplemented with 2–5% FBS and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). For the PRNT, a low final concentration of FBS ranging from 2 to 5% should be used for virus and cell growth, and for dilution of samples.

ii) 4% agarose stock solution (dissolved in distilled water). To prepare overlay medium for plaque formation, agarose solutions are typically used at 1–2% final concentrations.

iii) 0.1% neutral red solution. To visualise the plaque, a vital dye such as neutral red is added to the overlay medium. Neutral red is cytotoxic at high concentrations and light sensitive, therefore a low dye concentration is recommended. Dissolve the neutral red powder in distilled water at a concentration of 0.1 % (w/v) and, after autoclave, store the solution in light-tight container at 4°C until use.

2.1.4. Preparation of overlay medium

The composition of the first overlay medium:

Reagents	Amount
α-MEM containing antibiotics	14 ml
FBS (5% final concentration)	1 ml
4% agarose stock solution	5 ml
Total volume	20 ml

The composition of the second overlay medium:

Reagents	Amount
α-MEM containing antibiotics	12.8 ml
FBS (5% final concentration)	1 ml
4% agarose stock solution	5 ml
0.1% neutral red solution	1.2 ml
Total volume	20 ml

All amounts and volumes are given on a 24-well plate format. Combine reagents immediately prior to use. Be sure to keep the overlay medium at 42°C prior to adding to wells.

2.1.5. Viral plaque assay

To achieve accurate measurement, the appropriate virus dose for challenge should be determined before performing PRNT. Therefore, the target number of plaques can be determined by viral plaque assay.

For viruses belong to *Flaviviridae*, a two-overlay method is mostly used for this assay.

Test procedure (24-well plate format)

i) Prepare 90–100% confluent monolayer cells in a 24-well format.

ii) Prepare a 7-log serial dilution (10^{-1} to 10^{-7}) of the clarified JEV stock in a complete α-MEM. To do this, sequentially dilute 0.2 ml of the viral stock in 1.8 ml of medium in microtube.

iii) After labelling the plates, discard the medium from each well and immediately replace with 0.1 ml of the appropriate virus dilution. As a negative control, add a complete medium without virus.

iv) Incubate the cells with virus for 1 hour at 37°C in a CO_2 incubator.

v) Following 1 hour's incubation, discard medium containing virus from the wells and replace with 0.5 ml of agarose containing first overlay medium.

vi) Allow agarose overlay to be hardened for 1 hour at room temperature, and incubate the plates upside down to minimise water condensation in the wells in a 37°C incubator for 48 hours to allow virus plaques to develop.

vii) Add 0.5 ml of the second overlay medium containing 0.1% neutral red to each well and allow agarose overlay to be harden for 1 hour in the light-tight incubator.

viii) Incubate the plates upside down in a CO_2 incubator at 37°C for 48 hours to allow the cells to be maximally stained.

ix) Count the plaques by naked eyes and calculate the titre of viral stock. The titre can be calculated by using the following formula.

Titre (plaque-forming units [PFU]/ml) = number of plaques × dilution factor × 1 ml of inoculum per well.

2.1.6. PRNT for JEV antiserum

Test procedure

i) Prepare 90–100% confluent monolayer cells in a 24-well format.

ii) Prepare serial two-fold or four-fold dilutions of test sera and positive and negative control sera. The test sera should be heat-inactivated at 56°C for 30 minutes before the course of the assay. All test sera for assay should be initially ten-fold diluted by a complete α-MEM prior to making two-fold diluents.

Test group allotted by serum type:

a) Sera collected from unvaccinated pigs and horses;

b) Sera collected from vaccinated pigs and horses;

c) Immuno-positive swine or equine serum against JEV;

d) Negative serum: FBS or immuno-negative swine serum or equine against JEV.

iii) Prepare 200 PFU/0.1 ml of virus dilutions. The virus plaque dose in diluent should be previously determined by the viral plaque assay. 20 PFU/0.1 ml of virus dilution should be also prepared for the comparison (used as cut off value of virus titre).

iv) Add an equal volume of the serum dilution to the diluted virus stock for a final virus concentration of approximately 100 PFU/0.2 ml. In case of 20 PFU/0.1 ml of virus dilution, the final concentration of virus stock will be at 10 PFU/0.2 ml.

v) Incubate the plate containing the mixture of serum and virus for 1 hour at 37°C. After incubation, transfer 0.1 ml of the mixture to the cell.

vi) Following 1 hour's incubation, discard medium containing virus from the wells and replace with 0.5 ml of agarose containing first overlay medium.

vii) Allow agarose overlay to harden for 1 hour at room temperature, and incubate the plates upside down to minimise water condensation in the wells in a CO_2 incubator at 37°C for 48 hours to allow virus plaques to develop.

viii) Add 0.5 ml of the second overlay medium containing 0.1% neutral red to each well and allow agarose overlay to harden for 1 hour in the light-tight incubator.

ix) Incubate the plates upside down in a CO_2 incubator at 37°C for 48 hours to allow the cells to be maximally stained.

x) Count the plaques by naked eyes and calculate the titre of viral stock.

xi) Calculate the average number of plaques in the serum-free control wells and determine the pfu threshold at 50% and 90% reduction levels: 50% reduction = 0.5 × pfu/well (no serum) 90% reduction = 0.1 × pfu/well (no serum)

xii) Calculate the 50% and 90% end-point titre for the test sera, being the dilution of serum closest to the reduction level relative to average plaque number in the serum free control wells.

2.2. Haemagglutination inhibition

The HI test is widely used for the diagnosis of Japanese encephalitis, but has cross-reactivity with other flaviviruses. For this test, the sera must first be treated with acetone or kaolin, and then adsorbed with homotypic RBCs to remove any nonspecific haemagglutinins in the test sera. The RBCs of geese or of 1-day-old chickens are used at the optimum pH (see the table below). The optimal pH is dependent on the JEV strain used. The test should be conducted with the treated sera and 8 units of standard antigen; this is commercially available in some countries.

2.2.1. Haemagglutination (HA)

i) Preparation of virus antigen

1. Sucrose–acetone extraction of antigen from infected suckling mouse brains (SMB)

a) Homogenise infected SMB with 4 volumes of 8.5% sucrose.

b) Add the homogenate drop-wise to 20 times its volume of cold acetone.

c) Centrifuge (500 ***g*** for 5 minutes), then remove the supernatant.

d) Resuspend the sediment with the same volume as above of cold acetone, and keep in an ice bath for 1 hour.

e) Centrifuge (500 ***g*** for 5 minutes), then remove the supernatant.

f) Pool the sediment with cold acetone in a single tube.

g) Centrifuge (500 ***g*** for 5 minutes), then remove the supernatant.

h) Spread the sediment inside the tube and vacuum dry for 1–2 hours.

i) Dissolve the dry sediment with saline: 0.4 volume of original homogenate.

j) Centrifuge (8000 ***g*** for 1 hour, 4°C). The supernatant is ready for use.

ii) Preparation of goose red blood cells

1. Solutions

a) Acid-citrate-dextrose (ACD)

11.26 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$); 4.0 g citric acid ($H_3C_6H_5O_7.H_2O$); 11.0 g dextrose ($C_6H_{12}O_6$); distilled water to a final volume of 500 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes.

b) Dextrose-gelatine-veronal (DGV)

0.58 g veronal (Barbital); 0.60 g gelatine; 0.38 g sodium veronal (sodium barbital); 0.02 g (0.026 g) $CaCl_2$ (for $CaCl_2.2H_2O$); 0.12 g $MgSO_4.7H_2O$; 8.50 g NaCl; 10.0 g dextrose; distilled water to a final volume of 1000 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes (five times stock volume is easier to prepare).

2. Blood collection

1.5 ml of ACD + 8.5 ml of blood (0.5 ml of ACD + 2.8 ml of blood).

3. Washing (sterile)

a) Total blood + 2.5 volume of DGV. Centrifuge (500 ***g*** for 15 minutes), then remove the supernatant.

b) Resuspend the sedimented RBCs in three volumes (total blood) of DGV.

c) Centrifuge (500 ***g*** for 15 minutes), then remove the supernatant. Repeat steps 2 and 3 twice more (total four spin cycles).

d) Transfer the final RBC suspension to a flask with aluminium foil cover.

4. Adjusting the RBC concentration

a) 0.2 ml of the RBC suspension + 7.8 ml of 0.9% NaCl (1/40 dilution).

b) Read the optical density $(OD)_{490}$ in a spectrophotometer with 10 mm tube.

c) Adjust the RBC stock so that 1/40 dilution gives 0.450 of OD_{490}. (Final volume = Initial volume × absorbance OD_{490}/0.450.)

d) Store the RBC stock in a refrigerator for up to 1 week.

e) Before use, resuspend the RBCs gently and dilute 1/24 in virus-adjusting diluent (VAD).

iii) Antigen dilution

1. Stock solutions (should be kept at 4°C)

a) 1.5 M NaCl

87.7 g NaCl and distilled water to a final volume of 1000 ml.

b) 0.5 M boric acid

30.92 g H_3BO_3 and hot distilled water to a final volume of 700 ml (dissolve boric acid and cool down).

c) 1 N NaOH

40.0 g NaOH and distilled water to a final volume of 1000 ml.

d) Borate saline (BS), pH 9.0

80 ml 1.5 M NaCl, 100 ml 0.5 M H_3BO_3, 24 ml 1.0 N NaOH, and distilled water to a final volume of 1000 ml.

e) 4% bovine albumin

4 g bovine albumin fraction V, 90 ml BS, pH 9.0, adjust pH to 9.0 with 1 N NaOH, and BS, pH 9.0, to make a final volume of 1000 ml.

2. Antigen diluent

0.4% bovine albumin/borate saline (BABS): 10 ml 4% bovine albumin, pH 9.0, and 90 ml BS, pH 9.0.

3. Serial dilution

Two-fold serial dilution of antigen with BABS on U-bottom microtitre plate.

iv) Addition of goose red blood cells

1. Stock solutions

1.5 M NaCl

0.5 M Na_2HPO_4: 70.99 g Na_2HPO_4 (for Na_2HPO_4, 12 H_2O: 179.08 g), and distilled water to a final volume of 1000 ml.

1.0 M NaH_2PO_4: 138.01 g $NaH_2PO_4.H_2O$ (for NaH_2PO_4, $2H_2O$: 156.01 g), and distilled water to a final volume of 1000 ml.

2. Working solution: virus adjusting diluent (VAD)

VAD (pH)	1.5 M NaCl	0.5 M Na_2HPO_4	1.0 M NaH_2PO_4	
6.0	100	32	184	
6.2	100	62	160	Add distilled
6.4	100	112	144	water to a
6.6	100	160	120	final volume of
6.8	100	192	104	1000 ml
7.0	100	240	80	

Values of VADs are not the pH of each VAD, but the pH after each VAD is mixed with an equal volume of BABS, pH 9.0.

3. Procedures

a) 1 volume of stock goose RBCs + 23 volumes of VAD (1/24 dilution).

b) Add 25 µl of diluted RBCs to each well on microtitre plate containing diluted antigen (25 µl/well).

c) Incubate at 37°C for 30 minutes, then read the result.

++ Complete agglutination (uniformly thin pellicle of RBCs following the curvature of the well bottom)

\+ Partial agglutination (a ring associated with a rough or thinner pellicle)

± Minimal agglutination (a button on a thin or scattered pellicle)

– Negative agglutination (clearly defined button with no RBC film)

End point is the last dilution (highest dilution) in which ++ or + is observed.

Titre: the reciprocal of the end point dilution.

2.2.2. Haemagglutination inhibition

i) Preparation of test sera

1. Blood collection and separation of the sera

a) Incubate blood specimen at 37°C for 1 hour and then at 4°C overnight. If the test must be performed immediately, incubating the sample for 2–3 hours at 37°C can replace the overnight incubation.

b) Centrifuge (2000 ***g*** for 15 minutes) to separate the serum from the clot.

c) Heat inactivate at 56°C for 30 minutes.

d) Store at –20°C if not processed immediately.

2. 2-mercaptoethanol treatment (perform this step when IgM antibody titres should be determined)

a) Place 50 µl of the sera into two small test tubes.

b) Add 150 µl of 0.13 M 2-mercaptoethanol in PBS into one test tube, and 15 µl PBS into another tube.

c) Incubate at 37°C for 1 hour, then cool in an ice bath.

3. Acetone extraction

a) Add 2.5 ml of cold acetone to serum in a test tube. Cap with rubber stoppers, mix well and extract for 5 minutes in an ice bath.

b) Centrifuge cold (1500 ***g*** for 5 minutes), then remove the supernatant.

c) Repeat steps i and ii once more.

d) Spread the sediment inside tubes and vacuum dry at room temperature for 1 hour.

e) Add 0.5 ml of BS, pH 9.0, to each tube. Apply rubber stoppers. Dissolve the sediment overnight at 4°C to make 1/10 dilution of the sera.

4. Kaolin extraction as an alternative to acetone extraction

a) 25% acid-washed kaolin in BS, pH 9.0.

b) 1 volume of sera + 4 volumes of BS + 5 volumes of 25 % kaolin.

c) Extract at room temperature for 20 minutes with occasional shaking.

d) Centrifuge (1000 ***g*** for 30 minutes). The supernatant is 1/10 dilution of the sera.

5. Adsorption with goose RBCs

a) To each treated serum add 1/50 volume of packed goose RBCs.

b) Adsorb for 20 minutes in an ice bath.

c) Centrifuge (800 ***g*** for 10 minutes). The supernatant is ready for the HI test (1/10 dilution).

ii) Haemagglutination inhibition test

1. Primary haemagglutination titration of antigen

Dilute the antigen to make 8 units/50 µl.

2. Serial two-fold dilution of test sera on microtitre plate

a) Serum–antigen reaction

Add 25 µl of diluted antigen into each well containing diluted test sera. Place the remainder of the antigen in empty wells and incubate at 4°C overnight or 1 hour at 37°C.

3. Secondary haemagglutination titration of the antigen

a) Serially dilute the prepared antigen (8 units/50 µl) two-fold in a 25 µl system.

b) Add 25 µl of BABS to each well to make 50 µl/well.

4. Addition of goose RBCs

a) Dilute RBC stock (1/24) in VAD.

b) Distribute 50 µl into each well containing 50 µl of serum antigen mixture or secondary titration of antigen.

c) Incubate at 37°C for 30 minutes then read the result.

Serum HI titre: the reciprocal of the highest dilution of the test sera showing complete inhibition of HA.

5. Interpretation of the results

Four-fold difference between the titre in the acute and convalescent sera is considered to be a significant rise or fall and is diagnostic of infection with a virus antigenically related to that used in the test.

2.3. Complement fixation

Complement fixation (CF) is sometimes used for serological diagnosis. The antigen for this test is extracted with acetone/ether from the brains of inoculated mice.

2.3.1. Antigen preparation

i) Extract and weigh the brains of the inoculated dead mice.

ii) Add to the brains 20 volumes of cold acetone, kept at –20°C, and homogenise.

iii) Centrifuge the suspension at 5000 ***g*** for 5 minutes at 4°C, and remove the supernatant.

iv) Add to the pellet the same volume of cold acetone as used in step ii above, and mix well.

v) Extract with acetone by keeping the pellet at –20°C for 20 minutes, and repeat the centrifugation described in step iii above.

vi) Repeat steps iv and v.

vii) Repeat steps iv and v, but this time use cold acetone/ether (equal volume mixture).

viii) Repeat steps iv and v twice using cold ether.

ix) Remove the supernatant by aspirator and spread the pellet over the centrifuge tube.

x) Vacuum dry for 1–2 hours.

xi) Dissolve the pellet in cold saline (2 ml/g of brain) and keep at 4°C overnight.

xii) Centrifuge at 5000 ***g*** for 1 hour. The supernatant is the antigen.

2.3.2. Test procedure

i) Heat-inactivate the test sera at 1/4 dilution in gelatin–veronal buffer.

ii) Serially dilute the sera two-fold in a 96-well microtitre plate (25 µl).

iii) Add 25 µl of 4 units of antigen and mix by vibration.

iv) Add 50 µl of 2 units of complement (pooled fresh guinea-pig serum).

v) Mix by vibration and incubate at 4°C for 18 hours.

vi) Leave the microtitre plate at room temperature for 15 minutes.

vii) Add 25 µl of sensitised sheep RBCs to each well.

viii) Mix by vibration and incubate at 37°C for 30 minutes, then read the result.

ix) The highest dilution of test sera showing no haemolysis is the titre of the sera by CF test. A rise or drop of four-fold or more in the titre is considered to be significant.

2.4. Enzyme-linked immunosorbent assay

Various ELISA formats have been used to detect antibodies to JEV in horses and pigs. An epitope-blocking ELISA using a JEV-specific monoclonal antibody has been reported that can detect IgG in pigs (Pant *et al.,* 2006; Williams *et al.,* 2001) and horses (Lam *et al.,* 2005), although antibodies to closely-related flaviviruses can still cross-react). An IgM capture ELISA has been reported for testing pig sera (Pant *et al.,* 2006). An indirect ELISA for prevalence studies of JEV antibody in pigs has also been described (Yang *et al.,* 2006). These assays have been used for sero-prevalence studies and for diagnostic investigations. Conventional serological methods cannot differentiate antibodies induced by natural infection from those induced by vaccination. To detect antibodies induced by natural infections but not those induced by inactivated vaccines, an ELISA method that detects antibodies against non-structural 1 (NS1) protein of JEV, which is induced only by infection, has been developed (Konishi *et al.,* 2004).

C. REQUIREMENTS FOR VACCINES

1. Background

Two types of vaccines are commercially available in several Asian countries for humans and animals. For humans, inactivated vaccines prepared from infected mouse brains have been used for many years, but because of safety concerns, these have largely been replaced by cell culture-derived live-attenuated or inactivated vaccines. A live attenuated vaccine grown in hamster kidney cells has been widely used in China (People's Rep. of). An inactivated vaccine derived from Vero cell culture was licensed in 2009 in several countries. A recombinant live attenuated vaccine based on the yellow fever virus 17D vaccine strain and comprising JEV structural proteins was licenced for use in 2012 in Thailand and Australia.

The vaccine for the prevention of Japanese encephalitis in horses is prepared by formalin-inactivation of a virus suspension derived from cell cultures. For pigs, both inactivated and live-attenuated vaccines derived from cell cultures are used.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1.1. Rationale and intended use of the product

Inactivated vaccines have been used to protect horses from encephalitis and possible subsequent death caused by JEV infection. In pigs, both inactivated and live attenuated vaccines have been used to protect pregnant sows from stillbirth.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Beijing-1 and Nakayama strains of JEV have been used for production of inactivated JE vaccines for horses and pigs. Other JEV strains may also be used. The Anyang 300 and at222 strains have been used as live-attenuated vaccines in pigs and horses. The virus strains of inactivated vaccines must be lethal for 3-week-old mice within 14 days when inoculated intraperitoneally, and must be able to grow in a primary culture of porcine kidney or susceptible cell lines. SA-14-14-2, S^{-} Anyang 300 and sometimes other JEV strains are used (Fugisaki *et al.,* 1975). The virus strain for live attenuated vaccine must be lethal for 2- or 3-day-old mice when inoculated intracerebrally, but there must be no viraemia when inoculated in 1-month-old piglets and it must not infect fetuses when inoculated into pregnant sows during the first month of gestation. Attenuated JE vaccine strains are also able to grow in a primary culture of porcine kidney or susceptible cell lines. JE vaccine strains have the capacity to haemagglutinate the RBCs of geese, 1-day-old chickens or pigeons. The viruses must be able to be neutralised by a standard antiserum to JEV.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The seed virus must be free of contaminating bacteria, fungi, mycoplasmas and viruses. Tests for sterility and freedom from contamination of biological materials intended for veterinary use are found in chapter 1.1.9.

2.1.3. Validation as a vaccine strain

Although there are five genotypes of JEV based on nucleotide squence analysis, there is only one serotype of JEV (Tsarev *et al.,* 2000; Wills *et al.,* 1992). The JEV vaccine strain should have the genetic and biological properties mentioned in Section B.1. *Identification of the agent*.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic

If a new strain that is antigenically district from existing vaccine strains occurs, it may be necessary to develop a new vaccine strain from a representative field isolate. Before the new MSV

can be accepted, the biological characterisation of the MSV should be demonstrated in accordance with Section C.2.1.1.

In emergency situations where there is insufficient time to complete full testing of the MSV, provisional acceptance of a new strain should be based on a risk analysis of the possibility of contamination of the antigen. This risk assessment should include the inactivation of the antigen.

2.2. Method of manufacture

2.2.1. Procedure

The seed virus is inoculated into susceptible cell culture and the fluids are later harvested separately from each batch when virus replication is at its maximum. This fluid is filtered, or centrifuged at 1500 ***g*** for 30 minutes, and the supernatant fluid is processed as the virus suspension.

For inactivated vaccine, formalin (0.2%) or BEI (binary ethylenimine, 0.05 M) is added to the suspension to inactivate any live virus; this is considered to be the 'undiluted virus suspension'. Adjuvant may be added to enhance its immunogenicity.

For live attenuated vaccine, the fluid mixed with stabiliser is dispensed into vaccine bottles and lyophilised.

The passage levels of vaccine strain should not exceed three more than the original virus and two more than the seed virus. It is recommended that the original and seed viruses be maintained below –70°C, or below 5°C after lyophilisation.

2.2.2. Requirements for ingredients

Formalin or BEI is added to a virus suspension to inactivate the vaccine. The time and temperature of inactivation must be validated to demonstrate the inactivation kinetics.

2.2.3. In-process control

The virus suspension should be examined for bacterial and fungal contamination by culture techniques and for virus infectivity by inoculation into cell cultures. The inactivated undiluted virus suspension should be re-examined for contamination by cell culture and by microscopy after staining, to ensure complete inactivation of the virus by the formalin or BEI.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Final product of live attenuated JE vaccine is neutralised for 1 hour by mixing with the same amount of standard JEV antiserum. The mixed solution is inoculated into susceptible cells and observed for 5 days; the cells must not show any cytopathic effect (CPE) or haemagglutination in goose erythrocytes.

iv) Batch potency

Inactivated vaccine: The product is diluted 1/10 in PBS. Thirty mice aged 2–3 weeks are inoculated intraperitoneally with 0.1 ml of the diluted product twice at 3-day intervals. There should be an equivalent uninoculated control group. Ten mice of each group are challenged intraperitoneally with ten-fold dilutions (1/10, 1/100 and 1/1000) of the appropriate virus such as Nakayama strain 8 days following the first inoculation, and observed for 14 days. The survival rate should be more than 40% in the immunised group and the mortality rate in the control group should be more than 90%. The titre of challenge virus should not be less than 10^3 LD_{50} (50% lethal dose) per 0.2 ml.

Live attenuated vaccine for pigs: 1-month old piglets that are negative for antibody to JEV are used. Three piglets are inoculated with one dose of final product and one pig is used as a control. The pigs used for the potency test should be observed for 3 weeks and free from abnormalities. The blood of pigs used for the potency test is collected at 3 weeks and subjected to the HI test and PRNT. HI and PRNT titre in pigs inoculated with the final product should be over 1:20, but in the control pig should be less than 1:10.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For vaccine registration, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and Section C.2.*2 Method of manufacture*) should be submitted to the Authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

1-month-old piglets inoculated with two doses of live attenuated vaccine should show no clinical signs, and fetuses should not be infected when pregnant sows are inoculated with the same dose in the first month of gestation. For inactivated vaccine, ten 3-week-old mice are inoculated intracerebrally with 0.03 ml of the product and no death must be observed after 14 days.

i) Target and non-target animal safety

The live attenuated vaccine for pigs should be safe in pregnant pigs. The safety test for the inactivated vaccine is carried out in 3-week-old mice.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Ten 3-week-old mice are inoculated intraperitoneally with 0.3 ml dose of the live attenuated vaccine and observed for 14 days. The mortality rate of the mice should not exceed 20%.

iii) Precautions (hazards)

Vaccine should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger. Persons involved in vaccine production and vaccination should be vaccinated with a JEV vaccine for humans.

2.3.3. Efficacy requirements

As JEV is maintained among vector mosquitoes, pigs and wild birds, control and eradication of JEV using vaccines is difficult. Vaccines are used to protect horses from encephalitis and pregnant sows from stillbirths.

Methods for potency testing of JEV vaccine for animals have been reported. Efficacy tests for both inactivated and live attenuated vaccines are to be followed as mentioned in Section C.2.2.4 *Final product batch tests*, iv) *Batch potency*.

2.3.4. Method for distinguishing attenuated JEV vaccine strain from wild JEV

Virus isolate from naturally infected or vaccinated animals is inoculated into 3-week-old mice to confirm pathogenicity. If more than 20% of mice inoculated intraperitoneally with 0.3 ml die, the isolate is considered to be pathogenic. In addition, if colostrum-deficient piglets at 3–5 days of age are inoculated subcutaneously with 1 ml of the isolate and exhibit severe encephalitis, it is confirmed as pathogenic JEV. PCR and sequencing may also be performed to confirm the identity of virus isolate by comparison with reference JEV gene or genomes sequences (see Section 1).

2.3.5. Duration of immunity

The immune responses following vaccination have been investigated and appear to last over 1 year. As part of the registration/licensing procedure, the manufacturer should be required to demonstrate the duration of immunity of a given vaccine by measuring neutralising antibody at the end of the claimed period of protection.

2.3.6. Stability

When stored under the recommended conditions, the final product should maintain its potency for at least the designated shelf life of the product.

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* *

NB: There is a WOAH Reference Laboratory for Japanese encephalitis
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratory for any further information on
diagnostic tests, reagents and vaccines for Japanese encephalitis

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.1.11.

LEISHMANIOSIS

SUMMARY

Description and importance of the disease: *Leishmaniosis is not a single entity but comprises a variety of syndromes caused primarily by at least* 20 Leishmania *species affecting humans, transmitted by phlebotomine sand flies belonging to the genera* Phlebotomus *and* Lutzomyia*. Leishmaniosis is associated with and limited by the geographic distribution of its sand fly vectors. Fifteen nosogeographical entities have been classified worldwide, of which 13 have an established or putative zoonotic nature. In recent years, the number of regions becoming* Leishmania*-endemic has grown significantly, accompanied by an increased number of animal and human cases.*

In humans, the clinical spectrum ranges from asymptomatic infections to those with high mortality, with three distinct forms being classically described: visceral (*VL*)*, cutaneous* (*CL*) *and mucocutaneous* (*MCL*)*. Dogs are commonly affected by* L. infantum *and* L. chagasi (*now regarded as synonyms*)*, which causes a chronic viscero-cutaneous disease in this host* (*canine leishmaniosis, CanL*)*. Asymptomatic infection in dogs is widespread and contributes to maintaining the long-term presence of the parasite in endemic regions. The clinical appearance and evolution of leishmaniosis is a consequence of complex interactions between the parasite and host immune response. The outcome of infection depends on the ability of host macrophages to effectively destroy the parasite.*

Detection of the agent: *When clinical signs and characteristic lesions are present in affected humans and animals, the demonstration of the parasites in stained smears of splenic, bone marrow and lymph node aspirates, of skin scrapings, and in tissue biopsies, gives a positive diagnosis. If the infection is low grade, detection of parasites is possible only by attempting* in-vitro *isolation or by polymerase chain reaction* (*PCR*)*. As there are very few morphological differences among various species, the identification of any isolated* Leishmania *organism relies on biochemical and/or molecular methods. Several centres throughout the world are presently using isoenzyme and DNA characterisation to identify the agent.*

Serological tests: *Serology is the preferred method for diagnosis of VL and CanL, even during the early stages of the disease. In subclinical forms, seropositive cases are confirmed by parasitological diagnosis or PCR. Serology is of less value for CL and MCL. Of the several serological techniques available, the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay are the most suitable. Crude antigens for serodiagnostic tests can be prepared in the laboratory from cultured parasites. Tests based on recombinant antigens may also be used and have a high specificity although can be less sensitive.*

The rapid immunochromatographic assay is easy to carry out and can be performed in veterinary clinics, but has lower diagnostic efficiency than the indirect fluorescent antibody test (*IFAT*) *and enzyme-linked immunosorbent assay* (*ELISA*)*.*

Requirements for vaccines: *There is no effective vaccine available worldwide at present for use in dogs or humans. A number of vaccines for use in animals are under evaluation, and four have been authorised for use in dogs* (*two in Brazil and two in Europe*)*. Beyond other issues to be evaluated, the use of these vaccines is posing present and future challenges to the fields of diagnosis, epidemiology and surveillance of the parasite, especially in countries where the parasite does not occur. Leishmanin skin test antigen is no longer available worldwide and lacks standardisation.*

A. INTRODUCTION

Description of the disease: Leishmaniosis comprises a variety of syndromes caused by members of the protozoan parasite *Leishmania* (Kinetoplastida: Trypanosomatidae), which are transmitted to mammal hosts by the bite of

infected phlebotomine sand flies belonging to the genera *Phlebotomus* (Old World)[1] and *Lutzomyia* (New World). The disease results from the multiplication of amastigote forms in macrophages of the reticuloendothelial system. The epidemiology and clinical manifestations of the diseases are largely diverse, being usually grouped into two main entities: zoonotic leishmanioses, where domestic or wild animal reservoirs are involved in the transmission cycle and humans play a role as an accidental host, and anthroponotic leishmanioses, where humans are the sole reservoir and source of the vector's infection (Esch & Petersen, 2013). T lymphocytes and cytokines, play a crucial role in determining whether infection evolves toward a protective immune status or a progressive and manifest disease. In inbred mouse models, two different T helper (Th) cell subsets are involved, termed Th1 and Th2, which differ in their profile of secreted cytokines by a polarised activation; the Th1 response confers protection, whereas the Th2 response renders the host susceptible to infection. However, in human patients and dogs with clinically apparent infections, Th1 and Th2 type responses are not characteristically polarised, as both activating (e.g. interferon gamma, interleukin 12) and suppressive cytokines (e.g. interleukin 10, interleukin 13, interleukin 4, TGF beta) are detected (Murray *et al*., 2005).

Various forms of clinical manifestations of human leishmaniosis have been described and divided into three major clinical entities: visceral leishmaniosis (VL, kala azar), cutaneous leishmaniosis (localised, diffuse or disseminated CL, oriental sore, uta, pian bois, chiclero's ulcer) and mucocutaneous leishmaniosis (MCL, espundia) (World Health Organization [WHO], 2010). The diseases are mainly zoonoses with two exceptions, that of CL due to *L. tropica* in urban areas of the Middle East, and that of VL due to *L. donovani* in the Indian sub-continent (northern India, Nepal and Bangladesh) and in some parts of Eastern Africa (e.g. Ethiopia and Sudan). Canine leishmaniosis (CanL) is a chronic viscero-cutaneous disease caused by *L. infantum*, for which the dog acts as the source reservoir. Resistant asymptomatic dogs may exceed >50% of the infected canine population. Typical external signs recorded in susceptible dogs that evolve towards full-blown disease are lymph node enlargement, weight loss, exfoliative dermatitis, onychogryphosis, alopecia, ulcers and ocular alterations. Feline leishmaniosis (FeL) caused by *L. infantum* appears to be an emerging feline disease; in the past two decades it has been more and more frequently reported in endemic areas and sporadically also seen in non-endemic areas in re-homed cats (Pennisi *et al.,* 2015; Richter *et al*., 2014; Rüfenacht *et al*., 2005). Sporadic tegumentary cases have been reported in equids and cattle (Gramiccia *et al.,* 2011). Likewise, wild canid (wolf, fox and jackal) populations can show similar infection rates as in dogs, but the prevalence of progressive clinical signs in these species is substantially lower and thus they may not be as infectious as dog populations to supporting a primary transmission cycle. In an outbreak of visceral leishmaniosis in Spain, hares (*Lepus granatensis*) and rabbits (*Oryctolagus cuniculus*) were described as a reservoir (Jimenez *et al.,* 2014; Molina *et al.,* 2012).

Causal pathogen: Approximately 20 recognised *Leishmania* species are agents of human leishmanioses. In the New World, tegumentary forms are caused by *L. braziliensis*, *L. guyanensis*, *L. panamenisis*, *L. shawi*, *L. naïffi, L. lainsoni*, *L. lindenbergi*, *L. peruviana*, *L. mexicana*, *L. venezuelensis* and *L. amazonensis*; visceral and, more rarely, cutaneous forms, are caused by *L. infantum*. In the Old World, cutaneous forms are caused by *L. tropica*, *L. major* and *L. aethiopica*; visceral and, more rarely, cutaneous forms, are caused by *L. infantum* and *L. donovani*. *Leishmania infantum* and *L. chagasi* have been found to be identical by genotyping and should be regarded as synonyms (Kuhls *et al*., 2011). In addition, the taxonomic position of other *Leishmania* agents of tegumentary forms (*L. killicki* in the Old World; *L. pifanoi*, *L. garnhami* and *L. colombiensis* in the New World) is still under discussion. Other *Leishmania* species not pathogenic to humans include New and Old World rodent parasites and an agent of cutaneous leishmaniosis in Australian macropods (Dougall *et al*., 2009). Dogs are mainly affected by *L. infantum*, but, other *Leishmania* species are found in dogs in the New World (*Leishmania amazonensis, L. braziliensis, L. mexicana, L. venezuelensis*) (Pennisi *et al.,* 2015; Solano-Gallego *et al.,* 2009). *Leishmania tropica* and *L .major* are rarely reported in dogs and are found mainly associated with skin or mucocutaneous lesions (Baneth *et al.,* 2017).

Zoonotic risk and biosafety requirements: Direct contact with infected hosts or handling of biological samples and parasite cultures from these hosts is of low risk, because of the sand fly-borne nature of the infections and the lack of resistant forms in the environment. All laboratory manipulations with *Leishmania* spp. should be performed at an appropriate biosafety and containment level determined by biorisk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

Differential diagnosis: In dogs, the most common leishmaniosis signs may be confounded with ehrlichiosis, babesiosis and vector-borne or intestinal helminthiases.

1 In this chapter, the term 'New World' refers to the Americas, and the term 'Old World' refers to Africa, Asia and Europe (WHO, 2010).

Role of the vector: The phlebotomine vectors of leishmaniosis are, in some cases, only permissive to the complete development of the species of *Leishmania* that they transmit in nature, while in other instances they can transmit more than one species of *Leishmania* (Bates, 2007). There are emerging species of *Leishmania* infecting humans and animals for which the vector has not been identified There are some 500 known phlebotomine species, but only about 30 have been found to transmit leishmaniosis. Only the female sand fly transmits the parasites (WHO, 2010). In temperate areas the transmission of *Leishmania* occurs only during warm months, coinciding with the activity period of adult sand flies, but due to the variable and lengthy pre-patent period, seasonal variation in the disease incidence in dogs and humans may not be apparent. By contrast, in warmer regions such as much of endemic Brazil, sand fly activity and human and canine transmission of zoonotic VL is year round. Temperature affects the development time and overwintering of sand flies and the extrinsic incubation period, which is likely to be reflected in their duration of infectiousness.

B. DIAGNOSTIC TECHNIQUES

Leishmaniosis diagnosis must be based on an integrated approach that takes into account signalling, medical history, physical findings, clinical and pathological changes, and results of diagnostic tests. The diagnostic methods currently available are described below.

The list and fitness of each test for different purposes are given in Table 1.

Table 1. Test methods available for diagnosis of leishmaniosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Cytological examination	–	–	–	++	–	–
Histological examination	–	–		++	–	
Isolation in culture	–	+		++	–	
Molecular methods	++	+++		++	++	
Detection of immune response						
IFAT	+++	++	–	++	+++	–
ELISA	+++	++		++	+++	
Direct agglutination test	++	++		++	++	
Rapid immuno-chromatographic assay	–	–		++	+	

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; IFAT = Indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

1.1. Cytological examination

Cytological examination allows the presence of amastigotes in macrophages or extracellular fluid to be highlighted after appropriate staining (May Grunwald–Giemsa staining). Amastigote are small intracellular rounded or oval body, 1.5–3 × 2.5–6.5 μm in size, found in vacuoles within the cytoplasm of the macrophages. There is no free flagellum. The organism has a relatively large nucleus and a kinetoplast consisting of a rod-like body and a dot-like basal body.

The cytological investigation should therefore be performed on the following samples:

i) Papular, nodular and ulcerative skin lesions: sampling by fine needle aspiration or impression smear; ischemic ulcerative lesions may, however, be negative;

ii) Bone marrow and lymph nodes in the presence of clinical signs or clinical pathological changes referable to the involvement of parasites (anaemia, lymphadenomegaly, etc.);

iii) Other locations: biological fluids taken from sites with lesions (e.g. synovial fluid in the case of arthritis/polyarthritis, cerebrospinal fluid in the case of neurological signs, etc.). In the absence of sampling lesions, the organs or tissues in which parasites are most likely to be found are represented, in decreasing order of diagnostic sensitivity, by spleen, bone marrow, lymph node and blood (Mylonakis *et al.,* 2005; Saridomichelakis *et al.,* 2005).

In the case of a negative cytological result, the material used for cytology can be stored and sent to the laboratory for testing by polymerase chain reaction (PCR) (see Section B.1.4 *Molecular methods*). The specificity of microscopy for the diagnosis of visceral leishmaniosis is high, but its sensitivity varies according to the sampled tissues: 93–99% for the spleen, 52–85% for bone marrow, and 52–58% for lymph node aspirates. However, splenic aspiration is not recommended, given the very high risk of life-threatening bleeding. Bone marrow aspiration is less sensitive but safer and is currently considered the best method for obtaining a sample of tissue to analyse.

1.2. Histological examination

The parasite can be highlighted in sections of lesions stained with haematoxylin-eosin. In association with the parasite, the CanL-compatible alterations can also be highlighted, represented by granulomatous inflammation and/or vasculitis affecting different organs, ischaemic dermatopathies, lymphoplasmacytic dermatitis of the dermo-epithelial junction, lymphoid hyperplasia of the spleen and lymph nodes. The use of histological examination is always advisable when, despite a negative cytological examination, the strong suspicion of CanL persists, especially in the presence of dermatitis and in cutaneous forms characterised by focal lesions. If histological alterations such as those described above are detected in haematoxylin-eosin stained sections, but without detectable parasites it is advisable to proceed with immunohistochemical staining for *Leishmania* antigens. If this approach is also negative, the biopsy sample can be used for genetic analyses (PCR, sequencing, loop-mediated isothermal amplification) (see Section B.1.4 *Molecular methods*) (Muller *et al.,* 2003; Roura *et al.*, 1999a).

1.3. Isolation in culture

Isolation in culture is the most specific test because the development in culture of vital promastigotes is solely attributable to the genus *Leishmania* in the case of samples taken in endemic areas of the Old World. The parasitological investigation should be performed on the following samples: lymph node aspirate, bone marrow aspirate, skin scrapings and biopsies. In VL, blood can also be used (by taking the buffy coat), with reduced sensitivity. However, isolation in culture has the disadvantage of requiring long execution times and is performed only in specialised laboratories. The choice of the isolation and culture methods will depend on the immediate circumstances and on the technical capability and experience of the laboratory staff (WHO, 2010). Unfortunately, there is still no 'universal' culture medium in which all the different leishmanias will grow easily, and it is almost impossible to predict which medium will be best suited to the growth of a particular isolate of *Leishmania*. Individual laboratories have to find the most suitable medium among biphasic blood agar media and tissue culture media supplemented with fetal calf serum (Evans, 1987). When attempting primary isolation of unknown organisms, a blood agar-based medium should be used – preferably NNN medium (Novy, McNeil and Nicolle), otherwise brain–heart

infusion (BHI) agar medium or EMTM (Evans' modified Tobie's medium) should be used. For bulk cultivation of established isolates, suitable media are reported in Section B.1.3 below (see Evans, 1987 for media composition). The organisms from patients with chronic CL and MCL can be very difficult to cultivate. The parasites sometimes die when subcultured, even when the initial isolation is successful. This seems especially common when the initial isolation has been into a rich medium. Often this can be overcome if subcultures are made into less nutritionally rich media, such as NNN, or one of the semisolid media such as 'sloppy Evans' or semisolid Locke blood agar. There are many references available offering new alternative culture media to be used for cultivation of the parasite (Castelli *et al.,* 2014; Santarém *et al.,* 2014).

1.4. Molecular methods

PCR methods are available for diagnosis and/or identification of *Leishmania* from different types of human and animal samples. The molecular investigation should be performed on the following samples in decreasing order of sensitivity: bone marrow/lymph node, skin, conjunctiva, buffy coat, peripheral blood. In resistant dogs, *Leishmania* inoculation may not be followed by parasite dissemination therefore, in endemic areas, a positive PCR result on a skin sample in the absence of skin lesions does not necessarily mean that the dog is infected and will develop infection (Gradoni*,* 2002). Similarly bone marrow PCR positives may subsequently test negative (Oliva *et al.,* 2006). It is always better to use fresh or frozen material or material fixed in 95% ethyl alcohol. Formalin-fixed or paraffin-fixed samples can be used but give lower diagnostic yields.

Essentially, techniques developed either to identify established isolates of *Leishmania* or to detect organisms from several samples, include: (a) digestion of material with proteinase K and DNA extraction. These steps can be either performed using in-house protocols and reagents, or by commercial kits that are widely available; (b) standard PCR amplification using oligonucleotide sequences (primers) selected from the small-subunit rRNA gene (Mathis & Deplazes, 1995), kinetoplast DNA minicircles (Maarten et al., 1992), or other highly repetitive genomic DNA sequences (Bulle *et al*., 2002; Piarroux *et al.,* 1993); (c) analysis of amplification products by 1–2% agarose gel. PCR is a very sensitive method, especially if the PCR targets are "multi-copy" genomic sequences, those present in high numbers in every single parasite, such as the kinetoplast minicircles DNA (Cortes *et al.,* 2004).

The three most used techniques are:

i) Conventional or traditional PCR: *Leishmania* DNA is amplified using a pair of primers (complementary base sequences to the target sequence contained in *Leishmania* DNA); (Lachaud *et al.,* 2002; Muller *et al.,* 2003);

ii) Nested PCR: a modification of traditional PCR, more sensitive but less specific, as increasing the number of steps tends to increase the risk of contamination by foreign DNA and, therefore, of false positive results (Fisa *et al.,* 2001; Roura *et al.,* 1999b);

iii) Real-time PCR (quantitative): the use of fluorescent molecules or probes makes it possible to quantify the number of DNA copies present in a biological sample. Real-time PCR has a sensitivity similar to the nested PCR, but if performed with 'closed' systems, it is more specific because the sample undergoes a smaller number of manipulations and is therefore less prone to contamination. Real-time PCR can also provide information useful in the monitoring phase, e.g. number of parasites (Manna *et al.,* 2008).

Studies have also demonstrated the potential of loop-mediated isothermal amplification (LAMP) as a point of care diagnostic technique for CanL particularly in resource limited endemic areas (Nzelu *et al.,* 2019).

2. Serological tests

Seroconversion occurs within a few months of infection: on average 5 months (range 1–22) for natural infections and 3 months (range 1–6) for experimental infections (Moreno & Alvar*,* 2002). The diagnostic techniques available vary. Some, such as Western blotting, while showing excellent diagnostic performance, are not used on a large scale because of time constraints and cost. The most widely available are indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), direct agglutination tests (DAT), and the rapid immunochromatographic assay (dipstick or strip-test).

2.1. Indirect fluorescent antibody test

The IFAT is widely used because it is easy to perform. The test is genus specific, although significant cross-reactions have been reported in individuals infected with *Trypanosoma cruzi*. For these subjects, serological tests based on specific recombinant *Leishmania* antigens would be more appropriate (see Sections B.2.2.2 and B.2.2.4 below). In Chagas' disease-free areas, the IFAT for the diagnosis of clinical VL or CanL has a sensitivity of 96% and specificity of 98%, which is similar to the ELISA. Although amastigotes from frozen sections or smears of infected organs can be used as antigen, cultured promastigotes represent the commonest antigen source.

2.1.1. Antigen preparation

i) Harvest 3–4 ml of the liquid media of a 3-day-old culture showing flourishing promastigote growth (see Section B.1 for culture media).

ii) Wash the organisms three times with phosphate-buffered saline (PBS), pH 7.2–7.4, by centrifugation at 350 ***g*** for 15 minutes at room temperature.

iii) Resuspend the final cell pellet in PBS and adjust the promastigote concentration to approximately 4×10^6/ml with the aid of a haemocytometer.

iv) Distribute 30 µl of the promastigote suspension on to each circle of a multi-spot slide and allow to dry at room temperature.

v) Fix the promastigotes in cold acetone for 10 minutes, then put the slides into a plastic box and keep in a deep freezer (–35°C) for no longer than 2–3 months.

2.1.2. Test procedure

i) Wash the frozen antigen-coated slides in PBS and allow to dry at room temperature.

ii) Inactivate the sera for 30 minutes in a water bath at 56°C.

iii) Make doubling dilutions of test sera from 1/80 to 1/10,240 for human VL, and from 1/40 to 1/5120 for CanL. Positive and negative control sera, at dilutions of 1/80 and 1/160 for human VL, and of 1/40 and 1/80 for CanL, are also included in the test. No standard sera are available, but internal standards should be prepared and titrated.

iv) Distribute 30 µl of diluted serum samples on to each slide circle and incubate for 30 minutes at 37°C.

v) Remove the serum samples by vigorous washing in PBS, followed by immersion of the slides in PBS for 10 minutes. Allow the slides to dry.

vi) Distribute 30 µl of diluted fluorescein isothiocyanate (FITC)-conjugated anti-immunoglobulin on to each slide circle and incubate for 30 minutes at 37°C. FITC-conjugated anti-human and anti-dog immunoglobulins are commercially available. Follow the instructions for the appropriate dilution.

vii) Repeat step v and mount with a cover-slip in a few drops of PBS/glycerol (50% [v/v] of each).

viii) Read the slides under a fluorescent microscope. The highest dilution showing fluorescent promastigotes is taken to be the antibody titre.

2.1.3. Interpretation of the results

In acute human VL, the threshold titre usually ranges from 1/80 to 1/160. Asymptomatic or subclinical human disease usually results in titres below 1/80. In CanL the threshold titre ranges from 1/40 (indicative of exposure but not necessarily of established infection) to 1/160 (indicative of established infection), whereas a titre of 1/320 or above can be indicative of the disease in clinically suspected dogs (Paltrinieri *et al.,* 2010). As regards other domestic mammals (e.g. cats) no standardised IFA threshold limits are available. As IFAT performance may vary in different laboratories, it is better for each laboratory to define its own threshold titre using defined positive and negative reference sera.

2.2. Enzyme-linked immunosorbent assay

The ELISA can be carried out on serum or on a measured volume of blood. The blood is collected by needle-prick on to suitable absorbent paper strips and allowed to dry. The sample is eluted and tested at a single dilution previously determined to give an acceptable sensitivity and specificity. This test can be used for seroepidemiological surveys under field conditions.

In the classical method, the antigen is prepared as follows: promastigotes harvested from cultures are washed four times with PBS, pH 7.2, at 1000 ***g*** for 15 minutes. The packed promastigotes are resuspended in twice their volume of distilled water, and then sonicated at medium amplitude in an ice bath. The suspension is left at 4°C overnight to allow the proteins to come into solution. After a final centrifugation at 4000 ***g*** for 10 minutes to eliminate the cellular debris, the overlay, representing the concentrated soluble antigen, is dispensed into vials and stored at –20°C until required. For use in the test, it is reconstituted with PBS to the predetermined optimal protein concentration (around 20 μg/ml) as measured by Lowry's method. Enzyme (usually horseradish peroxidase)-conjugated reagents consist of anti-dog goat immunoglobulins or Protein A (Hamarsheh *et al*., 2012).

The ELISA is useful for the diagnosis of Old and New World leishmaniosis. Cross-reaction with *Babesia canis* and *Trypanosma* spp. has been reported (Gottstein *et al,* 1988). According to the *Leishmania* strain used, sensitivity of the ELISA can range from 86% to 99%.

A detergent-soluble promastigote antigen has been used in ELISA instead of the crude lysate, for the diagnosis of CanL. The detergent was Triton X-100 and the proteic extract was protected with protease inhibitors. Using this method, ELISA sensitivity increased to 99.5%, while its specificity was comparable with that of the IFA test (97%) (Mancianti *et al*., 1995).

The ELISA methods described above are based on crude antigenic preparations. A recombinant antigen from a cloned protein of *L. infantum*, called rK39, has been reported to be highly reactive to sera from human and canine visceral leishmaniosis cases when run in an ELISA format. Using 25–50 ng of the antigen, 99% specificity and sensitivity was consistently found for dogs with parasitologically proven disease (Scalone *et al*., 2002). In HIV-positive patients, K39-ELISA showed higher sensitivity (82%) than the IFA test (54%) (Houghton *et al*., 1998). The K39 antigen, which shows remarkable stability and reproducibility, is commercially available. More recently, a K9-K39-K26 recombinant chimeric antigen has been evaluated as a single ELISA protocol for serological diagnosis of both human and canine *Leishmania* infections (Daprà *et al*., 2008). In dogs, test specificity and sensitivity were reported to be 99.5% and 98.5%, respectively, with high concordance (K value: 0.98) with standard IFA test.

2.3. Direct agglutination test

The DAT has been described for the diagnosis of VL and CanL. After test improvement, DAT has been validated as a specific and sensitive assay for field investigations (Boelaert *et al*., 1999; Cardoso *et al*., 2004; Ozbel *et al*., 2000). The antigen consists of promastigotes harvested from cultures, washed in PBS, pH 7.2, treated with 0.4% trypsin (for 45 minutes at 37°C and then washed again), and stained with 0.02% Coomassie brilliant blue. Twofold serial dilutions of serum in PBS are made in V-bottomed microtitre-plate wells; 50 μl of antigen preparation is added to each well, and the plate is then carefully shaken by hand and left for 18 hours at room temperature. The test is read visually against a white background. Positive reactions are indicated by typical light-blue aggregates, while negative samples give a clear sharp-edged blue spot.

A modified DAT for detection of specific anti-leishmanial antibodies in canine reservoir hosts is considered to be highly suitable for wide-scale epidemiological and ecological field work and diagnosis of CanL, having 100% sensitivity and 98.9% specificity (Harith *et al*., 1988; 1989). The reliability of the test was improved by treating the test sera with 0.2 M 2-mercaptoethanol and incubating them at 37°C.

2.4. Rapid immunochromatographic assay (dipstick or strip-test)

The rapid immunochromatographic assay is easy to carry out and can be performed in veterinary clinics, but has lower diagnostic sensitivity than the ELISA and IFAT: specificity is medium-high but sensitivity is low (30–70%) (Gradoni, 2002; Mettler *et al.*, 2005; Reithinger *et al*., 2002) and therefore it can give false negative results. When there is a strong suspicion of a false negative result using a rapid immuno-

chromatographic assay, one of the other serology tests must be used. The value of a positive result is limited because the test does not allow antibody titre to be evaluated, however positive results can be useful for identifying individuals with parasite dissemination and for monitoring therapeutic response.

A rapid immunochromatographic assay using rK39 as antigen (K39 dipstick or strip-test, commercially available) has been evaluated in different endemic settings of VL. The nitrocellulose membrane of the test kit holds an absorbent pad at one end, a band of immobilised anti-protein A antibody (used to detect IgG) at the other (control region), and a band of rK39 antigen in the middle (test region). A protein-A-colloidal gold conjugate is used as the immunochromatographic detection reagent. One small drop (20 μl) of the serum to be examined is placed on the absorbent pad before two large drops (100 μl) of test buffer are added to the pad, and the mixture is allowed to migrate up the strip by capillary action. After 2–10 minutes, the result is positive if two distinct red lines appear (one in the test region and another in the control region), it is negative when no red line appears in the test region, and it is invalid if the control line fails to appear.

In clinical cases of human VL, two commercial brands of K39 dipstick showed 99–100% sensitivity and 95–100% specificity in India (Sundar *et al*., 2006), 90% sensitivity and 100% specificity in Brazil (Carvalho *et al*., 2003), and 100% sensitivity and specificity in the Mediterranean basin (Brandonisio *et al*., 2002). In dogs with both asymptomatic and symptomatic cases of CanL, the sensitivity and the specificity of the K39 dipstick were 97% and 100%, respectively (Otranto *et al*., 2005).

3. *Leishmania* species, subspecies or strains identification

Morphological identification enables identification of *Leishmania* at the genus level, but not at the species or subspecies level. Several techniques may be used to identify the different *Leishmania* species, subspecies or strains. Fifteen recognised *Leishmania* Identification Centres were listed by WHO in 2010.

3.1. Isoenzyme characterisation

Also known as multi-locus enzyme electrophoresis (MLEE), isoenzyme characterisation is the reference method for species identification (Rioux *et al.,* 1990; WHO, 2010), although this technique requires cultivation of a large number of parasites (5×10^9–1×10^{10}). The principles of enzyme electrophoresis are as follows: soluble enzymes are extracted from the organisms grown in media for bulk cultivation (BHI medium, MEM/FCS/EBLB [minimal essential medium/fetal calf serum/Evans' blood lysate broth] medium, Schneider's Drosophila medium). A small amount of the extract is then placed in an inert supporting substance, the matrix, containing a buffer at a fixed pH. The matrix is usually starch gel, but it could equally well be absorbent cellulose acetate, acrylamide or agarose. The pH of the buffer in the matrix is usually chosen so that the isoenzymes are negatively charged. A direct current is passed through the matrix carried by the ions in the buffer. When electrophoresis is completed, most proteins will have moved in the matrix towards the anode, depending on the amount of negative charge. If stained at this stage with a general protein stain, many bands will be seen. However, the high substrate and cofactor specificity of enzymes make it possible to stain only these proteins. Hence, the electrophoretic mobility of one particular enzyme can be compared among several organisms. The stained matrix with its collection of stained isoenzyme bands is known as a zymogram. Normally one or more extracts from reference organisms, in which the enzyme banding patterns are well documented, are included in the gel to aid the interpretation of results. Most enzymes used for characterisation purposes are stained by methods incorporating a dehydrogenase reaction. At least 12 enzymes should be examined; organisms showing identical zymograms are classified into zymodemes of a given species.

3.2. Molecular characterisation

Different molecular techniques have been developed that allow *Leishmania* characterisation at the species or strain level such as (a) PCR-restriction fragment length polymorphism (RFLP) analysis, in which the PCR products are digested by appropriate restrictions enzymes and the resulting restriction fragment patterns are analysed (Marfurt *et al.*, 2003; Minodier *et al*., 1997; Montalvo *et al*., 2012; Volpini *et al*., 2004); (b) multi-locus microsatellite typing (MLMT) (Kuhls *et al*., 2011) and (c) multi-locus sequence typing (MLST) (Mauricio *et al.,* 2006). In these cases, repeated and polymorphic DNA sequences are targeted, such as ribosomal internal transcribed spacer 1 (ITS1), cysteine protease B, kinetoplast DNA minicircles, surface glycoprotein 63, heat-shock protein 70, mini-exons and microsatellites (Reithinger & Dujardin, 2007; Schönian *et al*., 2008).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Vaccines

1.2. Outline of production and minimum requirements for vaccines

Currently there are no available laboratory-made antigens for *Leishmania* vaccine production. There are four licensed inactivated vaccines against CanL, which are patent-protected. The first vaccine was developed in Brazil. Outlines and requirements for production were approved by the Brazilian Ministry of Agriculture, Livestock and Food Supply. The vaccine consists of the glycoprotein-enriched fraction of *L. donovani* known as 'fucose-mannose ligand' (FML). Field studies showed about 80% clinical protection conferred by the antigen administered with QuilA saponin as adjuvant, and also good immunotherapeutic efficacy when used in sick dogs (Palatnik-de-Sousa *et al*., 2008). A second vaccine was also developed in Brazil, which uses the recombinant A2 antigen of *L. donovani* in association with a saponin adjuvant. It demonstrated 43% protection against a culture positive state in an artificial challenge model. The third vaccine is licensed in Europe. Outlines and requirements for production have been approved by the European Medicines Agency. Purified excreted-secreted proteins of *L. infantum* and with QA-21 saponin significantly reduces the risk of progressing to active infection or overt disease, with a clinical efficacy of 68%. In vaccinated dogs that developed disease and that were exposed to the bites of reared *P. perniciosus* vectors, the reduction in parasite transmission was significant when compared with matched controls. However, vaccine does not enable differentiation of vaccinated from infected animals. In addition, some safety concerns have been reported dogs vaccinated with purified excreted-secreted proteins (Oliva *et al.,* 2014). The fourth vaccine, available in the EU, contains the active substance protein Q, which is made of different fragments of proteins from *L. infantum.* A dog vaccinated with recombinant protein Q from *L. infantum* MON-1 has five times less risk of developing clinical disease than a non-vaccinated dog. There is a lack of information concerning the interruption of *Leishmania* transmission by dogs vaccinated with recombinant protein Q from *L. infantum* MON-1 is lacking (Cotrina *et al.,* 2018). Surveillance programmes, especially those based solely on serology in dogs should be revised to take into account the impact of vaccination and to ensure epidemiological data is interpreted appropriately.

At present, a number of promising anti-leishmanial vaccines are under experimental evaluation (Coler & Reed, 2005; Das & Ali, 2012). A chimeric antigen generated from three recombinant *Leishmania* antigens screened for their ability to elicit cellular immune responses (known as Leish-111f, patent-protected) and adjuvanted with monophosphoryl lipid A – stable emulsion (MPL-SE), represents the first defined vaccine for leishmaniosis in humans, having completed phase 1 and 2 safety and immunogenicity testing in healthy subjects (Coler *et al*., 2007). The same polyproteinic antigen and adjuvant failed to protect dogs from *L. infantum* infection in a phase 3 trial (Gradoni *et al*., 2005), while conferred some protection from disease when used as an immunotherapeutic agent in dogs with mild CanL (Trigo *et al*., 2010). Recently, a novel chimeric antigen generated from four recombinant antigens (KSAC, patent-protected) has been found promising for vaccine protection in both human VL and CanL (Goto *et al*., 2011).

2. Diagnostic biologicals (antigens for skin tests)

As the skin test is not used for diagnosis of animal infections, and the leishmanin antigen has not been internationally standardised, no recommendations can be made by WOAH.

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* *

NB: There is a WOAH Reference Laboratory for Leishmaniosis
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratory for any further information on
diagnostic tests, reagents and vaccines for leishmaniosis

NB: FIRST ADOPTED IN 1991 AS LEISHMANIASIS. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.1.12.

LEPTOSPIROSIS

SUMMARY

Description of the disease: *Leptospirosis is a transmissible disease of animals and humans caused by infection with any of the pathogenic members of the genus* Leptospira*. Acute leptospirosis should be suspected in the following cases: sudden onset of agalactia (in adult milking cattle and sheep); icterus and haemoglobinuria, especially in young animals; meningitis; and acute renal failure or jaundice in dogs. Chronic leptospirosis should be considered in the following cases: abortion, stillbirth, birth of weak offspring (may be premature); infertility; chronic renal failure or chronic active hepatitis in dogs; and cases of periodic ophthalmia in horses.*

Laboratory diagnosis of leptospirosis can be complex and involves tests that fall into two groups. One group of tests is designed to detect anti-leptospiral antibodies, and the other group is designed to detect leptospires, leptospiral antigens, or leptospiral nucleic acid in animal tissues or body fluids. The particular testing regimen selected depends on the purpose of testing (e.g. herd surveys or individual animal testing) and on the tests or expertise available in the area.

Detection of the agent: *The isolation or demonstration of leptospires in:*

a) *several of the internal organs (such as liver, lung, brain, and kidney) and body fluids (blood, milk, cerebrospinal, thoracic and peritoneal fluids) of clinically infected animals gives a definitive diagnosis of acute clinical disease or, in the case of a fetus, chronic infection of its mother;*

b) *the kidney, urine, or genital tract of animals without clinical signs is diagnostic only of a chronic carrier state.*

Isolation of leptospires from clinical material and identification of isolates is time-consuming and is a task for specialised reference laboratories. Isolation followed by typing from renal carriers is important and very useful in epidemiological studies to determine which serovars are present within a particular group of animals, an animal species, or a geographical region.

The demonstration of leptospires by immunochemical tests (immunofluorescence and immunohistochemistry) is more suited to most laboratory situations. However, the efficacy of these tests is dependent on the number of organisms present within the tissue, and these tests lack the sensitivity of culture. Unless specially prepared reagents are used, immunochemical tests do not identify the infecting serovar and results must be interpreted in conjunction with serological results. Reagents for immunofluorescence are best prepared with high IgG titre anti-leptospire sera, which are not available commercially. Rabbit leptospiral-typing serum or monoclonal antibodies can be used for immunohistochemistry and are available from leptospiral reference laboratories.

Genetic material of leptospires can be demonstrated in tissues or body fluids using a variety of assays based on the polymerase chain reaction (PCR), either in real-time or traditional formats. PCR assays are sensitive, but quality control procedures and sample processing for PCR are critical and must be adjusted to the tissue, fluid and species being tested. Like immunochemical tests, PCR assays do not identify the infecting serovar, although some will identify the infecting species.

Serological tests: *Serological testing is the most widely used means for diagnosing leptospirosis, and the microscopic agglutination test (MAT) is the standard serological test. Antigens selected for use in the MAT should include representative strains of the serogroups known to exist in the particular region as well as those known to be maintained elsewhere by the host species under test.*

The MAT is used to test individual animals and herds. As an individual animal test, the MAT is very useful for diagnosing acute infection: a four-fold rise in antibody titres in paired acute and

convalescent serum samples is diagnostic. To obtain useful information from a herd of animals, at least ten animals, or 10% of the herd, whichever is greater, should be tested and the vaccination history of the animals documented.

The MAT has limitations in the diagnosis of chronic infection in individual animals and in the diagnosis of endemic infections in herds. Infected animals may abort or be renal/genital carriers with MAT titres below the widely accepted minimum significant titre of 1/100 (final dilution).

Enzyme-linked immunosorbent assays (ELISAs) can also be useful for detection of antibodies against leptospires. Numerous assays have been developed and are primarily used for the detection of recent infections, the screening of experimental animals for use in challenge studies, and, in cattle, health schemes to assess levels of infection of serovar Hardjo – either as tests on individual animal blood or milk or as bulk milk tank tests. Animals that have been vaccinated against the serovar of interest may be positive in some ELISAs, thus complicating interpretation of the results.

Requirements for vaccines: *Vaccines for veterinary use are most often suspensions of one or more serovars of* Leptospira *spp. inactivated in such a manner that immunogenic activity is retained. While a range of experimental vaccines based on cellular extracts has been tested, commercial vaccines are whole cell products. The leptospires are grown in suitable culture media, which often contain serum or serum proteins. If used, serum or serum proteins should be removed from the final products. Vaccines may contain suitable adjuvants.*

A. INTRODUCTION

Leptospirosis is a transmissible disease of animals and humans caused by infection with the spirochete *Leptospira*.

Causal pathogen: In the past, all the pathogenic leptospires were classified as members of the species *Leptospira interrogans*, however the genus has been reorganised. The genus *Leptospira* consists of 66 species that can be classified across four subclades. Pathogenic spirochetes of the genus *Leptospira* belong primarily in the P1 subclade of the new classification (Casanovas-Massana *et al.,* 2020; Vincent *et al.,* 2019). Serologically, there are more than 300 distinct leptospiral serovars recognised and these are arranged in 30 serogroups[1].

In theory, any parasitic *Leptospira* may infect any animal species. Fortunately, only a small number of serovars will be endemic in any particular region or country. Furthermore, leptospirosis is a disease that shows a natural nidality, and each serovar tends to be maintained in specific maintenance hosts. Therefore, in any region, a domestic animal species will be infected by serovars maintained by a species or by serovars maintained by other animal species present in the area. The relative importance of these incidental infections is determined by the opportunity that prevailing social, management, and environmental factors provide for contact and transmission of leptospires from other species. An example of a host-maintained infection is serovar Hardjo infection in cattle. Limited host ranges allows for the development of control/eradication schemes.

Description of the disease: The use, interpretation, and value of laboratory diagnostic procedures for leptospirosis vary with the clinical history of the animal or herd, the duration of infection, and the infecting serovar. Acute leptospirosis should be suspected in the following cases: sudden onset of agalactia (in adult milking cattle and sheep); icterus and haemoglobinuria, especially in young animals; meningitis; and acute renal failure (in dogs). Chronic leptospirosis should be considered in the following cases: abortion, stillbirth, birth of weak offspring (may be premature); infertility; chronic renal failure (in dogs); and cases of periodic ophthalmia in horses. Two major chronic microbiological sequelae of leptospiral infection present particular diagnostic problems: the localisation and persistence of leptospires in the kidney and in the male and female genital tract. Chronically infected animals may remain carriers for years or life and serve as reservoirs of the infection for other animals and humans.

Zoonotic risk and biosafety requirements: *Leptospira* represent a moderate risk for human infection. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

1 https://leptospira.amc.nl/leptospira-library/

Differential diagnosis: 1) Diseases where acute milk drop may occur, such as acute viral infections and sudden absence of drinking water: 2) diseases with hepato-renal failure: and 3) diseases characterised by reproductive wastage – abortion, reduced litter size, stillbirth and infertility e.g. brucellosis, *Neospora*, Q fever and bovine viral diarrhoea infection in cattle, chlamydiosis and toxoplasmosis in sheep, Q fever in goats, etc.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of leptospirosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Isolation and identification	–	+++	+	+++	–	–
PCR	–	++	–	++	–	–
Detection of immune response						
MAT	–	+++	+	++	+++	+
ELISA	+++	–	+++	+++	++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; MAT = microscopic agglutination test; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

The demonstration of leptospires in blood and milk of animals showing clinical signs suggestive of acute leptospirosis is considered to be diagnostic. However, isolation from blood is not often successful because bacteraemia is transient and not always accompanied by clinical signs. Dogs are often treated with antibiotics before samples are collected for testing for *Leptospira,* which further decreases the likelihood of identifying the agent in blood. The demonstration of generalised leptospiral infection in a range of organs taken at necropsy is also considered to be diagnostic. However, if the animal lives long enough or has been treated with antibiotics, it may be difficult to detect intact organisms systemically; immunohistochemistry can be particularly helpful in identifying residual leptospiral antigen in these cases. Demonstration of leptospires in the genital tract, kidneys, or urine only must be interpreted with full consideration of the clinical signs, and serological results as these findings may merely indicate that the animal was a carrier.

Failure to demonstrate leptospires in the urine of an animal does not eliminate the possibility that the animal is a chronic renal carrier, it merely indicates that the animal was not excreting detectable numbers of leptospires at the time of testing. Collection of urine following treatment of the animals with a diuretic enhances the chances of detecting the organism. In important cases involving individual animals (e.g. clearing an infected stallion to return to breeding), negative tests on three consecutive weekly urine samples have been considered to be good evidence that an animal is not shedding leptospires in the urine.

The demonstration of leptospires in body fluids or internal organs (usually kidney, liver, lung, brain, or adrenal gland) of aborted or stillborn fetuses is considered to be diagnostic of chronic leptospirosis of the mother, and is evidence of active infection of the fetus.

1.1. Isolation of *Leptospira*

In experienced hands, the isolation of leptospires is one of the most specific methods of demonstrating their presence, provided that antibiotic residues are absent, that tissue autolysis is not advanced, that tissues are processed for culture rapidly after collection, and – in the case of urine – at a suitable pH. It should be noted that diagnostic sensitivity of agent isolation is low. However the method does give the laboratory access to the isolated strain, which provides important information about the epidemiology of the disease that can then be used to improve the diagnosis and the production of *Leptospira* vaccines.

If tissues or fluids cannot be transported promptly to the laboratory for leptospiral culture, the sample should be kept at 2–5°C to prevent overgrowth with other bacteria and autolysis of tissue samples. Liquid culture medium or 1% bovine serum albumin (BSA) solution containing 5-fluorouracil at 100–200 μg/ml should be used as transport medium for the submission of samples.

Culture should be carried out in a liquid or semisolid (0.1–0.2% agar) medium containing BSA and either Tween 80 (e.g. EMJH[2]) or a combination of Tween 80 and Tween 40. Contamination may be controlled by the addition of a variety of selective agents, e.g. 5-fluorouracil, nalidixic acid, fosfomycin, and a mixture of rifamycin, polymyxin, neomycin, 5-fluorouracil, bacitracin, and actidione. However, use of selective agents may reduce the chances of isolation when there are only small numbers of viable leptospires, and some strains of leptospires will not grow in selective media containing multiple antibiotics. Addition of 0.4–5% rabbit serum to semisolid culture medium enhances the chances of isolating fastidious leptospiral serovars.

Cultures should be incubated at 30 ± 2°C for at least 16 weeks, and preferably for 26 weeks. The time required for detection of a positive culture varies with the leptospiral serovar and the numbers of organisms present in the sample. Less fastidious serovars (e.g. Pomona and Grippotyphosa) may result in positive cultures as soon as 7–10 days after inoculation; other serovars (e.g. Hardjo and Bratislava) may take much longer. Cultures should be examined by dark-field microscopy every 1–2 weeks. It is important to use a 100 watt light source and a good quality dark-field microscope.

1.2. Immunochemical staining techniques

Leptospires may also be demonstrated by a variety of immunochemical staining techniques, e.g. immunofluorescence, and various immunohistochemical techniques (Wild *et al*., 2002). These are useful in diagnosing infection in pathological material that is unsuitable for culture or where a rapid diagnosis is required. As the success of these techniques is dependent on the number of organisms present, they are less suitable for diagnosing the chronic carrier state, where the numbers of organisms may be very low or localised. Leptospires do not stain satisfactorily with aniline dyes, and silver-staining techniques lack sensitivity and specificity, although they are a useful adjunct for histopathological diagnosis.

1.3. Nucleic acid detection methods

Polymerase chain reaction (PCR)-based assays are increasingly used for the detection of leptospires in tissues and body fluids of animals because of their perceived sensitivity and capacity to give an early diagnosis. Real-time PCR is faster than regular PCR and less sensitive to contamination (Picardeau, 2013). Assays fall into two categories based on the detection of genes that are universally present in bacteria, for example, *gryB, rrs* (16S rRNA gene) and *secY*, or the detection of genes restricted to pathogenic *Leptospira*, for example, *lipL21*, *lipL32*, *lipL41*, *ligA* and *ligB* (Thaipadunpanit *et al.,* 2011). These assays do not identify the infecting serovar, although some primer sets may permit further identification to the species or strain level if the PCR amplicons are sequenced. This further analysis is not a routine diagnostic method. Many of the PCR primer sets have been designed and evaluated for use in human rather than animal specimens and general agreement about the PCR primers to be used for testing of animal samples is lacking, although those based on the *lipL32* gene are the most commonly reported. Validation remains one of the outstanding issues surrounding the use of PCR in the diagnosis of animal leptospirosis (see Table 1), with the individual laboratory being responsible for the validation of the particular assay they use for the tissue, fluid, and species being tested. To date, only three real-time PCR have been validated (Ahmed *et al*., 2009; 2012b; Slack *et al*., 2007; Thaipadungpanit *et al*., 2011). The

2 EMJH: Ellinghausen-McCullough-Johnson-Harris

presence of amplification inhibitors in clinical samples can cause false-negative results, particularly in animal specimens that may be compromised by contamination with faeces or autolysis. Quality control of PCR assays used for leptospirosis diagnosis requires careful attention to laboratory design and workflow to prevent contamination of reagents, and appropriate control samples should be used (McCreedy & Callawayth, 1993). In addition, sample processing for PCR is critical and must be suited to the tissue, fluid, and species being tested.

1.4. Identification of leptospiral isolates

The identification of leptospiral isolates is a task for specialised reference laboratories. For complete identification, a combination of procedures is used to determine: 1) if the isolate is a pathogen or a saprophyte; 2) the species of *Leptospira* to which the isolate belongs; and 3) the serogroup and serovar of the isolate. A pure leptospiral culture may be identified as belonging to a pathogenic or saprophytic species by a variety of tests: the ability to infect animals; the relative resistance to 8-azaguanine; lipase activity; salt and temperature tolerance; and G+C content of DNA.

Speciation is based on DNA–DNA hybridisation analysis but increasingly other more rapid molecular techniques are used (Ahmed *et al*., 2012a) of which the multilocus sequence typing (MLST) (Ahmed *et al*., 2006) and *ppk* gene sequencing (Vincent *et al.,* 2019) appear robust. Different isolates belonging to a single serovar usually belong to the same species, but this is not always the case.

Strains belonging to *Leptospira* can be differentiated to the serogroup level (a concept that no longer has any taxonomic validity but which is a useful preliminary step in identification and antigen selection for vaccines and serological tests) by cross-agglutination reactions. Subsequent differentiation to the serovar level was traditionally by cross-agglutination absorption, although for most isolates this is now being done using less time-consuming methods such as monoclonal antibodies (MAbs). A variety of molecular methods can give results that may be concordant with serotyping. These methods are not valid for establishing new serovars, however they provide useful guidance on identification and can provide useful molecular epidemiological information at the sub-serovar level. PCR-based approaches include multi-locus variable number of tandem repeats (Slack *et al.,* 2006; Zuerner & Alt, 2009), amplification of insertion elements, amplified fragment length polymorphisms (AFLP) and fluorescent-labelled AFLP (Vijaychari *et al*., 2004) and arbitrarily primed PCR. Bacterial restriction-endonuclease DNA analysis (BRENDA) has proved very useful in epidemiological investigation of leptospires of food producing animals.

2. Serological tests

Serological testing is the laboratory procedure most frequently used to confirm the clinical diagnosis, to determine herd prevalence, and to conduct epidemiological studies. Leptospiral antibodies appear within a few days of onset of illness and persist for weeks or months and, in some cases, years. Unfortunately, antibody titres may fall to undetectable levels while animals remain chronically infected. To overcome this problem, sensitive methods are needed to detect the organism in urine or the genital tract of chronic carriers.

A wide variety of serological tests that show varying degrees of serogroup and serovar specificity, have been described. Two tests have a role in veterinary diagnosis: the microscopic agglutination test (MAT) and the enzyme-linked immunosorbent assay (ELISA).

2.1. Microscopic agglutination test

The MAT using live antigens is the most widely used serological test. It is the reference test against which all other serological tests are evaluated and is used for import/export testing. For optimum sensitivity, it should use antigens representative of all the serogroups known to exist in the region in which the animals are found and, preferably, strains representing all the known serogroups. The presence of a serogroup is usually indicated by frequent reaction in serological screening but can only be definitively identified by isolation of a serovar from clinically affected animals. The sensitivity of the test can be improved by using local isolates rather than reference strains, but reference strains assist in the interpretation of results between laboratories.

The specificity of the MAT is good; antibodies against other bacteria usually do not cross-react with *Leptospira* to a significant extent. However, there is significant serological cross-reactivity between

serovars and serogroups of *Leptospira* and an animal infected with one serovar is likely to have antibodies against the infecting serovar that cross-react with other serovars (usually at a lower level) in the MAT. Therefore, serology cannot be used to identify definitively the infecting serovar in an individual infection or outbreak – this requires isolation of the agent. However, in areas where the serovars of *Leptospira* present have been well described by isolation studies, serological examination of the infected animal(s) may suggest, but not definitively identify, the infecting serovar. In addition, animals that have been vaccinated against leptospirosis may have antibodies against the serovars present in the vaccine used. Therefore, it is particularly important to consider the vaccination history of the animals under test. Methods for carrying out the test have been described in detail (Faine *et al*., 2000; Goris & Hartskeerl, 2014; USDA, 1987).

2.1.1. Test procedure

i) The strains selected should be grown in liquid leptospiral culture medium (e.g. EMJH or other suitable medium) at 30 ± 2°C and the culture should be at least 4 days old, but no more than 10 days and this may depend on the growth and density of the cultures. Live cultures with densities of approximately 2×10^8 leptospires per ml are to be used as the antigens. For standardisation of culture density, adjusting cultures to a concentration of 2×10^8 leptospires per ml prior to testing may be considered. The density of *Leptospira* cultures can be determined by: (1) counting in a suitable counting chamber (Helber or Petroff-Hausser, cell depth 0.02 mm), (2) measuring optical density of the culture by spectrophotometry at 420 nm, (3) using the McFarland scale, and (4) estimating the number of leptospires per field by dark-field microscopy.

ii) The number of antigens to be used is determined and a screening test may be performed with a 1/50 serum dilution (or a different starting dilution based on the purpose of the test).

iii) A volume of each antigen, equal to the diluted serum volume, is added to each well, making the final serum dilution 1/100 in the screening test.

iv) The microtitration plates are incubated at 30 ± 2°C for 1.5–4 hours.

v) The plates are examined by dark-field microscopy.

The endpoint is defined as that dilution of serum that shows 50% agglutination, leaving 50% free cells compared with a control culture diluted 1/2 in phosphate buffered saline. The result of the test may be reported as the endpoint dilution of serum (e.g. 1/100 or 1/400) or as a titre that is the reciprocal of the endpoint serum dilution (e.g. 100 or 400).

2.1.2. Quality control of antigens used in the MAT

Identity of antigens is a crucial factor in conducting the MAT. Antigens should be evaluated for identity using hyperimmune rabbit sera, MAbs, or a molecular method that confirms passages over time, preferably each time the test is run, but at least twice a year. Hyperimmune rabbit serum for this purpose can be obtained from a reference laboratory or prepared using a protocol such as that given by the Subcommittee on the Taxonomy of *Leptospira*. Briefly, healthy rabbits weighing 3–4 kg that lack detectable anti-leptospiral antibodies are selected. Each rabbit is given an intravenous injection in a marginal vein of the ear with a well-growing live or formalin-treated culture with a density of approximately 2×10^8 leptospires/ml. It may be necessary to include a washing step as BSA can cause shock. The culture should be grown in Tween 80 BSA medium or another appropriate medium. Five injections of 1 ml, 2 ml, 4 ml, 6 ml, and 6 ml each are given at 7-day intervals. One week following the final injection, the homologous antibody titre is determined by MAT. If the titre is ≥1/12,800, the rabbit is anaesthetised and bled by cardiac puncture 7 days later (i.e. 14 days after the final injection). If the titre is <1/12,800, a further injection of 6 ml of culture can be given; 7 days after this injection the homologous titre is again determined. Unless the titre is ≥1/12,800 the procedure should be repeated with another rabbit. Two rabbits are used to prepare each antiserum. If the titres are satisfactory in both rabbits, the sera may be pooled. To preserve potency, it is preferable to freeze-dry the antiserum in 2-ml volumes and store it at 2–5°C. Alternatively, the serum can be stored in 1-ml volumes at –15 to –70°C. All animal inoculations should be approved and conducted according to the relevant standards for animal care and use. Other immunisation protocols may be considered based on the intended use of the antiserum and the need to reduce the number of rabbits used.

Purity of antigens used in the MAT should be checked regularly by culture on blood agar and in thioglycolate broth. Stock cultures of antigens may be stored at –70 to –80°C or in liquid nitrogen. There will be a low survival rate of leptospires after lyophilisation. Repeated passage of antigens in liquid medium results in a loss of antigenicity. In this case, a new liquid culture should be derived from the stock culture.

An annual international MAT proficiency scheme is available through the International Leptospirosis Society[3].

2.1.3. Interpretation and limitations of the MAT

A titre of 1/100 is taken as a positive titre, but given the high specificity of the MAT lower titres can be taken as evidence of previous exposure to *Leptospira*.

As an individual animal test, the MAT is very useful in diagnosing acute infection; the demonstration of a four-fold change in antibody titres in paired acute and convalescent serum samples is diagnostic. In addition, a diagnosis of leptospirosis is likely, based on the finding of very high titres in an animal with a consistent clinical picture. The test has limitations in diagnosis of chronic infection in individual animals, both in the diagnosis of abortion and in the identification of renal or genital carriers (Ellis, 1986). This is particularly true with the host-adapted leptospiral infections, e.g. serovar Hardjo infection in cattle: when a titre of 1/100 or greater is taken as significant, the sensitivity of the test is only 41%, and even when the minimum significant titre is reduced to 1/10, the sensitivity of the test is only 67% (Ellis, 1986). The demonstration of antibodies in fetal blood is diagnostic, but the titres are often very low, i.e. 1/10, requiring a modified testing procedure for most laboratories.

As leptospirosis is a herd problem, the MAT has much greater use as a herd test. To obtain useful information, samples should be taken from at least ten animals, or 10% of the herd, whichever is the greater. In a study of Hardjo infection in cattle, a 10-cow sample usually indicated the presence or absence of infection in a herd. Increasing the sample size markedly improved epidemiological information, investigations of clinical disease, and public health tracebacks.

In making a serological diagnosis of leptospirosis, the infecting serovar and the clinical condition involved must be fully considered. In the case of serovar Pomona-induced abortion in cattle, a high titre is commonly found at the time of abortion because the clinical incident occurs relatively soon after infection. Abortion in cattle due to serovar Hardjo is a chronic event; in this case, the serological response at the time of abortion is more variable, with some animals seronegative and others showing high titres. Cattle may experience a drop in milk production during the acute phase of Hardjo infection and this clinical sign is associated with high titres. Vaccination history must also be considered in the interpretation of MAT results as widespread vaccination contributes significantly to the number of seropositive animals and may mask the presence of chronic infections in the herd – particularly with serovar Hardjo.

2.2. Enzyme-linked immunosorbent assays

ELISAs for detection of anti-leptospiral antibodies have been developed using a number of different antigen preparations, assay protocols and assay platforms, including plate tests and dipstick tests. The antigen preparations have mainly been either whole cell preparations or outer membrane protein (OMP) preparations, with recent emphasis on developing tests using recombinant OMPs. The antigen used dictates the specificity of the ELISA. Recombinant OMP-based ELISAs are broadly reactive to antibodies to all pathogenic leptospires and so are of no value in epidemiological investigations. In contrast, lipopolysaccharide antigen-based ELISAs are serogroup specific and have value in epidemiological investigations and control schemes. IgM ELISAs have been shown to be useful in the diagnosis of acute infection. A total-Ig ELISA is useful in the identification of fully susceptible animals suitable for experimental challenge work. ELISAs have also been developed for use in milk from individual cows or in bulk tank milk for the detection of serovar Hardjo antibodies. These tests have been helpful in identifying Hardjo-infected herds and in serovar Hardjo control/eradication programmes (Pritchard, 2001). However, herds that are vaccinated against serovar Hardjo will also be positive in these various ELISAs, decreasing

3 Available at: https://leptosociety.org/proficiency_testing

their usefulness in regions where vaccination is a routine practice. OMP based tests are not yet widely available. While they may have a role in the diagnosis of incidental infections they are unlikely to have a role in control programmes for host maintained infections, such as serovar Hardjo, where naturally infected cattle produce weak or no response to outer membrane proteins, but where the major serological response is to outer envelope lipopolysaccharide antigens (Ellis *et al.,* 2000).

Problems with validation are a major constraint in assessing most ELISAs. Almost all have been validated against the MAT (using MAT titres of 1/100 or greater), which is an imperfect test, having a sensitivity of less than 50% in some chronic infections. Human investigators have attempted to over-come this problem by the use of Bayesian latent class models and random-effects meta-analysis studies (Limmathurotsakul *et al.,* 2012; Signorini *et al*., 2013), but the best validation possible is using sequential sera from culture positive cases (Goris *et al*., 2012). A small number of ELISAs for animals have been validated using sequential serum samples from experimental animals but not beyond 6 months post-challenge, while one commercial Hardjo ELISA has been validated against single serum samples from culture positive cattle.

C. REQUIREMENTS FOR VACCINES

1. Background

Leptospiral vaccines for veterinary use are suspensions of one or more strains of pathogenic *Leptospira* inactivated in such a manner that immunogenic activity is retained. Commercial vaccines are whole-cell products and are available globally for cattle, pigs and dogs. The leptospires are grown in suitable culture media that may contain serum or serum proteins. If used, serum or serum proteins should be removed from the final product. Vaccines may contain suitable adjuvants.

Vaccines are used in animals to protect both the animals and in-contact humans. They are a key tool in control or eradication programmes. Vaccines will not eliminate infection from an already infected host and therefore should be given prior to exposure. Commercial vaccines vary in their efficacy. A number of monovalent products used in cattle have been shown to produce clinical and microbiological protection for up to year, in contrast, a number of multivalent products have been shown to stimulate poor immunity. Vaccination programmes must be tailored to the target population and the efficacy of the product to be used. Ideally cattle should be vaccinated prior to possible exposure, and thereafter annually, with vaccination timed to precede major risk periods. A successful vaccination programme requires epidemiological studies to assess the incidence of different *Leptospira* serovars in a given population (Adler & de la Pena Moctezuma, 2010).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Proper selection of vaccine production strains is of utmost importance. Immunity induced by vaccination is largely serovar specific. A vaccine should be formulated for use in a particular animal species in a particular geographical region. It should contain only those serovars – and preferably those genotypes – that cause problems in the animal species, or that are transmitted by the animal species to other species in the region. Strains selected for use as master seed culture should be cloned on solid medium to ensure the absence of saprophytic *Leptospira* contaminants and uniformity of the culture.

Suitable strains should be further selected by their ability to grow to high yields under batch culture conditions.

Each component strain to be included in the final vaccine should be grown separately in liquid medium; preferably in a protein-free or low-protein medium.

The volume of each master seed culture should be amplified by growth for 2–10 days at 30°C ±2°C in a series of subcultures until a volume sufficient for use as a production seed culture is achieved. Cultures should be aerated and agitated as required.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Each subculture of the master seed culture should be checked for purity and for satisfactory growth. Purity can be checked by inoculating a loopful of culture into blood agar plates or into thioglycolate broth for incubation at 35–37°C for 2–5 days, and by examining a Gram-stained smear of culture sediment. Growth can be checked by dark-field microscopy. Each production seed culture should also be checked against its' homologous rabbit antiserum to ensure purity and homology. MAbs may also be used for this purpose.

2.1.3. Validation as a vaccine strain

There is a large volume of literature describing the efficacy of leptospiral vaccines. In most cases, vaccines provide significant protection against disease produced by homologous challenge under field conditions.

Vaccines are less efficacious at preventing infection in animals and a percentage of vaccinated animals will become infected with the relevant serovar and may shed the organism in their urine despite a lack of clinical signs of disease.

Efficacy trials and vaccine validation must be conducted in the target species for the vaccine. The vaccine should be administered as recommended on the label, and immunity should be tested by challenge with virulent field strains of each serovar by natural routes of infection, i.e. by conjunctival and/or vaginal challenge. Validation studies have often been conducted with challenge of immunity by intravenous or intramuscular injections of leptospires. Vaccines validated in this way have not always been shown to be protective against field challenge, which occurs by exposure of mucous membranes of the eye, mouth, and genital tract to leptospires. Most notably, commercial leptospiral vaccines containing serovar Hardjo have not always protected cattle from conjunctival or field challenge with serovar Hardjo. A draft monograph for the efficacy testing of serovar Hardjo vaccines has been prepared and specifies the use of more natural routes of challenge (European Pharmacopoeia monograph).

2.2. Methods of manufacture

2.2.1. Procedure

Manufacture is carried out by batch culture in appropriately sized fermentor vessels. These should be equipped with ports for the sterile addition of seed culture, air, and additional medium. They should also have sampling ports so that the purity and growth of the production culture can be monitored.

Ideally, low-protein or protein-free media are used for production. However, some strains require the presence of animal protein to achieve suitable yields; this is usually supplied as BSA. All media components that are not degraded by heat should be heat sterilised. This reduces the risk of contamination by water-borne saprophytic leptospires that are not removed by filter sterilisation.

After addition of the seed culture, the growth of the production culture is monitored at frequent intervals for the start of log-phase growth. Once this is observed, the vessel is then agitated and aerated. The final yield can often be improved by the addition of more Tween 80 to the culture when log-growth is first observed to be slowing down. Adequate growth may require up to 10 days of incubation at 30°C ±2°C.

Inactivation is usually by the addition of formalin, but phenol, merthiolate, and heat inactivation have also been used; the regulations of each country should always be taken into account.

After the appropriate inactivation period, the culture may be concentrated and extraneous protein material may be removed by ultrafiltration. Suitable volumes of the various strains to be included in the final vaccine can then be blended, and adjuvant and preservative added, if appropriate.

2.2.2. Requirements for ingredients

All products of biological origin, in particular BSA, must originate from a country with negligible risk of transmissible spongiform encephalopathies (see chapter 1.1.8).

2.2.3. In-process control

During production, daily or twice daily subsamples should be taken and monitored for growth of leptospires and absence of contaminants. Growth is monitored either by counting leptospires in a counting chamber under dark-field microscopy or by a nephelometer. The absence of contamination can be monitored by the microscopic examination of Gram-stained preparations of centrifuged culture.

Immediately prior to inactivation, a sample should be taken for checking against its homologous antibody in a MAT. The inactivated culture must be checked for freedom from viable leptospires. This is done by inoculating aliquots of inactivated culture into an appropriate growth medium, such as the medium of Johnson & Harris, incubating at 30°C ±2°C for at least 4 weeks, and examining weekly by dark-field microscopy for the presence of viable leptospires.

After blending, the levels of free inactivating agents, minerals present in adjuvants (such as aluminum), and preservative (such as thiomersal) must be within prescribed limits.

2.2.4. Final product batch control

i) Sterility

Selected samples of the completed vaccine should be tested for the absence of viable bacteria and fungi (European Pharmacopoeia, 2002a; 2002b; 9CFR 113.26). Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Identity checks should be carried out on the product before inactivation, usually by checking with appropriate antisera,

iii) Safety

Samples of completed product should be tested for safety. Methods for this have been described elsewhere (European Pharmacopoeia, 2002a; 9CFR 113.38). The test should be carried out for each route of inoculation indicated on the label and in two healthy animals of each category (e.g. pregnant animals, young stock) for which the vaccine is intended. The animals must be susceptible to the serovars used in the vaccine and their sera must be free from agglutinating antibodies to those serovars. Each animal is given an injection of the vaccine by the recommended route with twice the recommended dose, as stated on the label. The animals are observed for 14 days and should show no adverse local or systemic effects attributable to the vaccine.

iv) Batch potency

The use of target animal batch release safety tests or laboratory animal batch release safety tests should be avoided wherever possible. Samples of completed vaccine should be tested for potency in hamsters or guinea-pigs. Potency is usually measured by the vaccine's ability to prevent the death of the animal when challenged with a lethal dose. With some serovars that are not hamster or guinea-pig lethal, such as serovar Hardjo, potency is measured against prevention of renal infection when the animals are challenged with between 10 and 10,000 ID_{50} (50% infectious dose) or by induction of a suitable antibody titre in rabbits.

An example protocol is to inject 1/40 dog dose of the vaccine into each of ten healthy hamsters no more than 3 months old. After 15–30 days, each vaccinated hamster, and each of ten unvaccinated hamsters of the same age, is injected intraperitoneally with a suitable quantity of a virulent culture of leptospires of the serovar used to make the vaccine (or a suspension of liver or kidney tissue collected from an experimentally infected animal). In the

case of bivalent vaccines, each serovar is tested separately. For the vaccine to pass the test, at least 80% of the controls should die showing typical signs of *Leptospira* infection and at least 80% of the vaccinated animals should remain in good health for 14 days after the death of the controls. Other protocols may apply to cattle and pig vaccines, which contain as many as five or six components. The European Pharmacopoeia uses five vaccinated and five control animals.

In-vitro potency tests for leptospiral vaccines are being developed based on quantifying the protective antigen in the vaccine using MAbs in a capture ELISA and are currently coming into use (Klaasen *et al.*, 2013).

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

2.3.2. Safety requirements

Tests use single dose and repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) containing the maximum permitted payload and according to the case, the maximum number of vaccine strains.

i) General safety in the target animal

One example is to use no fewer than ten healthy target animals that do not have antibodies against *Leptospira*. Administer to each animal a double dose of the vaccine by the method recommended on the label. Observe the animals each day for 14 days. If adverse reactions attributable to the biological product occur during the observation period, the vaccine is unsatisfactory. If adverse reactions occur not attributable to the biological product, the test shall be declared inconclusive and has to be repeated (European Pharmacopoeia, 2002a; 9CFR; United States Department of Agriculture Standard Requirements § 9. CFR, 113).

ii) Safety in pregnant animals

If the vaccine is intended for use in pregnant animals, use no fewer than ten healthy animals at the stage of pregnancy that accords with the recommended schedule or at different stages of pregnancy. Administer to each animal a double dose of the vaccine by the method recommended on the label. Observe the animals at least until 1 day after parturition. The vaccine complies with the test if the animals do not show abnormal local or systemic reactions, signs of disease or die for reasons attributable to the biological product and if no adverse effects on the pregnancy or the offspring are noted.

iii) Precautions (hazards)

Vaccine should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label or leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure that it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

A minimum of ten vaccinates and ten controls (in the case of cattle) and eight vaccinates and eight controls (in the case of dogs) should be challenged with each serovar included in the vaccine. Animals should be vaccinated according to the proposed field use. Challenge should take place by a natural route. Animals should be of an age and reproductive status appropriate to any subsequent claims. Animals should be slaughtered (if they have not previously died) 28–35 days post-challenge and appropriate tissues cultured. Daily clinical examinations should be carried out. Blood should be cultured on days 4–7 post-challenge and on any day pyrexia is detected. Urines should be examined for the presence of *Leptospira* 14, 21 and 28 days post-challenge. Kidney and urine should be cultured at slaughter. If protection of the genital track is to be included in a protection claim, uterus and oviduct should also be cultured at slaughter. In the event of an animal dying, urine, kidney and liver should be cultured. For a claim of efficacy, 80% of vaccinates should be protected and at least 80% of controls infected.

2.3.4. Duration of immunity

Duration of immunity should be determined in the animal species for which the vaccine is intended using natural routes of challenge. Duration of immunity should not be estimated based on the duration of MAT titres in vaccinated animals as protection against clinical disease may be present with very low titres. Vaccinal immunity should persist for at least 6 months or longer depending on the label claim.

2.3.5. Stability

When stored under the prescribed conditions, the vaccines may be expected to retain their potency for 1–2 years. Stability should be assessed by determining potency after storage at 2–5°C, room temperature, and 35–37°C.

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NB: There are WOAH Reference Laboratories for Leptospirosis
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for leptospirosis

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.1.13.

MAMMALIAN TUBERCULOSIS (INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS* COMPLEX)

SUMMARY

Mammalian tuberculosis is a chronic bacterial disease of animals and humans caused by members of the Mycobacterium tuberculosis *complex* (*MTBC*). *Within the complex, important variants include* M. bovis, M. caprae *and* M. tuberculosis. *The disease is global in distribution, with only a few countries considered free. It is a major infectious disease of cattle, other domesticated animals, and certain wildlife populations. Zoonotic tuberculosis resulting from transmission to humans constitutes a public health problem.*

Aerosol exposure to the agent is the most frequent route of infection, but infection by ingestion of contaminated material also occurs. Characteristic tuberculous lesions occur most frequently in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes. Tuberculosis is typically a chronic disease, with clinical signs that may appear after several months to years. Infection is often subclinical; when present, clinical signs are not specifically distinctive and can include weakness, anorexia, emaciation, dyspnoea, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis. Tuberculosis in cattle, goats and deer is usually diagnosed in the live animal by tests for cellular immunity (*skin test or gamma interferon test*). *Enzyme-linked immunosorbent assay* (*ELISA*) *and lateral flow tests for serum antibodies can be useful for wildlife studies. After death, infection is diagnosed by necropsy, histopathological, bacteriological and nucleic acid detection techniques.*

Detection of the agent: *Bacteriological examinations may consist of the demonstration of acid-fast bacilli by microscopic examination of tissues or secretions, which provides presumptive identification. Confirmatory tests include the isolation of mycobacteria on selective culture media and their subsequent identification by cultural and biochemical tests or DNA techniques. The polymerase chain reaction* (*PCR*) *test may be used for the detection of members of the MTBC in clinical specimens. Spacer oligonucleotide typing* (*spoligotyping*), *mycobacterial interspersed repeat units–variable number tandem repeat* (*MIRU-VNTR*) *and whole genome sequencing are used for genotyping members of the MTBC for purposes of strain tracking and epidemiology.*

Delayed hypersensitivity test **(*tuberculin skin test*):** *This test is the standard method for detection of tuberculosis in live cattle, small ruminants, deer, pigs and camelids, among other species. The single test involves measuring the skin response after intradermal injection of tuberculin* (*purified protein derivative – PPD*). *The comparative tuberculin skin test with bovine* (*PPD-B*) *and avian* (*PPD-A*) *tuberculin is used mainly to differentiate between animals infected with the* M. tuberculosis *complex and those sensitised to tuberculin due to exposure to other mycobacteria or related genera. The decision to use the single or comparative test generally depends on the prevalence of tuberculosis infection and on the level of environmental exposure to other sensitising organisms. The recommended dose of bovine PPD-B in cattle is at least 2000 International Units* (*IU*) *and in the comparative tuberculin test, the doses should be no lower than 2000 IU each. Defined antigen skin tests are being evaluated and should prove useful for differentiation of infected from vaccinated animals.*

Blood-based laboratory tests: *Diagnostic blood tests include the interferon-gamma release assay* (*IGRA*), *which measures cellular immune responses to tuberculin or defined antigens, and the indirect ELISA and lateral flow assays, which detect antibody responses. The logistics and laboratory execution of some of these assays may be a limiting factor. The use of blood-based assays can be advantageous, especially with intractable cattle, zoo animals and wildlife, although interpretation of the test may be hampered by lack of validation data for some species.*

Requirements for vaccines and diagnostic biologicals: *The only currently available vaccine against* M. bovis *infections is bacille-Calmette-Guerin (BCG). However, BCG may sensitise animals to the tuberculin skin test and other tuberculin-based immunological tests. For this reason, vaccination of cattle is prohibited in many countries. Defined antigen skin tests or IGRA, once validated, should enable differentiation of infected from vaccinated animals. BCG vaccines have been granted regulatory approval for the immunisation of certain wildlife species such as the badger, and trials are in progress to evaluate the use of BCG in cattle in a number of countries.*

Methods for the production of bovine PPD tuberculins should comply with standard requirements for source materials, production procedures and precautions, added substances, freedom from contamination, identity, safety, potency, specificity and freedom from sensitising effect. The bioassays for biological activity are of particular importance, and the potency should be expressed in International Units (IUs) calibrated against the International Standard Bovine Tuberculin (ISBT).

A. INTRODUCTION

Mammalian tuberculosis is a chronic granulomatous disease of animals and humans that results from infection with pathogenic members of the *Mycobacterium tuberculosis* complex (MTBC). MTBC members belong to the family Mycobacteriaceae and are Gram-positive, acid-fast bacilli. The taxonomy of organisms in the MTBC is in flux. Recent genomic analyses suggest that all MTBC members belong to a single species – *M. tuberculosis*, with *M. africanum*, *M. bovis*, *M. caprae*, *M. microti* and *M. pinnipedii* considered heterotypic synonyms (variants) of *M. tuberculosis*; and *M. canettii*, *M. mungi*, and *M. orygis* recognised as strains of *M tuberculosis*. To retain linkage with historical nomenclature, the more widely recognised designations are used in this Chapter instead of infra-subspecific designations. Hence, we continue to use designations *M. bovis* or *M. caprae* instead of *M. tuberculosis* var. bovis or *M. tuberculosis* var. caprae, respectively, for ease of prior association.

While there is considerable evidence of host-association amongst members of the MTBC, currently all mammalian species are considered to be susceptible to tuberculosis, and nearly identical disease may potentially result in any host species from infection with any member of the complex. The primary pathogens associated with domesticated and wild animals include *M. bovis*, *M. caprae*, *M. microti, M. orygis* and *M. pinnipedii.* Human exposure to any member of the MTBC (apart from BCG vaccine strain) may result in zoonotic infection. *Mycobacterium tuberculosis* and *M. africanum* are primarily pathogens of humans, but these too are known to infect animals. While *M. canettii* has only been described in humans in Africa, it is considered to be a spillover transmission from a potential wild animal reservoir that has yet to be determined. *Mycobacterium mungi* has been recovered from banded mongooses (*Mungos mungo*), and *M. suricattae* from meerkats (*Suricata suricatta*), but neither organism has yet been described from other host species. *Mycobacterium microti* is often diagnosed in cats due to transmission from the rodent maintenance hosts. *Mycobacterium microti* has also been found widespread in wild boar. *Mycobacterium tuberculosis sensu stricto* may be found as anthroponoses in elephants and companion animals. Within the complex, *M. bovis*, *M. caprae* and *M. tuberculosis* are considered important variants in terms of broader host range, and public health perspective.

The disease is global in distribution. For up-to-date information, consult the WOAH WAHIS interface[1]. A number of countries are considered free of tuberculosis in livestock. In others, with active animal tuberculosis surveillance and control programmes, there are only infrequent disease reports. The disease remains endemic and largely uncontrolled in Africa, Asia, Latin America and most countries in the Middle East. Wildlife reservoirs of tuberculosis have been described in several countries, and members of the MTBC are frequently recovered from free-living and captive wildlife hosts. Spillover from suspected maintenance wildlife reservoir hosts to cattle and other livestock have been reported in certain regions of Canada (from elk, *Cervus canadensis*), the Iberian peninsula (wild boar, *Sus scrofa,* and several cervid species), Ireland and the United Kingdom (European badger, *Meles meles*), New Zealand (primarily the brush-tailed possum, *Trichosurus vulpecula*), South Africa (African buffalo, *Syncerus caffer*), and in certain regions of the United States (white-tailed deer, *Odocoileus virginianus*). In regions where MTBC circulates in wildlife, multiple spillover hosts ranging from rodents and small mammals to carnivores and ungulates may exist. In regions with high prevalence of human tuberculosis and close interface with livestock, humans may represent a maintenance host for livestock infections, with spillover from humans to animals reported for *M. tuberculosis, M. bovis* and *M. orygis*.

There is increasing evidence of regional and geographical variation in distribution of members of the MTBC reported amongst livestock species. For instance, while *M. bovis* appears the predominant cause of tuberculosis in

1 https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/

cattle in Africa, much of Western Europe, and the Americas, *M. caprae* appears to be the major cause of tuberculosis in cattle in central European countries. Similarly, there is increasing evidence of *M. tuberculosis*, *M. orygis*, and *M. caprae* representing major causes of TB in cattle in South Asia.

Tuberculosis is typically a chronic disease, with clinical signs that may appear after several months to years. Infected animals may remain quiescent for an extended duration and reactivate after several years or when immunosuppressed. Acute or per-acute infections occur rarely in animals, and if they do, are likely in animals with an underlying co-morbidity such as being immunosuppressed.

Infection typically occurs through the inhalation, ingestion or direct contact of infected material through mucus membranes or breaks in the skin. Risk of infection may depend upon dose, route of infection, or a number of pathogen, host or animal husbandry related variables. Younger animals are considered to be more susceptible to infection, as are immunosuppressed, pregnant or malnourished animals.

While inhalation of infected respiratory droplets is considered as the primary mode of transmission for MTBC in livestock species, infection through ingestion of contaminated milk (particularly in neonates), feed, or water as well as through contact with infected exudates, urine, faeces, as well as vaginal secretions and semen may also occur. Inhaled bacilli are phagocytosed by alveolar macrophages. Depending on factors including dose and route of infection along with pathogen and host covariates, the pathogen may not survive to initiate an active infection, or the macrophages may successfully clear infection. When the infection is not immediately cleared, the organism may remain quiescent, or actively proliferate. Active proliferation of the organism and the failed host response to contain the infection results in focal or disseminated lesions that consist of dead and degenerate macrophages surrounded by multiple lymphoid origin cells. These later coalesce into multinucleated giant cells, with caseation or calcification of the necrotic centre followed by formation of a granuloma surrounded by a fibrous capsule – the prototypical “tubercle”.

These granulomatous lesions vary considerably in size and are often encapsulated. They may be caseous, caseo-calcareous, or calcified in cattle and many other mammalian species. In cervids or other animals with a more rapidly progressing disease, pus-filled abscesses are apparent instead of the classic tubercles. While almost any organ system might appear lesioned, in animals where inhalation is the route of infection, tubercles are often observed in the lungs as well as in cranio-thoracic lymph nodes. In contrast, in animals in which the primary route of infection is ingestion, abdominal organs as well as mesenteric lymph nodes are more likely to harbour lesions. In some animals, including those with advanced disease, widely disseminated infections may occur and result in miliary tuberculosis with small foci scattered throughout a tissue.

The involvement of draining regional lymph nodes is common in cases of active TB. In a subset of animals, lesions remain localised, resulting in chronic disease and persistent infection. In some immunosuppressed animals or those with advanced infection, lymphatic or haematogenous dissemination may occur. This results in a generalised infection, or miliary TB, with nodular lesions in multiple organ systems including the lungs, liver, kidney, spleen, mammary glands, gastrointestinal tract and the central nervous system. In such advanced cases, the disease is invariably fatal if left untreated.

There are no pathognomonic or distinctive clinical features associated with mammalian tuberculosis. The disease has a typical slow onset, and infected animals may actively transmit infection without displaying any apparent signs. The most frequently observed clinical signs in cattle include a progressive weight loss, weakness, inappetence, low-grade elevated or fluctuating body temperature, coughing and lymphadenopathy.

The enlargement of superficial lymph nodes is often observed. In advanced cases, these may rupture and drain. Retropharyngeal lymph nodes may also be affected, and the enlargement of deeper lymph nodes may result in obstruction of blood vessels or lymphatics in the respiratory and or gastrointestinal tracts. Respiratory involvement is usually indicated by a moist, intermittent cough that is worsened in cold conditions or during exertion, with the onset of dyspnoea or tachypnoea in more advanced cases. Alterations in gastrointestinal function and motility, including diarrhoea or, less frequently, constipation may be observed. Red deer, other cervids and camelids often have an accelerated disease (as compared with cattle) with multi-organ involvement. Camelids and equids may present with more gastrointestinal signs. Elephant do not show typical signs until very late in disease.

The ante-mortem diagnosis of tuberculosis in animals may be performed by direct detection of the organism, or indirectly, by evidence of host immune response to infection using the tuberculin skin test (TST), the interferon-gamma release assay (IGRA) or antibody assays. In some animal species such as non-human primates and small companion animals, radiological examination and echography may be used to supplement other tests. Given the chronic nature of infection and absence of definitive clinical signs, evidence of tuberculosis in animals is often only identified post-mortem during slaughter surveillance or at necropsy by identification of characteristic lesions. Since

a substantial fraction of animals that are classified as reactors by the tuberculin skin test may not show visible lesions at necropsy, bronchial, mediastinal and other lymph nodes are routinely sampled during slaughter surveillance, for the confirmatory detection of infection.

Direct microbiological detection of tuberculosis is most often performed by culture of tissue samples, exudates and other suspect fluids or secretions, or trunk washes in elephants. Presumptive diagnoses may be made by microscopic identification of characteristic acid-fast bacilli in secretions or tissue samples or histopathological staining of multinucleated giant cells with central caseating necrosis; however, the microscopic test can often give negative results, even in sick animals, due to the limited presence of mycobacteria. Mycobacterial culture is performed by growth on selective solid or liquid media, followed by confirmatory assays that include identification of mycobacterial specific nucleic acid or other microbial biomarkers. Direct detection approaches such as culture are the definitive techniques for diagnosis of tuberculosis in animals, but are time-consuming and difficult to perform. For this reason rapid methods such as polymerase chain reaction (PCR) are increasingly used for the direct detection of DNA from biological specimens.

Evidence of immune response of animals to MTBC organisms is the most commonly used indirect approach for the identification of presumptively infected animals. The primary screening test in animals, the TST, is an *in vivo* measure of the delayed hypersensitivity response to antigens present in MTBC organisms. The assay is carried out by the intradermal injection of a purified protein derivative (or tuberculin) derived from a well-defined strain of *M. bovis* (PPD-B), and measurement of the subsequent increase in skin-fold thickness at the injection site. The site of test administration depends on species of animal. In regions with high exposure of animals to environmental mycobacteria, the difference in skin thickness to PPD-B and purified protein derivative from a *M. avium* strain (PPD-A) is used to improve specificity, but often at the cost of sensitivity. Given the historical use and success in enabling test and remove based control programmes, the TST is the preferred test for diagnosis of tuberculosis and for screening pre-trade.

The *in vitro* IGRA provides an alternative measure of the cellular immune response to infection. This assay measures the release of gamma interferon from blood cells stimulated with antigens derived from MTBC organisms. This assay is often used as an ancillary test to remove additional positive animals and may be more convenient than the skin test since it does not require repeated handling of the animal or extended intervals prior to retesting. However, since the IGRA requires time-bound stimulation of live blood cells, it may prove costly, cumbersome or difficult to implement, especially in remote or low resource settings.

Since specific antibodies to MTBC infection are thought to develop later during infection, serological or antibody tests are considered less sensitive than the cell mediated immune response assays. The most frequently used serological assays include enzyme linked immunosorbent assays (ELISA) or lateral flow assays that measure antibodies to specific MTBC antigens. Antibody assays may be particularly useful in detection of evidence of immune response to tuberculosis in wildlife species. In livestock as well as certain cervid and camelid species, the prior administration of the skin test antigen within a specified time period is needed to ensure assay sensitivity.

Vaccines such as bacille-Calmette-Guerin (BCG) have been evaluated for use in livestock and wildlife species. In cattle, BCG is reported to have only modest levels of direct efficacy based on meta-analyses of experimental challenge studies and field trials. However, taken together with potential indirect effects of reduced onward transmission, simulation models suggest that implementation of vaccine programmes in endemic regions where test and cull are not feasible, may considerably accelerate TB control efforts.

B. DIAGNOSTIC TECHNIQUES

Most members of the MTBC are zoonotic organisms. Animal specimens and bacterial cultures that may contain live mycobacteria should be handled at an appropriate biosafety and containment level determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). This should include use of biological safety cabinets where appropriate.

In cattle, clinical evidence of tuberculosis is usually lacking until very extensive lesions have developed. For this reason, its diagnosis in individual animals and establishment of surveillance and eradication programmes were not possible prior to the development of tuberculin by Koch in 1890. Intradermal inoculation of tuberculin, a concentrated sterile culture filtrate of tubercle bacilli, provides an indirect means of detecting previous exposure or active infection by measuring the delayed hypersensitivity immune response. In companion animals and in valuable exotic/zoo species, the application of radiography is frequently applied as a presumptive diagnostic.

The presence of species of the MTBC in clinical and post-mortem specimens may be demonstrated by examination of stained smears or tissue sections and confirmed by cultivation of the organism on primary isolation medium. Collection containers should be clean and sterile. Non-sterile sampling containers may result in the failure to identify members of the MTBC due to the rapid growth of contaminating environmental mycobacteria or other organisms. Single-use plastic, disposable containers, 50 ml in capacity, may be used for a variety of specimen types. Specimens that are to be sent to the laboratory must be cushioned and sealed to prevent leakage, and properly packaged to withstand breakage or crushing in transit. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) for shipping specimens from a suspected zoonotic disease must be followed as summarised in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*. Delivery of specimens to the laboratory within 48 hours greatly enhances the chances of cultural recovery of MTBC members. If delays in delivery are anticipated, specimens should be refrigerated or frozen to retard the growth of contaminants and to preserve the mycobacteria. In warm ambient conditions, or when refrigeration is not possible, the tissue may be stored in a saturated solution of sodium borate, but only for limited periods, no longer than 30 days.

The main diagnostic methods and their fitness for purpose are summarised in Table 1 and described in the following sections. This is not an exhaustive listing but indicates test appropriate for different purposes in different host species.

Table 1. Test methods available for use in cattle, goats, and camelids and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Acid-fast staining and microscopy	–	–	–	+	–	–
Bacterial isolation	++	–	++	+++	++	–
Histopathology and antigen detection	+	–	+	+	–	–
Real-time PCR (direct from specimens)	++	–	++	+++	++	–
Detection of immune response						
Delayed hypersensitivity skin test	+++ (**+++** / **+**)	+++ (**++** / **+**)	+++ (**++** / **+**)	++	+++ (**++** / **+**)	–
IGRA	++ (**++** / **+**)	++ (**+** / **+**)	+++ (**++** / **+**)	+ (**–** / **–**)	+++ (**++** / **+**)	–
ELISA antibody test	+ (**–** / **++**)	+ (**+** / **++**)	+ (**–** / **++**)	–	+ (**–** / **++**)	–
Lateral flow antibody test	+	+	+	–	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose. Methods whose fitness for purpose differ between cattle, goats and camelids are represented in black colour for cattle, and differences for **goats** and **camelids** shown in parentheses in red and blue colours, respectively.
PCR = polymerase chain reaction; IGRA = Interferon gamma release assay; ELISA = enzyme-linked immunosorbent assay.

1. Detection and identification of the agent

1.1. Microscopic examination

Mycobacterium spp. may be suspected microscopically on direct smears from clinical samples and on prepared tissue materials using the classic acid-fast Ziehl–Neelsen stain, but a fluorescent acid-fast stain may also be used. Immunoperoxidase techniques may also give satisfactory results. The presumptive diagnosis of mycobacteriosis can be made with haematoxylin and eosin staining if the tissue has characteristic histological lesions representing the animal's immune response to the infection (caseous necrosis, mineralisation, epithelioid cells, multinucleated giant cells and macrophages). As lesions are often paucibacillary, acid-fast organisms may not be detected in histological sections, even though MTBC organisms can be isolated in culture. However, large numbers of acid-fast organisms are seen in lesions in primates, felids, mustelids (badgers) and marsupials (brush-tailed possums).

1.2.Culture

Successful recovery of mycobacteria by bacteriological culture depends on the sampling technique at the abattoir, the type of tissue specimens collected and their preservation in transit to the laboratory (refrigeration or freezing), together with the chemical decontamination step and the type of culture media chosen to grow mycobacteria.

The best tissues for the isolation of MTBC organisms are mainly collected during post-mortem examination from animals tested positive to tuberculin or interferon gamma tests. Abnormal lymph nodes and parenchymatous organs with TB-compatible lesions should be collected for examination and culture. If pathological lesions are not detected then, specific lymph nodes from the head, respiratory and/or digestive locations should be taken for the same purpose. Pets or zoo animals with suspected tuberculosis can be tested with biopsy specimens from lymph nodes, oral swabs or tracheo-bronchial wash.

To process specimens for culture, the tissue samples are prepared by removal of extraneous material (e.g. fat), cut into small pieces, pooled, and homogenised using a stomacher or tissue grinder (e.g. mortar, pestle, blender, etc.) with sterile PBS, followed by a mandatory decontamination. The purpose of the decontamination step is to avoid growth of other bacteria present in the tissue samples. Due to the cell wall characteristic of mycobacteria, they can resist certain detergents or extreme changes in the pH. A variety of protocols has been described, for example treatment with 0.375–0.75% hexadecylpyridinium-chloride detergent (HPC), 2–4% sodium hydroxide, 5% oxalic acid or 4% sulfuric acid, among others. It is important to control the time of exposure of tissues to the decontaminant to avoid death of the mycobacteria. Depending on the amount of tissue and decontaminant it usually ranges between 10 and 20 minutes. Where acid or alkali methods are used they must then be restored to a neutral pH after decontamination at room temperature. The suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture and microscopic examination.

For primary isolation, the sediment is usually inoculated on to a set of solid and/or liquid media. Different solid egg-based media can be used such as Lowenstein–Jensen, Coletsos base or Stonebrink. These media should contain either pyruvate or pyruvate and glycerol. Solid agar-based media such as Middlebrook 7H10 or 7H11 or blood-based agar medium may also be used (Cousins *et al*., 1989, Gormley *et al.*, 2014).

Liquid culture systems are used routinely in some laboratories; in these systems growth is measured by fluorometric means. Liquid media offer a shorter time of growth and relatively rapid mycobacterial detection. However, their disadvantages are a higher contamination rate and the impossibility of making an initial diagnosis based on the colonies' morphological characteristics. PCR is a useful method to confirm bacterial growth in liquid media. Additionally, it should be considered that some decontaminants (e.g. HPC) are not compatible with some liquid systems.

Cultures are incubated for a minimum of 6 weeks, preferably for 10–12 weeks (and up to 16 weeks when there is a suspicion of *M. microti*) at 37°C with or without CO_2. The incubation period also depends on the use of liquid or solid media. The media should be in tightly closed tubes to avoid desiccation. When solid media are used slopes are examined for macroscopic growth at weekly intervals during the incubation period. When growth is visible, smears can be prepared and stained by the Ziehl–Neelsen technique.

Alternatively, DNA can be extracted from colonies and any specific PCR for mycobacteria or MTBC species can be performed (see section 1.3 Nucleic acid recognition methods). Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used, the presence of tuberculosis compatible lesions and/or the initial bacterium load in the tissue specimen.

If gross contamination of culture media occurs, the culture process should be repeated using retained inocula with an alternative decontaminating agent or modifying the concentration or time of exposure to the decontaminant. The limiting factor in isolation is often the poor quality of the samples submitted and every effort should be made to ensure that the laboratory receives good quality samples.

Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis; however every isolate needs to be confirmed. It is often necessary to distinguish *M. bovis* from the other members of the MTBC, including *M. tuberculosis, M. africanum M. caprae. M. microti* and *M. pinnipedii*.

Mycobacterium avium or other environmental mycobacteria may also be isolated from tuberculosis-like lesions in cattle. In such cases, definitive identification of the causative agent may be needed, and mixed infections excluded.

Isolates may be presumptively identified from their cultural and biochemical properties, although this can be time consuming due to the slow growth of the members of the MTBC. On a suitable pyruvate-based solid medium, colonies of *M. bovis* are smooth and off-white (buff) in colour. The organism grows slowly at 37°C but does not grow at 22°C or 45°C. One approach to differentiate MTBC from non-tuberculous mycobacteria is through growth in media containing 500 mg/litre p-nitrobenzoic acid. MTBC do not grow while most non-tuberculous mycobacteria do.

Mycobacterium bovis is a microaerophillic and nonchromogenic bacterium sensitive to thiophen-2-carboxylic acid hydrazide (TCH) and to isonicotinic acid hydrazide (INH). Drug susceptibility testing can be evaluated by different protocols, including growth on solid 7H10/7H11 Middlebrook agar medium, on egg-containing media, or liquid systems (ECDC, 2018). The egg medium should be prepared without pyruvate because it inhibits INH and could have a similar effect on TCH (which is an analogue of INH) and thus give false-positive (resistant) results. *Mycobacterium bovis* strains are also sensitive to para-amino salicylic acid and streptomycin. Effective drug concentrations are different for egg-based and agar-based media. Results for niacin production and nitrate reduction are negative in *M. bovis*. In the amidase test, *M. bovis* is positive for urease and negative for nicotinamidase and pyrazinamidase.

1.3. Nucleic acid recognition methods

1.3.1. Polymerase chain reaction

Rapid identification of isolates to the level of MTBC can be performed by use of polymerase chain reaction (PCR) targeting 16S–23S rRNA, insertion sequences such as IS*6110* or IS*1081*, or similar sequences representing MTBC-specific targets. Species-level identification of members of the MTBC may be achieved with molecular genetic approaches for detecting the presence or absence of genomic "regions of differences" or species-defining (single-nucleotide) polymorphisms. Sub-species identification may be achieved with molecular typing approaches such as spoligotyping or, increasingly, whole genome sequence analyses to aid in strain differentiation as well as in molecular epidemiologic analyses.

PCR is routinely used for the detection of members of the MTBC in clinical specimens (mainly sputum) in humans, and is increasingly applied for the diagnosis of tuberculosis in animals. A number of commercially available assays and various 'in-house' methods have been evaluated for the detection of members of the MTBC in fresh samples or fixed tissues. Of key importance is that the DNA extraction method to be employed includes a mechanical lysis step as well as the chemical lysis (Lorente-Leal *et al*., 2019). Various MTBC- or species-specific targets for amplification have been identified as noted above. The most common targets for PCR identification of members of the MTBC are the multicopy insertion elements IS*6110* or IS*1081* (or both). Several assays for real-time PCR-based detection of these two targets have been reported, with good to excellent performance characteristics as compared with either culture or histopathology (e.g. Courcoul *et al.,* 2014; Sanchez-Carvajal *et al.,* 2021). Examples of well validated primer and probe sequences include those in use for the USDA national animal tuberculosis surveillance program that target IS*1081*:

IS1081_F1 5'-GGC-TGC-TCT-CGA-CGT-TCA-TC;
IS1081_RI 5'-CGC-TGA-TTG-GAC-CGC-TCA-T;
IS1081_PI 5'-CTG-AAG-CCG-ACG-CCC-TGT-GC;

and for IS*6110*:

IS6110_F1 5'-CAG-GAC-CAC-GAT-CGC-TGA-TC;
IS6110_R1 5'- CTG-CCC-AGG-TCG-ACA-CAT-AG;
IS6110_P1 5'-CGT-CCC-GCC-GAT-CTC-GTC-CA,
amongst others (Dykema *et al.,* 2016).

Amplification products are preferably detected by hybridisation with probes in real-time assays or visualised by gel electrophoresis and staining. Commercial kits and the 'in-house' methods, in fresh, frozen or boric acid-preserved tissues often do not perform well during interlaboratory comparisons, and hence may require additional validation to ensure accuracy. False-positives, or false-negative results in specimens containing low numbers of bacilli, are often of concern. Pre-analytical sample processing including decontamination and DNA extraction procedures, elimination of polymerase enzyme inhibitors, etc., have considerable impact on assay performance. Use of standard operating procedures, internal and external extraction and amplification controls, procedures for the prevention of cross-contamination, and application of closed tube real-time PCR assays are highly recommended for the reliable molecular genetic identification of members of the MTBC.

1.3.2. DNA fingerprinting

A variety of DNA-fingerprinting techniques has been developed to distinguish members of the MTBC for molecular epidemiological purposes (Guimaraes & Zimpel, 2020; Merker *et al.,* 2017). These methods are useful in identifying and tracing sources of origin, as well as for tracking transmission and spread of MTBC members within herds and globally. Spacer oligonucleotide typing, or spoligotyping, is a PCR-based method that has been widely used for genotyping members of the MTBC (Kamerbeek *et al.,* 1997). The method involves amplification of chromosomally-encoded loci that contain a variable number of short direct repeat sequences interspersed with nonrepetitive spacers. Strain-dependent "spoligotype" patterns of the *in-vitro*-amplified DNA are then determined after hybridisation with multiple spacer oligonucleotides, and represented as digital codes. The implementation of standardised protocols and nomenclature for identification and designation of spoligotypes and the establishment of online searchable databases for pattern matching has greatly enabled interlaboratory comparison of spoligotypes for strain tracking and epidemiology[2] (see Couvin *et al.*, 2019). Spoligotyping has often been combined with mycobacterial interspersed repetitive units (MIRU)-variable tandem repeat (VNTR) typing to increase the discriminatory power of MTBC genotyping. Currently a 24-loci MIRU-VNTR PCR assay is used for *M. tuberculosis* (Supply *et al.,* 2006) but optimisation of the combination of loci for a specific region is recommended for *M. bovis* and *M. caprae* to decrease the cost and time spent performing the assays whilst maximising discriminatory power.

1.3.3. Whole genome sequencing

The genomes of all members of the MTBC have been sequenced, and whole genome sequencing is increasingly being used routinely to genotype and distinguish between isolates for epidemiological studies as well as for understanding transmission chains and dynamics and MTBC evolution (Guimaraes & Zimpel, 2020).

2. Delayed hypersensitivity test

2.1. The intradermal tuberculin test

The intradermal tuberculin test is the standard method for the detection of tuberculosis in a wide variety of mammals, including bovine, ovine, caprine and cervid species. The test involves the intradermal administration of tuberculin purified protein derivative (PPD) and the measurement of increase in skin

2 www.mbovis.org; www.pasteur-guadeloupe.fr:8081/SITVIT2/index.jsp

thickness at the site of injection resulting from a delayed hypersensitivity reaction after a specified time interval (for instance, 72 hours in cattle).

The TST is not recommended for companion animals such as cats and dogs, where radiographic examination has supplanted its use.

The TST may be performed by single intradermal test (SIT) using PPD-B alone, either as the single cervical test (SCT) or the caudal (tail) fold test (CFT); or by the comparative cervical test (CCT) that measures the difference in increased skin thickness between PPD-B and that to PPD-A. The SIT typically exhibits greater sensitivity than the CCT, while the CCT provides greater specificity. The latter is often used to account for potential sensitisation of animals to environmental mycobacteria or antigenically cross-reactive organisms other than the members of MTBC (Good *et al*., 2018).

Recently developed defined antigen skin tests (DST) may overcome some of the limitations of SIT and CCT with improved sensitivity as compared with CCT and specificity as compared with SIT. DST may also be used to differentiate infected from BCG-vaccinated animals (Srinivasan *et al.,* 2019).

The SCT and CCT are performed in the mid-cervical region of bovine, ovine, caprine and cervid species, whereas the CFT is performed in the caudal fold of the tail (but is not suitable for cervids). The hypersensitivity responses to tuberculin observed in the cervical region are greater than in the caudal fold. However, both SCT and CFT have been successfully applied for the surveillance, control and elimination of tuberculosis in cattle in a number of countries.

Tuberculin skin tests are not well-validated in species other than bovids and cervids. As a consequence, the approaches to application and interpretation of the SCT, CCT and CFT in bovines are often considered as the default in other species. Alternate sites for antigen administration and interval for measurement of hypersensitivity responses may be indicated in some species. For example, in swine, it is administered at the base of the ear, and the hypersensitivity reaction evaluated after 48 or 72 hours. For non-human primates, "old tuberculin" is administered intrapalpebrally to the upper eyelid (see below, also Chapter 3.10.10 *Diseases transmissible from non-human primates*).

While animals exposed to MTBC organisms exhibit hypersensitivity to tuberculin, a positive tuberculin skin test result is unable to distinguish actively infected animals from those that may have recovered from infection. The test must therefore be interpreted in context.

Delayed hypersensitivity reactions may not develop for a period of 3–6 weeks following infection and recently infected or immunosuppressed individuals may present as false-negative. Hence, declaring a herd to be "free from tuberculosis infection" requires negative results from several sequential intradermal tests performed at 6–8-week intervals of all animals in the herd. In certain instances, anergy or hypo-responsiveness to tuberculin may occur in chronically infected animals with severe pathology leading to false negative results. In addition, animals may become desensitised due to repeated administration of tuberculin, in particular when applied at intervals of fewer than 6 weeks, and true positives may be missed. In such suspected false negative cases, serological or defined antigen cell-mediated immune response assays may be used as alternate or confirmatory assays.

The decision to use the SCT, CFT and CCT is dependent on the local regulations and the overall context and goals for performing the test. The CCT with higher specificity is typically applied for surveillance purposes or at the start of a control programme in an endemic country. Once the presence of the disease is confirmed a higher sensitivity approach may be preferred to avoid missing infected animals. CCT is also useful in regions with high environmental *Mycobacterium* exposure. In contrast, SCT or CFT with higher sensitivity are frequently applied to confirm freedom from infection in low burden settings.

2.2. Test procedure

2.2.1. Intradermal administration of PPDs

Personnel conducting the test should be specifically trained and certified for testing of animals. Prior to antigen administration, the injection sites must be clipped and cleaned. While performing the mid-cervical test, the thickness of the skin fold at the intended injection site should be measured with callipers, and the site suitably marked. All measurements of skin thickness should

be recorded in whole millimetres as measurements in fractions of millimetres may provide a false sense of precision. The same individual should measure the skin fold thickness before the injection and after the specified time interval using the same callipers to avoid additional introduction of operator or equipment-related variation.

A short needle, bevel edge outwards and graduated syringe charged with antigen is then inserted obliquely into the deeper layers of the skin to assure the delivery of specific volume of tuberculin. Preferably this should be performed using a calibrated and well-maintained multi-dose syringe or multiple injection gun. Because of the thin and sensitive skin of cervids, sheep, goats, and other species, a narrow gauge needle (e.g. 25G), may be preferred in some cases. The potency of both PPD-B and PPD-A antigens should be estimated by biological methods comparing with reference standard PPDs. A minimum 2,000 International Units (IU) of PPD should be administered in a volume not exceeding 0.2 ml. A correct injection is confirmed by visualising and palpating a small pea-like swelling at the site of injection.

In case of the CCT, the distance between the two injection sites should be approximately 15 cm: to prevent errors, routinely PPD-A is injected in the top site and PPD-B in the lower site. In young animals or smaller breeds with insufficient space to prepare the two injection sites on the same side of the neck, one injection may be performed at identical locations in the centre of the middle third of both sides of the neck.

In the CFT, a short needle, bevel edge outwards, is inserted obliquely into the deeper layers of the skin on the lateral aspect of the caudal fold, midway along the fold and between the hairline and the ventral aspect of the fold. The injection site should be visually inspected closely and palpated carefully to detect changes from the normal.

The increase in skin-fold thickness at each injection site is then measured after 72 (± 4) hours interval or as prescribed depending on the species.

2.2.2. Interpretation of the test results in cattle

The interpretation of the skin test results is based on observation of clinical signs at the injection site and the recorded increase in skin thickness response following administration of antigen. Animals are considered as reactors when the increase in skin fold thickness exceeds a pre-specified threshold. The following guidelines may vary depending on the local context, and prevailing regulation. More stringent interpretations of test results, for example considering any palpable reaction as positive are permitted, and often recommended, e.g. in the CFT, to accelerate the local or national control programmes.

2.2.2.1. The single cervical test (SCT)

The single cervical test requires a single injection of PPD-B and the reaction is commonly considered to be negative if only limited swelling is observed, with an increase of 2 mm or less. The reaction is considered inconclusive if the increase in skin-fold thickness is more than 2 mm and less than 4 mm and considered positive if there is an increase of 4 mm or more.

ΔB	Interpretation of result
≤ 2mm	Negative
> 2 and < 4mm	Inconclusive
≥ 4mm	Positive

Animals that are inconclusive by the single intradermal test should be subjected to a second test after an interval of 6 weeks. Animals that repeatedly test as inconclusive should be considered as positive.

A more stringent interpretation is recommended, particularly in a high-risk population or in-contact animals.

2.2.2.2. The comparative cervical test (CCT)

In the interpretation of the CCT, a reaction to PPD-B is commonly considered negative if the increase in skin thickness is less than 2 mm. Depending on local legislation, the CCT is usually considered to be positive if the increase in skin thickness at the injection site of PPD-B is greater by more than 4 mm than the reaction shown at the site of the PPD-A. The reaction is considered inconclusive if the reaction to PPD-B is 2 mm or more, and is greater than the PPD-A reaction by 4 mm or less. All inconclusive reactors should be retested after 6 weeks. If the increase in skin thickness with PPD-B is less than or equal to the increase in that with PPD-A, the animal is classed as negative.

Animals that are inconclusive by the CCT should be subjected to a second test after an interval of 6 weeks. Animals that are positive or again inconclusive should be regarded as positive and be removed from the herd. To accelerate the clearance of TB from a known infected herd, a more stringent interpretation may be adopted wherein the inconclusive results may be interpreted as test positive in order to avoid the 6 weeks desensitisation waiting period. The presence/absence of clinical signs should also be considered in determining the herd status.

Interpretation of the comparative cervical test

First round of testing

ΔB (mm)	ΔB – ΔA (mm)	Interpretation of result
< 2	–	Negative
≥ 2	≤ 0	Negative
	> 0 and ≤ 4	Inconclusive
	> 4	Positive

Repeat testing inconclusives

ΔB (mm)	ΔB – ΔA (mm)	Interpretation of result
< 2	–	Negative
≥ 2	≤ 0	Negative
	> 0 and ≤ 4	Positive
	> 4	Positive

2.2.2.3. The caudal fold test (CFT)

The standard interpretation is that any swelling, sensitivity, or increase in skin thickness is considered to be a positive response to the bovine tuberculin. This interpretation may vary according to local regulations, for example in some countries an increase of >3mm may be used as the cut-off for positive reactions. The size of responses may vary and are not indicative of infectious status. Responses may be small, hard, pea-sized responses, diffuse responses, circumscribed responses, or large responses. If there is doubt about whether a response has occurred, the opposite side of the tail may be palpated to determine if there is a change from normal. Any observed change should be recorded. Test observation without palpation is unacceptable.

As is the case with the SIT, the use of the CFT as a screening test may result in false reactors to the test. Positive reactors to the CFT require secondary testing by using the CCT which must be performed within 10 days of the initial test, or else after 60 days.

2.2.3. Interpretation of test results in cervids

For cervid species (elk, reindeer, red deer, white tailed deer, sika, and others) only the mid-cervical test is used. In deer, both sites should be in the middle third of one side of the neck, the anterior site at least 100 mm behind the head and the posterior site approximately 130 mm from the other. In smaller deer, inoculation sites are used on each side of the neck.

Repeated skin tests should only be carried out at a 120-day interval to minimise desensitisation. In the EU, the same interpretation as for bovines is used in cervids for both the SCT and CCT. In the United States, the SCT is recommended as a primary test, and the CCT as a supplemental test in captive cervids. If an animal reacts to the SCT, a retest with the CCT after 120 days is indicated.

2.2.4. Interpretation of test results in sheep and goats

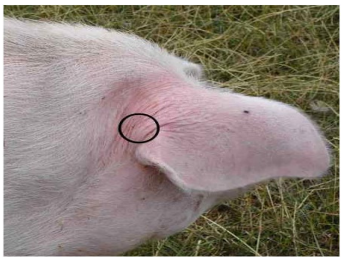
The skin test and its interpretation are less well validated in sheep and goats than in cattle and deer (see Roy *et al.*, 2020). The skin test can be performed in both species in the mid-cervical or the scapular area using the SCT or CCT or in the caudal fold. Other areas free of wool can be used, e.g. the upper inner areas of the rear legs, which avoids loss of wool quality. Given the small size of the neck in both species, the CCT is often performed by applying the PPDs on opposite sides of the neck. Evaluation of the SCT and CCT can be done by measuring and applying the same interpretation as for cattle, depending on the national legislation.

2.2.5. Interpretation of test results in camelids

The skin test can be performed at the axillary, cervical or prescapular regions. When both PPD-B and PPD-A are used, they should be injected intradermally on each side at identical sites (cervical, prescapular or axillary). In the cervical and prescapular sites, it may be possible to inject both PPDs on the same side if separated sufficiently. Evaluation of the SCT and CCT can be done by measuring and applying the same interpretation as for cattle, depending on national legislation.

Skin tests for camelids have shown lower sensitivity and specificity compared with that for cattle. In the EU, it is recommended to use as a complementary test to the serological protocol.

2.2.6. Interpretation in pigs



It is advisable to use the CCT with a dose of 2000 IU of each antigen. Reactions in pigs may be very pronounced and higher concentrations of PPDs may result in necrosis of the skin. The preferred site is the loose skin at the dorsal surface of the ear at the furrow between head and ear. The PPD-A and PPD-B are injected at the base of the left and right ear, respectively. The reaction can be read after 48 or 72 hours. Interpretation should follow either the same scheme as used for the CCT in cattle or by palpation, depending on local legislation.

2.2.7. Interpretation in exotic and zoo animals

For non-human primates, "mammalian old tuberculin" is administered intrapalpebrally in the middle of the upper eyelid adjacent to the edge of the lid, to detect tuberculosis caused by *M. tuberculosis* or *M. bovis.* If repeat testing is required, the eyelid should be alternated between each test. The readings are taken at 24, 48 and 72 hours post-injection. The reactions of grade 1 and 2 are negative, grade 3 inconclusive and grades 4 and 5 are interpreted as positive. See also chapter 3.10.10 *Diseases transmissible from non-human primates.*

Grade	Reaction
0	No Reaction
1	Bruise - extravasation of blood in the eyelid associated with the injection of tuberculin
2	Varying degrees of erythema of the palpebrum
3	Moderate swelling with or without erythema
4	Obvious swelling of the palpebrum with drooping with or without erythema
5	Necrosis of the eyelid with varying degrees of swelling, including eyelid partially or completely closed

Intradermal tuberculin tests are not recommended for use in elephants due to a high false-negative rate in culture-positive animals.

2.3. Defined antigen skin tests

Research directed toward identification and characterisation of the key antigenic components of field isolates of *M. bovis* and BCG vaccine strains has led to development of skin tests and blood tests that are based on detection of responses to molecularly defined antigens (Middleton *et al.*, 2021). These defined antigen diagnostic tests could potentially be utilised on their own or in combination with other tests, including conventional PPD tuberculin tests, to improve diagnostic sensitivity or specificity, as well as to

help differentiate *M. bovis*-infected animals from those which have been vaccinated or exposed to environmental mycobacteria (Srinivasan *et al.*, 2019).

3. Blood-based laboratory tests

Besides the intradermal tuberculin test, a number of blood tests that measure cellular or humoral immune responses of animals to MTBC have been developed. Due to cost and or complexity considerations these laboratory-based assays are often used as ancillary tests to improve detection of infected animals (parallel testing), or to confirm results of an intra-dermal skin test (serial testing). Administration of tuberculin skin tests is known to enhance the sensitivity of antibody-based tests, if serum is taken between 2-8 weeks after skin testing, leading to greater test accuracy. The IGRA measures cellular immunity while humoral antibodies are measured by serological methods such as ELISA and lateral flow assays.

3.1. Interferon-gamma release assay (IGRA)

In this test, the release of a lymphokine gamma interferon is measured in a whole-blood culture system. The assay is based on the release of interferon-gamma from sensitised lymphocytes during a 16- to 24-hour incubation period with specific antigen (PPD-tuberculin) (Wood *et al*., 1990). The test makes use of the comparison of interferon-gamma production following stimulation with avian and bovine PPD. The detection of interferon-gamma is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine interferon-gamma. It is recommended that the blood samples be transported to the laboratory avoiding extreme temperatures (e.g. a range of 17–27°C) and the assay set up as soon as practical, but not later than the day after blood collection (Coad *et al*., 2007). In some areas, especially where 'non-specificity' is prevalent, some concerns about the accuracy have been expressed when blood stimulation is performed with PPDs. However, because of the capability of the IGRA to detect early infections, the use of both IGRA and skin tests in parallel allows detection of a greater number of infected animals before they become a source of infection for other animals, as well as a source of contamination of the environment (Gormley *et al*., 2006). The use of defined mycobacterial antigens such as ESAT-6 and CFP-10 can improve the specificity (Buddle *et al*., 2001), and these antigens are employed in a number of countries such as the United Kingdom, New Zealand and France for serial testing. The use of such antigens may also offer the ability to differentiate BCG-vaccinated from unvaccinated animals. In animals that are excitable, difficult or dangerous to handle, the advantage of the IGRA over the skin test is that the animals need be captured only once. The IGRA has been approved for use in a number of national programmes including the EU, UK, USA, New Zealand, and Australia. In New Zealand and the United Kingdom for example, the IGRA is used for serial testing (to enhance specificity) and parallel testing (to enhance sensitivity). The test is available as commercial kits for bovine species and primates; however it has been validated in only a few species.

3.2. Serology for detection of specific antibodies

The application of serological assays for diagnosis of tuberculosis in animals has increased in the 21st century. ELISA is the most widespread technique, however alternative serological platforms have been developed (e.g. lateral flow tests, a multi-antigen print immunoassay or a multiplex chemiluminescent assay) (Bezos *et al*., 2014). Serological assays have been proposed as a valuable ancillary diagnostic tool to complement cell-based methods, increasing the detection of infected animals and helping to control tuberculosis in domestic and wild animals (Casal *et al*., 2017; Thomas & Chambers, 2021). In camelids it is recommended to use serology as a complementary test to the skin test and 15 to 30 days after skin testing. The advantages of these tests are their simplicity, low cost and lower logistical demands compared, for example, with the IGRA, as they do not require immunological stimulation with antigens, and samples can be stored for a prolonged time before processing. These tests could be especially helpful for detecting anergic animals that do not respond well to cell-based immune techniques. Their sensitivity, however, is lower than cell-based tests, particularly in recently infected animals, increasing in advanced stages of the disease. The use of combinations of specific antigens such as MPB83, MPB70, ESAT-6 and CFP-10 has been demonstrated to increase the sensitivity and specificity of serological tests (Thomas & Chambers, 2021).

In recent years, new antibody detection tests using different methodologies have also demonstrated better performance than skin tests in wildlife (wild boar, deer, badgers) and domestic animals (cattle, sheep, goats, alpacas, pigs). Examples include the P22 ELISA based on a multiprotein complex named P22 obtained by affinity chromatography from PPD-B, or a double-recognition ELISA that detects

specific antibodies against MPB83, using MPB83 protein as both an antigen coating the plate and as a conjugate (Cardoso-Toset *et al*., 2017; Casal *et al*., 2017; Infantes-Lorenzo *et al*., 2019). Moreover, the booster effect observed on the antibody titres caused by a recent intradermal tuberculin test in MTBC-infected animals can be used as an valuable diagnostic strategy to increase the sensitivity of serological tests at 15–30 days after PPD injection (Casal *et al*., 2014).

Serological tests are valuable for detecting MTBC infections in wildlife. A lateral flow test based on MPB83 protein and CFP10/ESAT-6 fusion protein, has been shown to be useful for detecting infection in domestic animals, but particularly in wildlife and zoo animals because it is easy to perform and gives immediate test results. Although their sensitivity is limited, these methods are useful where no cell-based tests are available and where skin testing has proven unreliable, (Greenwald *et al*., 2009; Lyashchenko *et al*., 2008; Thomas & Chambers, 2021).

WOAH has evaluated a number of tests for use in cattle serum and plasma as supplemental tools, together with other methods, for diagnosing and managing tuberculosis infection. See the WOAH Register of Diagnostic Kits for further information[3].

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

C1. Vaccines

Guidelines for the production of veterinary vaccines provided here and in Chapter 1.1.8, *Principles of veterinary vaccine production*, are intended to be general in nature, and may be supplemented by national and regional requirements.

The only currently available vaccine against *M. bovis* infections is bacille-Calmette-Guerin (BCG), a live attenuated strain of *M. bovis*. Trials have been conducted on a number of other vaccines, but none has been shown to induce a superior protection to BCG (Vordermeier *et al.,* 2016).

BCG has been shown to be safe in cattle and some other livestock as well as in free-living and captive wildlife. Studies have noted variable levels of efficacy in experimental and field trials in cattle. This variability has been attributed to factors including vaccine formulation, age of animal, route of vaccination, and exposure to environmental mycobacteria. The protection provided by BCG is due to a reduction in susceptibility of vaccinates to infection, as well as through reduction of onward transmission from vaccinated animals resulting from lowered pathology and infectiousness.

Similarly, to what is observed in humans, a systematic review and meta-analysis showed only modest (~25%) direct efficacy of BCG against bovine TB challenge in cattle (Srinivasan *et al.,* 2021). However, scenario analyses considering both direct and indirect effects suggest that disease prevalence could be substantially reduced up to official TB-free depending on the starting levels of infection, and 50–95% of cumulative cases averted over 50 years with BCG vaccination. For these reasons, BCG vaccination may help accelerate control of bTB in endemic settings, particularly with early implementation in the face of dairy intensification in regions that currently lack effective bTB control programs.

Experimental trials have established that protection wanes between one to two years post-vaccination, but revaccination when immunity has waned after two years boosted protection. BCG vaccination of infected cattle does not result in exacerbation of infection (Buddle *et al.,* 2016).

BCG vaccine strains including Danish 1331, Pasteur 1173P2 and Russia have been used at a dosage ranging from 10^4 to 10^6 colony-forming units (CFU) given subcutaneously. Since the use of vaccines may compromise tuberculin skin tests or other immunological tests, the use of molecularly defined tuberculin as diagnostic antigen is needed. Significant progress has been made in the development of so-called DIVA antigens that allow the differentiation of BCG-vaccinated from *M. bovis*-infected animals, particularly when used in the gamma-interferon test (Vordermeier *et al.,* 2016) or as skin test reagents (Srinivasan *et al.,* 2019) and are based on the use of gene products that are encoded on *M. bovis* gene regions that are deleted or not expressed in some BCG strains. Hence, feasibility of

3 https://www.woah.org/en/what-we-offer/veterinary-products/diagnostic-kits/the-register-of-diagnostic-kits/

implementation of BCG vaccination in cattle and other livestock species may be enabled by availability of DIVA tests once fully validated and the regulatory frameworks established.

BCG vaccines may also be used to reduce the spread of *M. bovis* in wildlife reservoirs of infection. BCG has been granted a limited regulatory approval in the UK for intramuscular vaccination of badgers at a dose of $2–8 \times 10^6$ CFU. Field trials have found that BCG significantly reduces infection from natural exposure to *M. bovis* in badgers, reduces the risk of infection in unvaccinated cubs in vaccinated social groups (Carter *et al.,* 2012) and in some cases may result in a lowering of cattle TB incidence (Martin *et al.,* 2020). Oral dosing of BCG to brush-tailed possums and European badgers in field trials have been shown to confer significant protection against natural exposure to *M. bovis* (Gormley *et al.,* 2017; Tompkins *et al.,* 2009). Oral dosing of possums and badgers with BCG vaccine has been shown to be safe, although transient shedding of BCG in faeces has been demonstrated (Perrett *et al.,* 2018).

C2. Production of tuberculin

Tuberculins are prepared from the heat inactivated liquid cultures of MTBC grown as pellicles on broth. These PPDs, precipitated using trichloroacetic acid (TCA) or ammonium sulphate, replaced the former heat-concentrated tuberculins, having a higher specific potency. Additional filtration steps to remove non-specific cell wall material further increased the specificity. In addition a mutant of *M. bovis,* named AN5, that showed "luxurious" growth as a pellicle on glycerol broth was selected to facilitate large scale economic production of PPD-B.

PPD-B produced from cultures of *M. bovis* AN5 was subsequently shown to be more specific in the intradermal assay in cattle than "human" PPD prepared from cultures of *M. tuberculosis* that had been used previously in eradication campaigns.

In the interests of standardisation, the *Terrestrial Manual* will continue to recommend use of PPD-B in the SCT, or in combination with PPD-A in the CCT, as the preferred reagent for intradermal skin testing and/or the IGRA in regions where BCG vaccination is not being considered. See Sections B.2 *Delayed hypersensitivity test* and B.3.1 *Gamma-interferon assay* for details of the test procedures. All PPDs used for national or local surveillance programmes should be validated and calibrated in comparison with established reference standards.

2.1. Seed management

Since the quality of PPD-B largely depends on the ability of the production strain to produce dominant T-cell antigens like ESAT-6, CFP-10 and Rv3615c as well as the ability to grow on glycerol broth, other virulent and well characterised *M. bovis* strains could be suitable for the production of PPD-B. However, in the interests of international standardisation and harmonisation of production and potency testing it is recommended that defined isolates of *M. bovis* AN5 or *M. bovis* Vallee should be used for PPD-B production. These have been in use for over 70 years and are well characterised. A record must be kept of their origins and subsequent history, including the number of passages from the original receipt from a Reference Laboratory (e.g. for AN5 since supplied by "Weybridge"), a spoligotype pattern and (where possible) whole genome sequence data. The strains of *M. bovis* used as seed cultures must be shown to be free from contaminating organisms.

2.2. Method of culture

If the source culture for production was a primary isolate grown on solid medium, it is necessary to adapt the organism to grow as a floating culture or pellicle (e.g. by incorporating a sterile piece of potato in the culture flasks of liquid media, such as Watson Reid's medium). When the culture has been adapted to pellicle growth on the liquid medium, it may be used to produce the Master Seed lot, which is preserved in freeze-dried form. All seed lots should be checked in a pilot study for their ability to support the production of a PPD-B of sufficient quality.

In accordance with GMP guidelines and to guarantee a constant quality of PPD-B, a Seed Lot System of Master and Working seeds for the well-characterised production strain will have to be established and stored at -70°C or below. The Working Seeds used to inoculate the production media must not be more than four culture passages from the Master Seed. As the Working Seeds lose the ability to produce a suitable PPD-B, they should be replaced at frequent intervals with new Working Seeds.

2.3. Method of manufacture and in-process control

For the production of PPD-B, Working Seeds are inoculated on glycerol broth (made from beef guaranteed free of TSEs) in Roux culture flasks and when a pellicle has been formed on top of the liquid medium, this pellicle will be divided and used to inoculate a larger number of penicillin flasks containing a synthetic production medium, e.g. Dorset-Henley, to avoid non-*M.bovis* proteins contaminating the final product.

During growth, which will take approximately 60 days, growth should be checked daily and when "abnormal" cultures are detected, e.g. showing changes in colour, signs of contamination, or sinking pellicles, these should be removed since they will negatively affect the quality of the final product. These abnormal cultures should be discarded after autoclaving.

Subsequently, the cultures are heat inactivated at 98–100°C for 2–3 hours and cellular debris is removed in a series of filtration steps, starting with coarse filters to remove larger particles and finally clarified using for example a Seitz series of filters with EK1 as the final sterilisation filter. The combination of different filters and their respective cut-off's as well as the number of each filter used depends largely on the volume to be processed but the removal of as much insoluble material is an essential step to maximise the specificity of the final product.

Proteins in the filtrate are precipitated by TCA (or ammonium sulphate depending on the producer). Precipitation is followed by a series of washing and centrifugation steps then alkaline treatment to bring the water soluble proteins to a neutral pH (6.5–7.0) in an isotonic glucose-phosphate buffer containing 0.04% w/v phenol.

The protein concentration of the final product or "concentrate" will be determined by the Kjeldahl method and is routinely presented as total nitrogen and TCA precipitable nitrogen.

The potency and specificity of the final product is determined in the guinea pig assay using the International standards for PPD-B and PPD-A, respectively.

Depending on the outcome of the potency assay, final dilutions are made for the commercial product with the same isotonic glucose-phosphate buffer pH 6.5–7.0 containing 0.04% w/v phenol, to obtain a minimal dose of 2000 IU in the intradermal assay. Currently, many commercially available PPD-Bs contain an estimated potency of 2500 or 3000 IU per dose to optimise sensitivity of the intradermal assay.

For the specificity of the PPD-B it is essential that the final protein concentration is kept as close as possible to 1.0 mg/ml, products with a higher protein concentration are known to be less specific, hence many countries demand a maximum protein concentration as part of their regulatory requirements.

2.4. Batch control

Samples should comply with the officially recognised standards for the production of tuberculin as set out in the *European Pharmacopoeia* or equivalent regulatory standards.

2.4.1. Sterility

Sterility testing is generally performed according to international guidelines (see also Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

2.4.2. Safety

Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and this must be injected

intraperitoneally into at least two guinea-pigs, dividing the dose between them. It is desirable to take a larger sample, such as 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and are then examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture.

2.4.3. Sensitising effect

To test the sensitising effect, three guinea-pigs that have not been treated previously with any material that could interfere with the test are injected intradermally on each of three occasions with the equivalent of 500 IU of the PPD-B sample in a 0.1 ml volume. Each guinea pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same PPD-B sample. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

2.4.4. Potency

In the 1980s the World Health Organization (WHO) developed and designated a new international standard to which it assigned a unitage of 32,500 IU/mg, which gave it equivalency to earlier local standards. This became known as the Bovine International Standard (BIS) tuberculin. This is available from the National Institute for Biological Standards and Control in the UK, for use by Veterinary Services and commercial manufacturers to calibrate national and corporate reference standards to ensure that production batches conform to international standards. As supplies of BIS are now limited, WOAH has initiated an international collaborative study to produce and calibrate a new reference reagent, to be known as the International Standard Bovine Tuberculin-2 (ISBT-2). An announcement will be made by WOAH when its validation has been completed.

Countries where bovine PPD tuberculin is produced should establish their own national reference preparations for bovine PPD as working standards. These national reference preparations should be calibrated in guinea-pigs against the official international standard (BIS/ISBT-2) for bovine PPD.

2.4.4.1. Standardisation of bovine PPD

a) Guinea-pigs sensitised with live *M. bovis*

The potency of a PPD-B sample is determined by comparison with a reference preparation of PPD-B, either the international standard or a derived national reference standard, in guinea-pigs infected with the production strain used e.g. *M. bovis* AN5.

The model, including variables such as breed and supplier of animals, infection dose, duration of infection needs to be validated prior to performing the assay: the combination of these variables and the expected potency of the serial dilutions of the PPD-B samples should result in lesions with a diameter of not less than 8 mm and not more than 25 mm for the assay to be valid. Infection doses can differ considerably between batches with regard to their virulence and each new batch will have to be carefully tested to avoid animal welfare issues as well as open tuberculosis which would result in excretion of bacilli and thus an increased health risk for the operators.

At least 8 albino guinea-pigs, each weighing 400–600 g, are infected with a low dose, e.g. 0.0001 mg wet mass of live bacilli of the homologous production strain e.g. *M. bovis* AN5 not less than 4 weeks prior to the assay. The bacilli are suspended in 0.5 ml of a 9 g/litre solution of sodium chloride and a deep intramuscular injection is made on the medial side of the thigh.

A suitable design for a potency assay is as follows: The potency of two PPD-B samples is estimated in the above infection model of 8 or 9 guinea-pigs against the standard for PPD-B, with three dilutions at five-fold intervals of each PPD-B, the 2 samples as well as the standard. The dilutions of the tuberculin preparations are made in isotonic phosphate-buffered saline (pH 6.5–7.5) containing 0.005 g/litre of polysorbate 80. The optimal concentrations are chosen in such a way that good readable skin reactions

are obtained with acceptable (8–25 mm) limits. As an example: amounts of 0.001, 0.0002 and 0.00004 mg PPD-protein corresponding to the international standard for PPD of 32, 6.4 and 1.28 IU, respectively, can be used. The injection volume is 0.2 ml.

In a single assay, two test PPDs are compared with the standard PPD in nine guinea-pigs, applying eight intradermal injections per animal and employing a balanced incomplete Latin square design.

Normally, the reading of the assays is done 24 hours after the injection of the tuberculins, but a second additional reading can be performed after 48 hours. The different diameters of erythema are measured with calipers in tenths of a millimetre and recorded on assay sheets. The results are statistically evaluated using standard statistical methods for parallel-line assays according to Finney (1978). A statistical program for the parallel line analysis, Combistats, is available from the European Pharmacopeia or EDQM.

The relative potencies of the two test tuberculins are calculated with their 95% confidence limits, the slopes of the log dose–response curves for each preparation (increase in mean reaction per unit increase in log dose) and the F ratios for deviations from parallelism.

According to the European Pharmacopoeia, the test is not valid unless the confidence limits (p = 0.95) are not less than 50 per cent and not more than 200 per cent of the estimated potency. The estimated potency is not less than 66 per cent and not more than 150 per cent of the stated potency. The stated potency is not less than 20,000 IU/ml.

b) Guinea-pigs sensitised with killed *M. bovis*

For laboratories that do not have biosecure facilities to house guinea-pigs infected with *M. bovis,* an alternative potency assay is often used, using heat-inactivated antigen to sensitise guinea-pigs. Because of differences in the sensitising antigens, results are not directly convertible between the two models. It is advised that interlaboratory comparisons are conducted to evaluate the potency of tuberculins assayed by the two methods.

As with the live AN5 assay, the "heat-inactivated" tuberculin potency assay should be validated to optimise the combination of sensitising dose and the potency of the PPD-B dilutions assayed in the model.

The assay is performed as follows: the PPD tuberculin is bioassayed in guinea-pigs sensitised with heat inactivated *M. bovis*, of the same strain used for PPD production, against the standard for bovine PPD tuberculin by an eight-point assay comprising four dilutions corresponding to about 20, 10, 5 or 2.5 IU. The injection volume is 0.1 ml. In this assay, two test PPD-Bs are compared with international/national standard PPD-B in eight guinea-pigs, applying eight intradermal injections per animal and employing a Latin square design. The guinea-pigs are sensitised with inactivated bacilli of *M. bovis,* 5–7 weeks before the assay. The heat inactivated bacilli are suspended in buffer and made into an emulsion with mineral oil adjuvant. A deep intramuscular injection is made on the medial side of both thighs, using a dose of 0.5 ml.

2.4.5. Specificity

A suitable assay for specificity is as follows: three bovine test tuberculins are assayed against the standard for avian PPD tuberculin (or three avian test tuberculins against the standard for bovine PPD tuberculin) by a four-point assay in heterologously sensitised guinea-pigs, comprising two dilutions at 25-fold intervals of each tuberculin. Quantities of 0.03 mg and 0.0012 mg of test tuberculoprotein, corresponding to approximately 975 and 39 IU, are chosen because these doses give good readable skin reactions. The injection doses of the standard are lower, namely 0.001 mg and 0.00004 mg. In one assay, three test tuberculins are compared with the standard tuberculin in eight guinea-pigs by applying eight intradermal injections per animal and employing

a balanced complete Latin square design. The reading of the results and the statistical evaluation are the same as with the potency test, but the interpretation should take into account that a satisfactory PPD should have a biological activity that does not surpass 10% of a standard with homologous sensitisation.

2.4.6. Stability

Provided the tuberculins comply with the legislative standards required for production and are stored at a temperature of between 2°C and 8°C and protected from light, they may be used up to the expiry date as specified in the licence for production of tuberculin. For long-term storage, it is recommended to keep the PPD in a concentrated form rather than the diluted form and the concentrate should also be stored in the dark.

2.4.7. pH control

The pH should be between pH 6.5 and 7.5.

2.4.8. Protein content

The protein content is determined as indicated in Section C.2.*3 In-process control*.

2.4.9. Storage

During storage, liquid bovine tuberculin should be protected from light and held at a temperature of 5±3°C. Freezing of the liquid product may compromise the quality. However, freeze-dried preparations can be prepared and they may be stored at higher temperatures (but not exceeding 25°C); they should be and protected from light. Periods of exposure to higher temperatures or to direct sunlight should be kept to a minimum.

2.4.10. Preservatives

Antimicrobial preservatives or other substances that may be added to a tuberculin must have been shown not to impair the safety and effectiveness of the product.

The maximum permitted concentration for phenol is 0.5% (w/v), and for glycerol it is 10% (v/v).

2.4.11. Precautions (hazards)

Appropriately diluted tuberculin injected intradermally in humans or animals, can result in a localised reaction at the injection site. Even in very sensitive individuals, severe, generalised reactions are extremely rare and limited. Operators with known sensitivity to tuberculin should carry out a risk assessment before handling the material.

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NB: There are WOAH Reference Laboratories for bovine tuberculosis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for bovine tuberculosis

NB: FIRST ADOPTED IN 1989 AS BOVINE TUBERCULOSIS. CHAPTER FIRST ADOPTED WITH CURRENT TITLE IN 2022.

CHAPTER 3.1.14.

NEW WORLD SCREWWORM (*COCHLIOMYIA HOMINIVORAX*) AND OLD WORLD SCREWWORM (*CHRYSOMYA BEZZIANA*)

SUMMARY

The New World screwworm[1] (*NWS*), Cochliomyia hominivorax (*Coquerel*), *and the Old World screwworm*[1] (*OWS*), Chrysomya bezziana *Villeneuve, are both obligate parasites of mammals, including humans, during their larval stages. Both species are in the subfamily Chrysomyinae of the family Calliphoridae of the order Diptera* (*true flies*). *Larvae feeding on the skin and underlying tissues of the host cause a condition known as wound or traumatic myiasis, which can be fatal. Infestations are generally acquired at sites of previous wounding, due to natural causes or to animal husbandry practices, but they may also occur in the mucous membranes of body orifices.*

Female flies are attracted to wounds, at the edges of which each female lays an average of 175 (*OWS*) *to 343* (*NWS*) *eggs. The larvae emerge within 12–24 hours and immediately begin to feed, burrowing head-downwards into the wound. After developing through three larval stages* (*instars*) *involving two molts, the larvae leave the wound and drop to the ground, into which they burrow to pupate. The duration of the life-cycle off the host is temperature dependent, being shorter at higher temperatures, and the whole cycle may be completed in less than 3 weeks in the tropics.*

Treatment is generally effected by application of organophosphorus insecticides into infested wounds, both to kill larvae and to provide a residual protection against reinfestation. Preventive measures include the spraying or dipping of susceptible livestock with organophosphorus compounds and, more recently, use of avermectins (*especially doramectin*) *as subcutaneous injections to animals 'at risk'. Strict control of the movement of animals out of affected areas also acts as a preventive measure.*

Identification of the agent*: The larvae of NWS and OWS can be easily confused with each other and with the larvae of other agents of myiasis. Accurate diagnosis involves the identification of larvae extracted from the deepest part of an infested wound. The mature, third instars are most reliable for this purpose, and those of NWS can be identified by their darkly pigmented dorsal tracheal trunks extending from the twelfth segment of the body forward to the tenth or ninth. This pigmentation is unique to the larvae of NWS among the species encountered in wound myiasis. Confirmation of OWS relies on the recognition of a characteristic combination of spinulation, the number of lobes on the anterior spiracles* (*4–6*), *and pigmentation of secondary tracheae.*

In the adult stage, species in the genus Cochliomyia *can be separated from other genera involved in wound myiasis by confirmation of a metallic body colour, ranging from light blue to green, with three dark longitudinal stripes always present on the thorax. The separation of NWS from the very similar* C. macellaria *and the identification of adult OWS are discussed in this chapter.*

Serological tests*: At present there are no applicable serological tests, nor are they indicated in the identification of this disease. However, serology may have a future role in studies of the prevalence of myiasis.*

1 In this chapter, the term 'New World' refers to the Americas and the term 'Old World' refers to Europe, Africa and Asia.

***Requirements for vaccines and biological control**: There are no vaccines or biological products available, except for the use of sterilised male flies in the sterile insect technique (SIT). In this technique, vast numbers of sterilised male flies are sequentially released into the environment, where their matings with wild females produce infertile eggs, leading to an initial population reduction and, progressively, eradication.*

A. INTRODUCTION

The New World screwworm fly (NWS), *Cochliomyia hominivorax* (Coquerel), and the Old World screwworm fly (OWS), *Chrysomya bezziana* Villeneuve, are species of two genera of the subfamily *Chrysomyinae* of the dipteran family *Calliphoridae* (blowflies). Both species are obligate parasites of mammals and, rarely, birds. The zoonotic implications are considerable because humans, especially the young, elderly or infirm, can be infested, with severe and sometimes fatal consequences (Spradbery, 1994). More recent reports of human cases of screwworm myiasis by *C. hominivorax* and *C. bezziana* include Olea *et al.* (2014) and Aggarwal *et al.* (2014), respectively. Despite being in different genera and geographically separated, the two species have evolved in remarkable parallel. They have almost identical life histories because they fill identical parasitic niches in their respective geographical zones. The following discussion will relate to both species, except where indicated.

Unlike most other species of blowflies, adult female screwworms do not lay their eggs on carrion. Instead, they lay them at the edges of wounds on living, injured mammals or at their body orifices. Virtually any wound is attractive, whether natural (from fighting, predators, thorns, disease, and/or tick and insect bites) or man-made (from shearing, branding, castrating, de-horning, docking, and/or ear-tagging). Commonly infested natural wounds are the navels of newborn animals, and the vulval and perineal regions of their mothers, especially if traumatised. If eggs are deposited on mucous membranes, the larvae can invade undamaged natural body openings such as the nostrils and associated sinuses, the eye orbits, mouth, ears, and genitalia.

Within 12–24 hours of the eggs being laid, larvae emerge and immediately begin to feed on the wound fluids and underlying tissues, burrowing gregariously head-downwards into the wound in a characteristic screwworm fashion. As they feed, tearing the tissue with their hook-like mouthparts, the wound is enlarged and deepened, resulting in extensive tissue destruction. Infested wounds often emit a characteristic odour, which can be the first indication that at least one animal in a group is infested. Although the odour is not always apparent to humans, it is obviously highly attractive to gravid females (Hall, 1995), which lay further batches of eggs, so increasing the extent of the infestation. A severe infestation that is left untreated may result in the death of the host.

Screwworm larvae pass through three stages (or instars), separated by cuticular molts that facilitate rapid growth, and they reach maturity about 5–7 days after egg hatch. They then stop feeding and leave the wound, falling to the ground into which they burrow and pupate. The pupa develops within the puparium, a barrel-shaped protective structure formed by hardening and darkening of the cuticle of the mature larva. On completion of development, adult flies usually emerge from the puparium in the morning and work their way up to the soil surface, where they extend their wings for hardening prior to flight. Males become sexually mature and able to mate within 24 hours, but the ovaries of females need to mature over 6–7 days, and females only become responsive towards males, mating when about 3 days old. About 4 days after mating, female flies are ready to oviposit. They seek a suitable host and lay their eggs, all oriented in the same direction, like a tiled roof, firmly attached to each other and to the oviposition substrate. The numbers of eggs laid per batch vary depending on many factors (e.g. fly strain, disturbance during oviposition), but the average first batch has in the order of 175 eggs for OWS and 343 for NWS (Spradbery, 1994). Following the first egg batch, further batches are laid at intervals of 3–4 days (Thomas & Mangan, 1989). Adult flies live on average for 2–3 weeks in the field during which time they feed at flowers, and the females also take in protein, e.g. from serous fluids at animal wounds and decomposing animals.

The rate of development of the immature stages is influenced by environmental and wound temperatures, being slower at low temperatures, although true diapause does not occur. This effect is most pronounced in the off-host pupal stage, which can vary from 1 week to 2 months' duration depending on the season (Laake *et al*., 1936). Thus, the complete life cycle of NWS may take 2–3 months in cold weather, whereas in temperate conditions with an average air temperature of 22°C, it is completed in about 24 days (James, 1947), and in tropical conditions averaging 29°C it is completed in about 18 days (Thomas & Mangan, 1989).

The degree to which NWS and OWS can tolerate cold has had a major influence on their distributions, best documented for NWS. Historically, the range of NWS extended from the southern states of the United States of

America (USA), through Mexico, Central America, the Caribbean islands and northern countries of South America to Uruguay, northern Chile and northern Argentina (James, 1947). This distribution contracted during the winter months but expanded during the summer months, producing a seasonality at its edges and year round populations in the central areas – the New World tropics. Use of the sterile insect technique (SIT) in major programmes has resulted in eradication of NWS from the USA, Mexico, Curacao, Puerto Rico, and the Virgin Islands and, in Central America, from Guatemala, Belize, El Salvador, Honduras, Nicaragua Costa Rica (Wyss, 2001) and Panama. Panama was recognised as free from NWS in 2006 and a permanent barrier zone was established primarily in the Darien province of eastern Panama. This serves as the northern limit of NWS in the Americas. A NWS eradication programme was also officially launched in Jamaica in July 1998, as part of a plan to eradicate the species from the entire Caribbean. This programme encountered severe setbacks due to a complex combination of management and technical difficulties (Vreysen *et al*., 2007), which eventually led to the failure of the programme on the island. Although NWS is a New World species, in 1988, it was detected in Libya in North Africa where it threatened to become firmly established. However, it was eradicated in 1991 by an intensive SIT campaign (Lindquist *et al*., 1992). The threat of spread of screwworms aided by modern rapid transport systems is ever present, necessitating constant vigilance from quarantine and other front-line animal health and medical officers in unaffected areas. Imported cases of NWS have been reported in Mexico, USA, and even in the United Kingdom.

An outbreak of NWS occurred in Florida, USA, in 2016–17 and was eliminated by use of the SIT from ground release chambers. Cases were found predominantly in wildlife (particularly Florida Key deer, *Odocoileus virginianus clavium*) with only a few in domesticated animals (dogs, cats and pet pigs) (USDA, 2017).

The distribution of OWS is confined to the Old World, as the name suggests, throughout much of Africa (from Ethiopia and sub-Saharan countries to northern South Africa), the Middle East Gulf region, the Indian subcontinent, and south-east Asia (from southern China [People's Rep. of] through the Malay Peninsula and the Indonesian and Philippine islands to Papua New Guinea) (James, 1947; Sutherst *et al*., 1989; Zumpt, 1965). OWS was reported from Hong Kong for the first time in 2000, infesting dogs, and a first human case was reported in 2003 (Ng *et al*., 2003). OWS myiasis has also been reported from Algeria (Abed-Benamara *et al*., 1997), in a local shepherd, and in Mexico (Romero-Cabello *et al*., 2010). However, in the absence of other reported cases, particularly animal cases, a continuing presence in either region seems unlikely and the original cases could have been misidentified, emphasising the need for correct identification of samples. The situation in the Gulf area and surrounding regions is dynamic, with reports confirmed from Iran, Iraq and, most recently, Yemen (Robinson *et al*., 2009). Epizootics of traumatic myiasis can follow introductions into such areas, especially where the livestock owners and veterinarians are unfamiliar with OWS (Siddig *et al*., 2005). The climatic requirements of the two screwworm species are very similar and their potential distributions, if unrestrained, would overlap considerably (Sutherst *et al*., 1989).

Treatment of infested wounds usually relies on the application of organophosphorus insecticides such as coumaphos (also dichlofenthion or fenchlorphos), taking due note of the manufacturer's safety instructions (Graham, 1979; Spradbery *et al.*, 1994). The insecticide should be applied at 2- to 3-day intervals until the wound has healed.

Prevention of screwworm infestation can be achieved by spraying or dipping of livestock, for example if member of the group was found to be infested, if animals were traversing or leaving an infested area, or following wound-inducing animal husbandry practices, e.g. shearing and castration.

Indirect prevention of screwworm infestation includes the avoidance of wounding procedures at the times of year when flies are numerous, the careful handling of livestock to minimise wounding, the removal of sharp objects (e.g. wire strands) from livestock pens, and the use of measures to reduce other wound-causing parasites, in particular ticks, e.g. by dipping and by insecticide impregnated ear-tags.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Morphology	+++	+++	+++	+++	+++	–
Hydrocarbon analysis	–	–	–	+	–	–
Mitochondrial DNA analysis	–	–	–	+	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.

1. Identification of the agent

Identification of the eggs and first instars of the agents of myiasis based on morphology can be difficult. First instar larvae submitted to a laboratory can be identified following the descriptions and identification key provided by Szpila *et al.* (2014).

Larvae collected for diagnosis should be removed from the deepest part of the wound to reduce the possibility of collecting non-screwworm species, which may infest the shallower parts of the wound. Living specimens should first be examined for pigmentation of the dorsal tracheal trunks (Figures 1 and 4) and then be preserved in 80% ethanol and returned to the laboratory for examination under a dissecting microscope at up to ×50 magnification (for further techniques see: Hall & Smith, 1993; Spradbery, 1991; Zumpt, 1965). If larvae are placed directly into most preservative solutions they contract and darken. However, optimal preservation of larvae, in their natural extended state, can be made by killing them in boiling water (15–30 seconds immersion) before storage in 80% ethanol. This killing method had no negative effect on subsequent extraction of mitochondrial DNA, amplified by polymerase chain reaction (PCR) (Wardhana *et al*., 2012), but it might impact other molecular techniques and this should be borne in mind.

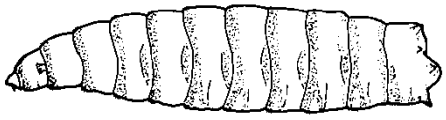
Second instars have only two spiracular slits in each of the posterior spiracular plates compared with the three slits of third instars (Figures 2 and 3). Second instars of NWS can be diagnosed by the presence of dark pigmentation of the dorsal tracheal trunks, for over half their length in the terminal segment. Other species have less extensive pigmentation of the dorsal tracheal trunks, for example, these trunks are pigmented for no more than one-third of their length in the twelfth segment of OWS. The anterior spiracles of second instar NWS have from seven to nine branches compared with about four branches in OWS (Kitching, 1974). More positive identification may be gained by rearing living, immature larvae to third instars. This can be done on the standard meat medium used for large-scale rearing of NWS before the introduction of gel diets, i.e. in the proportion of 1 litre water, 1.3 kg ground horse or beef meat, 50 g dried bovine blood, and 1.5 ml formalin (Taylor & Mangan, 1987), mixed and maintained at 35–38°C and 70% relative humidity. For simply rearing up larvae for identification, the exact meat and blood types are not essential, and more readily available fresh blood could be used instead of dried blood.

Remove larva from wound and examine gross surface structure

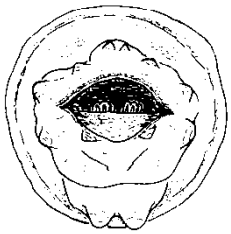
'Hairy' larva with obvious body processes

Chrysomya albiceps, C. rufifacies, C. varipes, C. villeneuvi, Fannia sp.

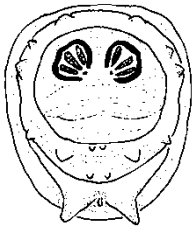
'Smooth' larva, with spine bands but no obvious body processes except on last segment



Posterior spiracles almost concealed in deep cavity on posterior 'face' of last segment



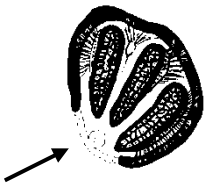
Posterior spiracles not in cavity but clearly exposed on posterior 'face' of last segment



Sarcophagidae

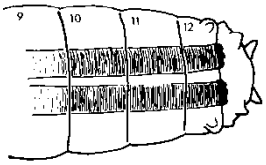
Peritreme of posterior spiracular plate closed

Peritreme of posterior spiracular plate open



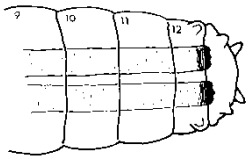
Muscidae and *Lucilia/Calliphora* species

Dorsal tracheal trunks darkly pigmented forwards from the 12th to the 10th or even 9th segment

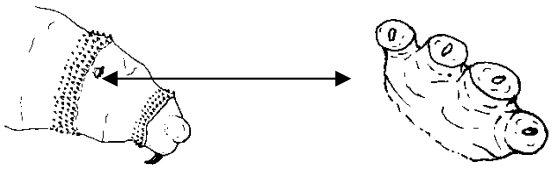


Cochliomyia hominivorax

Dorsal tracheal trunks not darkly pigmented except possibly in posterior half of 12th segment

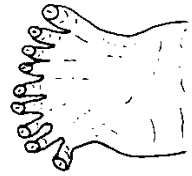


Anterior spiracle with 4–6, rarely 7, lobes



Chrysomya bezziana

Anterior spiracle with 9 or more, rarely 8, lobes



Other species of *Chrysomya, Cochliomyia*, *Phormia* or *Protophormia*

Fig. 1. Identification key for the diagnosis of third instars of Cochliomyia hominivorax and Chrysomya bezziana from cases of wound myiasis. To avoid misidentifications, it is essential that the key is worked through from the first step for each specimen.

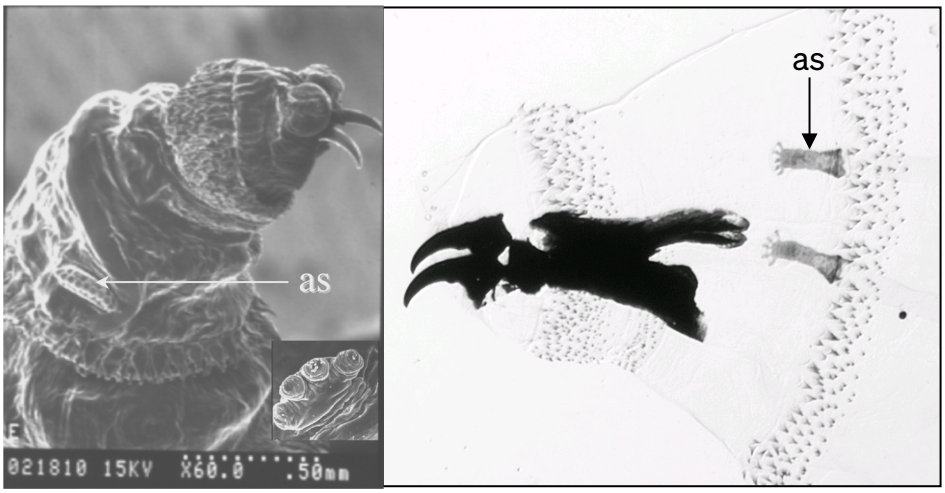


Fig. 2. Head and next two segments of third instar of Cochliomyia hominivorax (left, viewed by scanning electron microscopy, inset is the anterior spiracle of Chrysomya bezziana) and of Chrysomya bezziana (right, viewed by compound light microscopy, note the thorn-like spines and that this slide preparation has been cleared using 10% KOH so that the anterior spiracles on both sides of the first thoracic segment are visible); as = anterior spiracle.

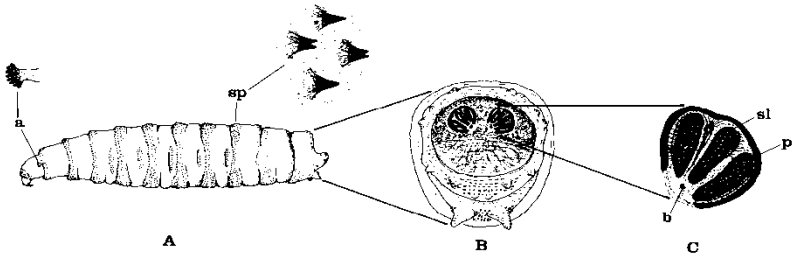


***Fig. 3. Characteristics of third instar of Cochliomyia hominivorax: (A) whole larva, lateral aspect; (B) posterior face of terminal segment; (C) posterior spiracular plate; a = anterior spiracle; b = button adjacent to opening in peritreme; p = peritreme; sl = spiracular slit; sp = spines.* (*After Laake et al.* [*1936*].)**

Third instars of both NWS and OWS have a robust, typical maggot shape, with a cylindrical body from 6 to17 mm long and from 1.1 to 3.6 mm in diameter, pointed at the anterior end (Laake *et al*., 1936; Spradbery, 1991). Fully mature larvae of both NWS and OWS develop a reddish-pink tinge over the creamy white colour of younger larvae. Both screwworm species have prominent rings of spines around the body and these spines appear large and conspicuous under a microscope, when compared with most non-screwworm species, the longest averaging 130 μm. In NWS the spines can be either single or double pointed, but in OWS they are always single pointed and thorn-like (Figure 2). The anterior spiracles of NWS each have from six to eleven well separated branches, but usually from seven to nine (Figure 2). In OWS, the anterior spiracles each have from three to seven branches, but usually from four to six (Figure 2). The latter character should not be used on its own to identify OWS, because third instars of the obligate myiasis-causing species *Wohlfahrtia magnifica* (Diptera: Sarcophagidae), whose distribution overlaps that of OWS in the Middle East, have similarly branched anterior spiracles. Hence, in using any identification key, such as that in Figure 1, it is essential that each specimen be taken through the whole key to avoid misidentifications. On the posterior face of the terminal segment of both NWS and OWS, the posterior spiracular plates all have a darkly pigmented, incomplete peritreme partially enclosing three straight, slightly oval-shaped slits, which point towards the break in the peritreme. These diagnostic features are illustrated in Figure 3. Of greatest diagnostic value are the dorsal tracheal trunks, which extend forwards from the posterior spiracular plates and are darkly pigmented up to the tenth or ninth segment in NWS (Figure 1; see also: Hall & Smith, 1993; James, 1947; Spradbery, 1991; Zumpt, 1965 for identification keys). This feature is seen most easily in living larvae. Those in preservative may need dissection to remove opaque tissues covering the trunks. The dorsal tracheal trunks of OWS are darkly pigmented only in the twelfth segment. However, in OWS the secondary tracheae branching off the dorsal tracheal trunks are pigmented from the twelfth segment forwards to at least the tenth segment (confirmed in specimens throughout the range, from Malaysia, Bahrain and Zimbabwe; M.J.R. Hall, unpublished). Conversely, in

NWS these secondary tracheae are not pigmented, only the dorsal tracheae are. Hence, the tracheal pigmentation appears almost reversed between the two screwworm species (Figure 4).

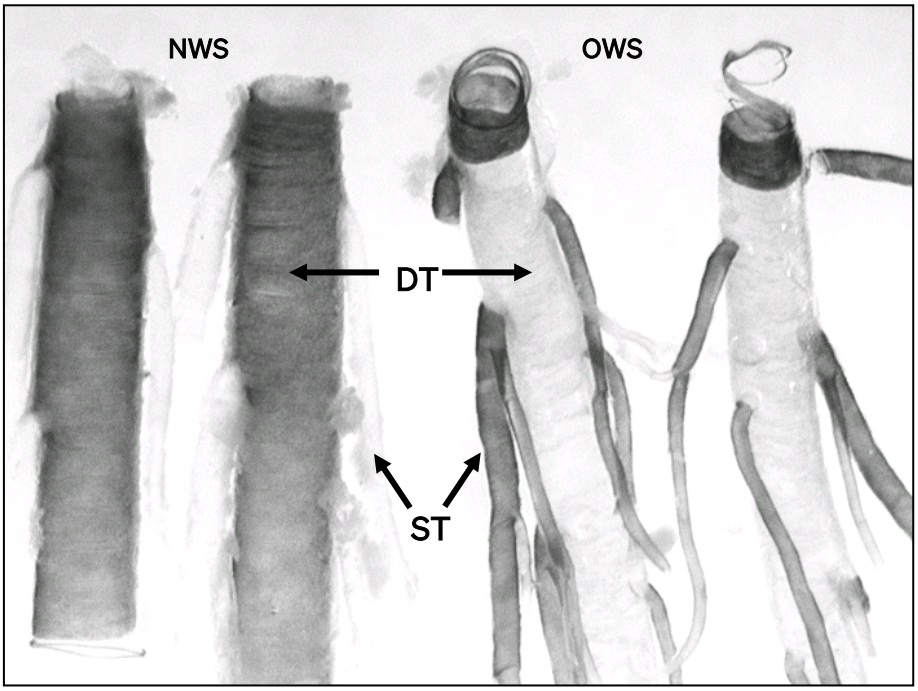


Fig. 4. Dorsal tracheal trunks of third instar of* Cochliomyia hominivorax (*left*) *and* Chrysomya bezziana (*right*) *dissected forwards from the posterior spiracles* (*top*) *to ninth abdominal segment* (*bottom*). *Note that the pigmentation of the main dorsal trunks* (*DT*) *and the smaller secondary tracheae* (*ST*) *is almost reversed between the species.

Adult: Adult flies needed for identification purposes are often collected using wind-oriented traps (Broce *et al*., 1977) and sticky traps (Spradbery, 1991) baited with a synthetic odour (Mackley & Brown, 1984). A modified bucket-trap combined with a newly developed attractant caught an average of 3.1 times as many OWS as a sticky trap baited with the earlier lure and was more selective for OWS (Urech *et al.,* 2012). Real-time PCR methods can detect OWS in such bulk fly traps even when the prevalence is as low as one OWS in 1,000 other flies (Jarrett *et al*., 2010). Alternative sampling systems, using electrocuting grids or sticky surfaces at odour-baited visual targets, have been used for research purposes (Hall, 1995). Identification of adult flies is seldom required for the diagnosis of myiasis, because the larval stages are those most apparent to livestock owners and veterinary personnel. However, a brief description follows.

i) **NWS**: The body length is usually 8–10 mm, with three dark longitudinal stripes on the dorsal surface of the thorax. Although this fly may generally be a deep blue to blue green metallic colour, colour is variable and can range from light blue to green. This combination of colour and pattern is not shared by any other species commonly involved in wound myiasis except the secondary screwworm of the New World, *Cochliomyia macellaria* (Fabricius). These two *Cochliomyia* species can be separated by the presence of black setulae on the fronto-orbital plates of the head of NWS compared with only light yellow hairs on the fronto-orbital plates of *C. macellaria*. The fifth (=fourth visible) abdominal tergite of NWS has only a very slight lateral pollinose dusting, whereas that of *C. macellaria* has a dense dusting, producing a pair of distinct, lateral, silvery-white spots. In addition, females of NWS have a dark brown-black basicosta, whereas those of *C. macellaria* have a yellow basicosta (Figure 5; see also: Dear, 1985; Laake *et al*., 1936; Spradbery, 1991).

ii) **OWS**: The body is up to 10 mm long and has a metallic blue, bluish-purple or blue-green colour, i.e. it is very similar to NWS, but without the thoracic stripes. The lower squama (s in Figure 5) also differs from NWS, being distinctly covered with fine hairs over its entire upper surface in OWS and other *Chrysomya* species, whereas in NWS it is hairless above, except near the base. Adults of OWS can be distinguished from other *Chrysomya* found in cases of myiasis by the combination of black-brown to dark-orange-coloured anterior thoracic spiracles (rather than pale yellow, creamy, or white), with waxy-white, lower squamae (rather than blackish-brown to dirty-grey) (Spradbery, 1991; Zumpt, 1965).

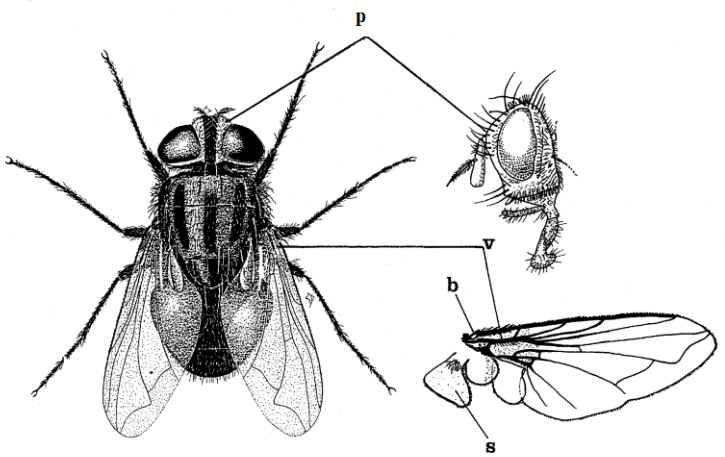


Fig. 5. Characteristics of adult* Cochliomyia hominivorax*; note longitudinal thoracic stripes; b = basicosta; p = fronto-orbital plate, indicated from above on whole* Cochliomyia hominivorax *and laterally on head of typical calliphorid fly; s = lower squama, surface hairless except at base; v = stem vein with hairs on dorsal posterior surface.

In addition to the standard morphological techniques discussed previously, more recent techniques for identification of screwworms and their geographical origins include cuticular hydrocarbon analysis (see in Spradbery, 1991) and analysis of mitochondrial DNA (Fresia *et al*., 2011; Wardhana *et al*., 2012). Problems with identification of larvae or adults from cases of myiasis can be referred to the WOAH Reference Laboratory for New World screwworm or the FAO Collaborating Centre on Myiasis-Causing Insects and Their Identification[2].

2. Serological tests

No standardised serological tests are presently available, nor are they indicated for diagnosis of this disease. However, experimental studies have shown that serological techniques have potential value in future investigations of the prevalence of screwworm infestations in animal populations to detect antibodies to screwworm post-infestation (Thomas & Pruett, 1992).

C. REQUIREMENTS FOR VACCINES AND BIOLOGICAL CONTROL

There are no biological products such as vaccines, available currently. However, research towards development of potential vaccines is being conducted (Sukarsih Partoutomo *et al*., 2000). The only proven method of eradication of NWS relies on a biological technique, the sterile insect technique, SIT (Lindquist *et al*., 1992), which has also been applied experimentally to OWS (Spradbery, 1994). In this technique, male flies sterilised in their late pupal stage by gamma or x-ray irradiation are sequentially released into the wild in vast numbers. All of their matings with wild females result in infertile eggs only, leading to a progressive population reduction and, eventually, eradication. In operational situations, SIT is supported by the insecticide treatment of screwworm-infested wounds in livestock, by strict control of livestock movement, by the quarantining of infested animals and by an active publicity campaign. SIT is very expensive because of the cost of continuous production and aerial dispersion of sterile flies. Historically, it has been considered cost effective only when used as an eradication strategy in situations where the geography would favour such a programme (e.g. Lindquist *et al*., 1992). Presently, there is only one production facility for sterile adults of New World screwworm, located in Pacora, Panama[3].

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3 For further information contact: USDA/APHIS, 4700 River Road, Riverdale, Maryland 20737, USA.

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NB: There is a WOAH Reference Laboratory for New World screwworm (*Cochliomyia hominivorax*)
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for New World screwworm (*Cochliomyia hominivorax*)

NB: FIRST ADOPTED IN 1991 AS NEW WORLD SCREWWORM; FIRST ADOPTED WITH CURRENT TITLE IN 2000.
MOST RECENT UPDATES ADOPTED IN 2019.

CHAPTER 3.1.15.

NIPAH AND HENDRA VIRUS DISEASES

SUMMARY

Hendra virus (*HeV*) *and Nipah virus* (*NiV*) *emerged in the 1990s as the causes of outbreaks of zoonotic diseases. The first reported outbreak of HeV occurred in Brisbane, Australia in 1994. In this case HeV caused severe respiratory disease and the death of 13 horses and a horse trainer at a stable. NiV appeared in the human population between September 1998 and April 1999 in Malaysia as the cause of fatal acute encephalitis, after spreading primarily as a respiratory disease of unknown aetiology in the pig population. HeV has caused the death of four of seven infected people in Australia while it has been reported that there have been more than 600 cases of NiV in humans, with more than 400 deaths, in Bangladesh, India, Malaysia, Philippines and Singapore. Fruit bats* (*flying-foxes*) *in the genus* Pteropus *are reservoir hosts of both viruses.*

HeV infection of horses does not have a pathognomonic presentation. Horses may present with respiratory, neurological or non-specific clinical signs. Respiratory signs include tachypnoea or frothy nasal discharge. Neurological signs include ataxia, head tilt, circling, seizures, depression or recumbency. Non-specific signs include high fever, tachycardia, inappetence or colic. HeV infection of horses is not uniformly fatal, nor does it appear to be highly contagious among horses. Close contact with infected flying-fox urine, saliva or birth products is necessary for its spill over from flying-foxes to horses. Infected horses on pastures have rarely transmitted the virus to other horses. Transmission appears to occur more readily in closed environments such as stables and veterinary clinics and is associated with contact with bodily fluids from infected horses.

NiV infection of pigs is highly contagious and characterised by fever with respiratory and sometimes neurological involvement, but many infections are subclinical. Some infected pigs display an unusual loud barking cough. Abortion has also been reported.

It is not currently known if the susceptibility of dogs and cats to infection is at a level to have potential for epidemiological significance.

Infection of humans is from animal contact, usually from an amplifier host rather than directly from the reservoir host: NiV from swine and horses and HeV from horses. Investigations of outbreaks of human cases of NiV in Bangladesh have indicated human infection from Pteropid bats directly without an intermediary/amplifier host. Human-to-human transmission has only been seen in outbreaks of NiV in Bangladesh and India.

HeV and NiV are closely related and are the founding members of the genus Henipavirus*, family* Paramyxoviridae*. There are two genotypes of NiV, namely NiV-M* (*Malaysia*) *and NiV-B* (*Bangladesh*)*. Recent genetic characterisation of NiV-B from bat and human cases in Bangladesh 2012–2018 have shown genetic divergence during 1995 forming two sublineages: NiV-B1 and NiV-B2* (*Rahman* et al., *2021*)*. Two genotypes of HeV have been recognised, namely HeV-g1 and HeV-g2. HeV and NiV are dangerous human pathogens. All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level.*

Detection of the agent: *Because of the zoonotic potential and high fatality rates associated with these viruses, diagnostic laboratories may decide, following a comprehensive risk assessment, to use molecular techniques such as real-time reverse transcription polymerase chain reaction* (*RT-PCR*) *for agent detection rather than attempting propagation of infectious virus. Both HeV and NiV can be propagated in a wide range of cultured cells. Virus isolation from field samples should be attempted, but only in situations where operator safety can be assured. Identification procedures include RT-PCR, immunostaining of infected cells, and neutralisation with specific viral antisera.*

Viral antigen is present in vascular endothelium, and in the case of NiV in pigs, the respiratory epithelium. A wide range of formalin-fixed tissues can be examined to detect HeV and NiV antigen by immunohistochemistry (IHC).

Serological tests: *Following a biological risk assessment, diagnostic laboratories may decide to avoid serological tests that use live virus. Virus neutralisation tests (VNT) and enzyme-linked immunosorbent assays (ELISA) are available. ELISA is currently being used as a screening tool and VNT is accepted as the reference procedure and confirmatory test. The ability of antibodies to HeV and NiV to cross-neutralise to a limited degree means that a single VNT using either virus does not provide definitive identification of antibody specificity.*

Requirements for vaccines: *There is a vaccine available for HeV approved for use in horses in Australia. There is no vaccine currently available for NiV.*

A. INTRODUCTION

Hendra virus (HeV) and Nipah virus (NiV) are classified in the family *Paramyxoviridae* subfamily *Orthoparamyxovirinae*, genus *Henipavirus*. They have morphological and physicochemical properties typical of paramyxoviruses. The viruses are pleomorphic in shape and enveloped, with herringboned nucleocapsids. Virions are 40–600 nm in diameter. Glycoprotein and fusion protein spikes project through a lipid envelope. HeV and NiV have a non-segmented, single-stranded, negative-sense RNA genome (18.2 kb) consisting of six genes which code for six major structural proteins, namely: N (nucleocapsid protein), P (phosphoprotein), M (matrix protein), F (fusion protein), G (glycoprotein) and L (large protein).

HeV and NiV occur naturally as viruses of fruit bats, also known as flying-foxes. These are members of the genus *Pteropus,* family *Pteropodidae*. Antibodies to HeV have been detected in all four Australian pteropus species with seroprevalence varying over time. To date, all HeV cases in horses have occurred within the geographical range of the *Pteropus* bats, which correlates to the east coast of Australia. Antibodies to NiV or closely related viruses have been detected in pteropid bats over much of their geographical range. HeV-g1 has been isolated from Australian flying-foxes (Halpin *et al*., 2000), with a novel HeV genotype (HeV-g2) detected in two flying-fox species in Australia (Wang *et al.,* 2021). NiV has been isolated from flying-foxes in Malaysia and Cambodia (Chua *et al*., 2002; Reynes *et al*., 2005). In Ghana, a small study showed 39% of *Eidolon helvum*, a non-pteropus fruit bat, had NiV reactive antibodies (Hayman *et al*., 2008). Henipavirus-like sequences were also obtained from *Eidolon helvum* in Ghana (Hayman *et al*., 2008). The detection of antibodies to and sequences of henipaviruses in African bats suggests that the range of potential NiV infections may be wider than previously thought.

HeV disease emerged in Brisbane, Australia, in September 1994 in an outbreak of acute respiratory disease that killed 13 horses and a horse trainer (Murray *et al*., 1995). The virus was initially called equine morbillivirus, but subsequent genetic analyses indicated that it was sufficiently different to belong in its own genus. There has been at least one spill-over event each year since 2006, with most events involving only a small number of horses. A retrospective investigation isolated HeV-g2 from a 2015 equine case (Annand *et al.,* 2022). To date seven human cases have resulted in four deaths (case fatality rate 57%). Clinical signs in humans range from an influenza-like infection to severe pneumonia or encephalitis leading to death (Yuen *et al.,* 2021). All infected people have had very close contact with infected body fluids from infected horses through performing invasive procedures and/or have not worn fully protective personal equipment.

In Malaysia, retrospective studies of archival histological specimens indicate that NiV had caused low mortality in pigs since 1996 but remained unknown until 1999 when it emerged as the causative agent of an outbreak of encephalitis in humans that had commenced in 1998 (Chua *et al*., 2000; Nor *et al*., 2000). Unlike respiratory disease caused by HeV in horses, which was frequently fatal but characterised by poor transmissibility in the field, respiratory disease caused by NiV in pigs was often subclinical but highly contagious in these intensively housed animals (Hooper *et al.,* 2001). This led to rapid virus dispersal through the Malaysian pig population with authorities choosing culling as the primary means to control spread (Nor *et al*., 2000). Over one million pigs were destroyed; approximately 40% of infected humans, mostly pig farmers in Malaysia and abattoir workers in Singapore who had direct contact with live pigs, died of encephalitis (Chua *et al*., 2000). A small number of cats, dogs and horses were also infected on infected pig farms during that outbreak (Hooper *et al*., 2001; Nor *et al*., 2000) but the infections were not epidemiologically significant.

Outbreaks of human NiV disease have occurred on an almost annual basis in Bangladesh since 2003, with a few outbreaks in West Bengal, in neighbouring India, and more recently in Kerala, on the western coast of India (Arunkumar *et al*., 2019). Drinking fresh date palm sap contaminated by fruit bat saliva, urine or excreta has been identified as the likely route of transmission from the wildlife reservoir to humans in the Bangladesh outbreaks (Luby *et al*., 2006). In some outbreaks, there has been human-to-human transmission. As a result of these ongoing outbreaks it is estimated that across Malaysia, Singapore, Bangladesh and India there have now been >600 cases of NiV in humans, with >400 deaths.

In 2014 in the Philippines, an outbreak of human cases of NiV was reported, with 9 deaths from 17 cases (Ching *et al.,* 2015). In this outbreak infected sick horseswere butchered and consumed by the people who later became infected. Subsequent human-to-human transmission was suspected. Cats and dogs were also affected.

Diagnosis of disease caused by henipaviruses is primarily by detection of viral RNA in clinical or post-mortem specimens, and virus isolation or demonstration of viral antigen in tissue samples (Daniels *et al*., 2001). Detection of specific antibody can also be useful particularly in pigs where NiV infection may go unnoticed. Identification of HeV antibody in horses is less useful because of the high case fatality rate of infection in that species. Human infections of both HeV and NiV have been diagnosed retrospectively by serology. Demonstration of specific antibody to HeV or NiV in either domestic animals or humans is of diagnostic significance because of the rarity of infection and the serious zoonotic implication of transmission of infection.

The henipavirus genus is expanding, with new viruses recently identified. Cedar virus was isolated from the urine of *Pteropus* bats in Australia in 2009. It remains to be seen if it has the capacity to spill over to other species, and if so, cause disease (Marsh *et al*., 2012). A number of other henipa-like viruses have been detected in wildlife by PCR and sequencing but have not yet been isolated by traditional virus isolation techniques (Wu *et al*., 2014). Two henipa-like viruses have been isolated from shrews in the Korea (Rep. of) (Lee *et al.,* 2021).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of henipaviruses and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Virus isolation	–	–	–	+++	–	–
RT-PCR & real-time RT-PCR	+	+	++	+++	+	–
IHC	–	–	–	++	–	–
IFA	–	–	–	++	–	–
Detection of immune response[(b)]						
ELISA	+++	+++	+++	+	+++	+++
VNT	+++	+++	+++	+	+++	+++
Bead assays	+++	+++	+++	+	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; IHC = Immunohistochemistry;
IFA = Indirect fluorescent antibody; ELISA = enzyme-linked immunosorbent assay; VNT = virus neutralisation test.
[(a)]A combination of agent identification methods applied on the same clinical specimen is recommended.
[(b)]Positive ELISA and bead-based assay results should be confirmed by the VNT unless the assay is validated for the purpose.

1. Laboratory biosafety

HeV and NiV are dangerous human pathogens with a high case fatality rate and for which there is no human vaccination or effective antiviral treatment. Transport of suspected specimens to laboratories, and all laboratory manipulations with live viral cultures (including serological tests such as virus neutralisation (VN) using live virus) or potentially infected/contaminated material such as tissue and blood samples must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). The safety of the laboratory workers must be assured by the biological risk management strategy adopted. Laboratories may adopt different biological risk management strategies depending on whether they are testing samples by PCR or attempting to propagate the agents. Molecular methods for agent detection are preferable as front-line tests as they carry less risk to the laboratory worker than virus isolation. Laboratories that do not have appropriate containment facilities for the handling of samples from suspect cases or for working with infectious virus should avail of the services of an WOAH reference or other specialist laboratory.

Virus propagation in particular must be conducted in high containment facilities under stringent conditions that will prevent accidental infection of personnel in the laboratory. During primary virus isolation from specimens collected from suspect cases, it must be appreciated and reflected in procedures that if a paramyxovirus-like cytopathic effect (CPE) develops in infected cultures, the level of risk has increased. Appropriate biosafety guidelines will emphasise good laboratory practice, the level of containment, the class of biosafety cabinet and the appropriate personal protective equipment required. Reverse-transcription polymerase chain reaction (RT-PCR) or immunofluorescent detection of henipavirus antigen in cells fixed with acetone may be used to identify the isolate as a henipavirus. Transfer of cultures to specialist laboratories should follow transportation standards as specified in Chapter 1.1.3 *Transport of biological specimens.*

2. Detection of the agent

2.1. Virus isolation and characterisation

Virus isolation greatly facilitates identification procedures but should only be undertaken where operator safety can be guaranteed. Isolation is especially relevant in any new case or outbreak, particularly in countries or geographical areas where infection by HeV or NiV has not been previously documented. Molecular detection techniques which do not require propagation of live virus, can be used to identify the presence of viral genome in samples. Implication of wildlife species as natural hosts of the viruses requires positive serology, PCR or virus isolation from wild-caught animals.

2.1.1. Sampling and submission of specimens

The range of tissues yielding virus in natural and experimental cases has been summarised (Daniels *et al*., 2001). In live animals, swabs (nasal or oro-naso-pharyngeal), EDTA (ethylene diamine tetra-acetic acid) blood and serum should always be submitted. Urine, brain, lung, kidney and spleen are also useful, and can be collected if appropriate biosafety precautions can be taken during sampling. In pregnant animals or in cases of abortion, uterus, placenta and fetal tissues should be included as appropriate. Specimens should be transported at 4°C if they can arrive at the laboratory within 48 hours; if the transport time will be over 48 hours, the samples should be sent frozen on dry ice or nitrogen vapours (≈ –78.5°C). Specimens should not be held at –20°C.

Diagnostic specimens should be submitted to designated laboratories in specially designed containers, in accordance with Chapter 1.1.3.

2.1.2. Isolation in cultured cells

Biosafety considerations are of absolute importance during henipavirus isolation, as noted in Section 1 above.

Virus isolation is aided by the fact that HeV and NiV grow rapidly to high titre in many cultured cells. African green monkey kidney (Vero CCL181) and rabbit kidney (RK-13) cells have been found to be particularly susceptible. HeV also replicates in suckling mouse brain and in embryonated hens' eggs, and laboratories using these isolation systems in the investigation of undiagnosed infections should be aware of this possibility.

In the laboratory conducting virus isolation, tissues are handled under sterile conditions, and 10% (w/v) suspensions are generated by grinding the tissues in a closed homogenisation system. All processes should be carried out under appropriate conditions as determined by a thorough biosafety risk assessment. Tubes used should have O-rings, and an external thread. Following clarification of the homogenate by centrifugation in a rotor with safety cap at 300 ***g*** for 3–5 minutes and 4°C, the supernatant is added to confluent cell monolayers.

A CPE usually develops within 3 days, but two 5-day passages are recommended before judging the attempt unsuccessful. After low multiplicity of infection, the CPE is characterised by formation of syncytia that may, after 24–48 hours, contain over 60 or more nuclei. Syncytia formed by NiV in Vero cell monolayers are significantly larger than those created by HeV in the same time period. Although the distribution of nuclei in NiV-induced syncytia early in infection resembles that induced by HeV, with nuclei aggregated in the middle of the syncytia, nuclei in mature NiV-induced syncytia are distributed around the outside of the giant cell.

2.1.3. Methods of identification

i) Immunostaining of fixed cells

The speed with which HeV and NiV replicate and the high levels of viral antigen generated in infected cells make immunofluorescence a useful method to rapidly identify the presence of henipaviruses using either anti-NiV or anti-HeV antiserum. The serological cross reactivity between HeV and NiV means that polyclonal antiserum to either virus or mono-specific antisera to individual proteins of either virus, will fail to differentiate between HeV and NiV.

a*)** ***Test procedure

Under appropriate laboratory conditions to manage biological risks, monolayers of Vero or RK-13 cells grown on glass cover-slips or in chamber slides are infected with the isolated virus, and the monolayers are examined for the presence of syncytia after incubation for 24–48 hours at 37°C. It is recommended that a range of virus dilutions (undiluted, 1/10, 1/100) be tested because syncytia are more readily observed after infection at low multiplicity. Once visible syncytia are detected, infected cells are fixed by immersion completely in a vessel filled with acetone, or paraformaldehyde. The vessel is sealed, and surface sterilised prior to removal to a laboratory environment where the slides on which the virus in now inactivated may be air-dried. Viral antigen is detected using anti-HeV or anti-NiV antiserum and standard immunofluorescent procedures. A characteristic feature of henipavirus-induced syncytia is the presence of large polygonal structures containing viral antigen that fluoresce.

ii) Immuno-electron microscopy

The high titres of HeV and NiV in cells *in vitro* permit their visualisation in the culture medium by negative-contrast electron microscopy without a centrifugal concentration step. Detection of virus–antibody interactions by immunoelectron microscopy provides valuable information on virus structure and antigenic reactivity, even during primary isolation of the virus. Other ultrastructural techniques, such as grid cell culture (Hanna *et al*., 2006), in which cells are grown, infected and visualised on electron microscope grids, and identification of replicating viruses and inclusion bodies in thin sections of fixed, embedded cell cultures and infected tissues complement the diagnostic effort. The details of these techniques and their application to the detection and analysis of HeV and NiV have been described (Hyatt *et al*., 2001).

2.2. Viral identification: differentiation of HeV and NiV

2.2.1. Comparative immunostaining

Further identification of a henipavirus isolate as either HeV or NiV is based on comparative immunostaining as described in this section. It is necessary to compare the isolate with standard cultures of both HeV and NiV, and so all work must be conducted using procedures to manage the biological risks. The control and test viruses are titrated on Vero cell monolayers in 96-well

plates and after 18–24 hours, foci of infection are detected immunologically in acetone-fixed cells using anti-viral antiserum. The virus titres are expressed as focus-forming units (FFU)/ml.

2.2.2. Immunofluorescence assay

A virus isolate that reacts with anti-HeV and/or anti-NiV antisera in an immunofluorescence assay is considered to be serologically identical to either HeV or NiV if it displays the same sensitivity to neutralisation by anti-HeV and anti-NiV antisera as do the HeV or NiV positive controls. Anti-HeV antiserum neutralises HeV at an approximately four-fold greater dilution than that which neutralises NiV to the same extent. Conversely, anti-NiV antiserum neutralises NiV approximately four times more efficiently than HeV (Chua *et al*., 2000).

2.2.3. Microtitre neutralisation

This procedure is dependent on the availability of anti-serum, specific for HeV and NiV, as well as cell-culture adapted viruses. Stock HeV and NiV and the unidentified henipavirus are diluted and replicates of each virus containing approximately 100 $TCID_{50}$ in 50 µl are added to the test wells of a flat bottom 96-well microtitre plate. The viruses are mixed with an equal volume of either Eagle's minimal essential media (EMEM) or a range of dilutions of anti-HeV or anti-NiV antiserum in EMEM. The mixtures are incubated at 37°C for 45 minutes and approximately 2.4×10^4 cells are added to each well to a final volume of approximately 200 µl. After 3 days at 37°C, the test is read using an inverted microscope and wells are scored for the degree of CPE observed. Those that contain cells only or cells and antiserum should show no CPE. In contrast, wells containing cells and virus should show syncytia and cell destruction. A positive well is one where all or a proportion of cells in the monolayer form large syncytia typical of henipavirus infection.

3. Molecular methods – detection of nucleic acid

The complete genomes of both HeV and NiV have been sequenced, and as more isolates come to hand their sequences have been deposited on GenBank. PCR-based methods are commonly used to detect virus. They have the biosafety advantage of not propagating live infectious virus and they have been validated in a number of laboratories. They are also highly sensitive and specific. Specimens sampled for PCR should be inactivated as part of the RNA extraction procedure before any further manipulations (PCR or sequencing). Advice on procedures is available from the WOAH Reference Laboratory[1].

3.1. Real-time reverse-transcription polymerase chain reaction

For the primary detection of henipaviruses, real-time RT-PCR is used (see Table 2). There is a range of test methods and primers published, such as the HeV-g2 M gene assay which is broadly reactive and detects HeV-g1 and HeV-g2 (Wang *et al.,* 2021), whereas the HeV-g1 M gene assay is Hendra-g1 specific (Smith *et al*., 2001). The HeV P gene assay detects both Hendra and Nipah (Feldman *et al*., 2009).

Table 2. Real-time RT PCR assays for the detection of HeV and NiV

Assay	Oligo	Name	Primer sequence (5' → 3')	Probe label (5' → 3')
HeV-g1_M gene (Smith *et al*., 2001)	Forward	HeV M 5755F	CTT-CGA-CAA-AGA-CGG-AAC-CAA	
	Reverse	HeV M 5823R	CCA-GCT-CGT-CGG-ACA-AAA-TT	
	Probe	HeV M 5778P	TGG-CAT-CTT-TCA-TGC-TCC-ATC-TCG-G	FAM-TAMRA
HeV-g2 M* gene (Wang *et al.,* 2021)	Forward	HeV-g2-M-F	CTG-ATC-TAC-GTT-ACG-GCA-AAC-CTT	
	Reverse	HeV-g2-M-R	GG-CCC-GCT-TCA-CCA-TCT-CTT-AC	
	Probe	HeV-g2-M-P	CAG-CAT-TGA-ATA-TTG-ACC-CGC-CAG-TCA	FAM-BHQ1

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3.

Assay	Oligo	Name	Primer sequence (5' → 3')	Probe label (5' → 3')
HeV-g2 N gene (Wang *et al.,* 2021)	Forward	HeV-g2-N-F	TGC-GAC-AGA-TCC-CAG-TAG-TAT-TAA-AT	
	Reverse	HeV-g2-N-R	GGC-AGC-TTA-TTC-GGC-AAA-AG	
	Probe	HeV-g2-N-P	CTC-TGG-TGA-CGG-AAC-ACA-AAT-GCA-AAT-TTC	FAM-BHQ1
HeV_P** gene (Feldman *et al*., 2009)	Forward	HeV_P_2698F	ACA-TAC-AAC-TGG-ACC-CAR-TGG-TT	
	Reverse	HeV_P_2794R	CAC-CCT-CTC-TCA-GGG-CTT-GA	
	Probe	HeV_P_2721P	ACA-GAC-GTT-GTA-TAC-CAT-G	FAM-MGBNFQ
HeV_N*** gene (Feldman *et al*., 2009)	Forward	HeV N119F	GAT-ATI-TTT-GAM-GAG-GCG-GCT-AGT-T	
	Reverse	HeV N260R	CCC-ATC-TCA-GTT-CTG-GGC-TAT-TAG	
	Probe	HeV N198-220P	CTA-CTT-TGA-CTA-CTA-AGA-TAA-GA	FAM-MGBNFQ
NiV_L gene**** (Wang unpublished)	Forward	NiV_L_11908F	GGT-ATG-ART-GTT-TTT-TGT-TTT-GGT-TTA-C	
	Reverse	NiV_L_11962R	CGG-CTT-TTG-YGA-ATT-CTT-GA	
	Probe	NiV_L_11937P	ATC-AAA-ACA-GAG-ATG-CGA-GC	FAM-MGBNFQ

*The HeV-g2 M gene assay is broadly reactive and detects HeV-g1 and HeV-g2
**The HeV P gene assay detects both HeV-g1 and HeV-g2 and NiV, but with slightly less sensitivity for HeV-g2.
***The HeV N gene assay detects HeV-g1. There are a few mismatches with HeV-g2 but this is overcome when viral loads are high.
****The NiV L gene assay detects both strains of NiV, namely NiV-M and NiV-B.

3.2. Conventional RT-PCR and Sanger sequencing

Two semi-nested conventional PCR assays, targeting the M gene and the P gene, can also be used for the detection of HeV. These two assays are used as supplementary tests to confirm the results from the real-time assays when unusual/atypical results arise. They are also used for characterisation of detected HeVs when followed by Sanger (di-deoxy) sequencing using the same primers (see Table 3).

Table 3. Primers used for conventional PCR and sequencing of HeV

Target	Assay	Type	Name	Primer Sequence (5'-3')	PCR product
HeV M* gene (Wang *et al.,* 2021)	Primary PCR	Forward	HeV M 5481F	GCC-CGC-TTC-ATC-ATC-TCT-T	300 bp
		Reverse	HeV M 5781R1	CCA-CTT-TGG-TTC-CGT-CTT-TG	
	Semi-nested PCR	Forward	HeV M 5481F	GCC-CGC-TTC-ATC-ATC-TCT-T	211 bp
		Reverse	HeV M 5691R2	TGG-CAT-CTT-TCA-TGC-TCC-ATC-TCG-G	
HeV P gene	Primary PCR	Forward	HeV P 4464F1	CAG-GAG-GTG-GCC-AAT-ACA-GT	335 bp
		Reverse	HeV P 4798R	GAC-TTG-GCA-CAA-CCC-AGA-TT	
	Semi-nested PCR	Forward	HeV P 4594F2	TCA-ACC-ATT-CAT-AAA-CCG-TCA-G	205 bp
		Reverse	HeV P 4798R	GAC-TTG-GCA-CAA-CCC-AGA-TT	

*The HeV M gene semi-nested conventional PCR detects both HeV-g1 and HeV-g2. The HeV-P gene semi-nested conventional PCR also detects HeV-g1 and HeV-g2, but with much lower sensitivity for HeV-g2.

3.2.1. HeV RT-PCR conditions

i) Primary RT-PCR

1× 48°C for 30 minutes, 94°C for 2 minutes

40× 95°C for 30 seconds, 53°C for 30 seconds, 68°C for 45 seconds

1× 68°C for 7 minutes

ii) Semi-nested PCR

1× 95°C for 5 minutes

30× 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds

1× 72°C for 7 minutes

A range of conventional PCRs for NiV have been described, most of which target the N gene. For more details see Wacharapluesadee & Hemachudha (2007). A semi-nested PCR targeting the L gene has been described by Feldman *et al*., 2009 and is shown below. This assay detects NiV-M and NiV-B and will also detect HeV.

Table 4. Primers used for conventional PCR and sequencing of both strains of NiV (Feldman et al., 2009)

NiV L gene	Primary RT-PCR	Forward	LFWD1	TGA GYA TGT ATA TGA AAG ATA AAG C	364 bp
		Reverse	LREV	TCA TCY TTA ACC ATC CCG TTC TC	
	Semi-nested PCR	Forward	LFWD2	ACC GAR CCA AGA TTG GT	266 bp
		Reverse	LREV	TCA TCY TTA ACC ATC CCG TTC TC	

3.2.2. NiV-L RT-PCR conditions

i) Primary RT-PCR

1× 48°C for 30 minutes

1× 95°C for 15 minutes

30× 94°C for 30 seconds, 42°C for 30 seconds, 68°C for 1 minute

1× 68°C for 5 minutes

ii) Semi-nested PCR

1× 95°C for 15 minutes

30× 94°C for 30 seconds, 46°C for 30 seconds, 72°C for 30 seconds

1× 72°C for 5 minutes

Laboratories wishing to establish molecular detection methods should refer to published protocols or consult the WOAH Reference Laboratory.

3.3. Henipavirus antigen detection in fixed tissue – immunohistochemistry

Immunohistochemistry is a powerful tool that allows the visualisation of viral antigen within cell and tissue structures. Nucleoprotein viral antigen in usually located within particulate structures of variable size and form within the cytoplasm. Because of the morphological aspect to the interpretation, colour signal can be effectively evaluated for its specificity. The test is done on formalin-fixed tissues, allowing the procedure to be done safely under non-microbiologically contained conditions.

Henipavirus antigen replicates in a range of cell types, including endothelium, vascular smooth muscle, lung parenchyma, kidney glomeruli, neuron cell bodies, lymphoid tissues and connective tissues (Hooper *et al.,* 2001; Marsh *et al*., 2011). Antigen is particularly dense in syncytia and in macrophages

within lesions. Therefore, suitable tissues for diagnosis of henipavirus infection include lung, brain, lymph nodes, spleen and kidney. In the absence of these tissues, it is worthwhile examining any tissue type, as antigen can be found in occasional blood vessels throughout the vascular bed. Unless full protective clothing can be worn and suitable disinfection protocols be implemented, it is safer to remove only small pieces of tissue through 'keyhole' sampling from suspect cases. Lung tissue and sub-mandibular lymph nodes are good tissues to remove in this manner.

Rabbit polyclonal antisera raised against recombinant henipavirus nucleoprotein are highly reliable for use as primary antibodies for diagnostic immunohistochemistry. Detection of phosphoprotein antigens is also suitable for diagnostic purposes, although phosphoprotein tends to be less expressed than nucleoprotein. There are various secondary detection systems on the market that can be used. The following is an example of an immunohistochemical procedure using an immunoperoxidase system and AEC chromogen. Other methods can be used, with slight variation of the method for different enzymes and chromogens.

3.3.1. Test procedure

i) Process the fixed tissues according to routine histological procedures into paraffin wax blocks and cut sections onto glass slides. Cut positive control sections and negative controls, if appropriate.

ii) Dewax the slides by immersion in three consecutive xylene baths for 3 minutes each. Hydrate sections through two changes of 98–100% ethanol, one change of 70% ethanol and running tap water to remove residual alcohol.

iii) Antigen retrieval can be done through heating in a citrate buffer (pH 9) for 20 minutes at 97°C, or by proteinase K digestion for 5 minutes.

iv) At this point and between each successive step till after step vii, wash the slides in TRIS buffer (pH 7.6) multiple times.

v) Block endogenous compound at this stage. This will depend on the detection system used, for example, if an immunoperoxidase system is used then endogenous peroxidase needs to be blocked with 3% aqueous H_2O_2 for 10 minutes.

vi) Add the primary antibody at a pre-characterised dilution for 45 minutes.

vii) Add the secondary antibody conjugate. Many different systems are available: the simplest and most robust consist of a single step. Consult the manufacturer's product guidelines for the correct use.

viii) Add the chromagen (for example, 3-amino-9-ethylcarbazole (AEC), or 3,3' diamino-benzidine (DAB) for 10 minutes. Refer to the product guidelines for the correct use.

ix) Wash in distilled water to stop colour development.

x) Counterstain in haematoxylin for 30 seconds to 3 minutes (depending on type).

xi) Rinse in tap water. Add Scott's solution (0.04 M sodium bicarbonate, 0.3 M magnesium sulphate), for 1 minute and wash well in running tap water.

xii) Mount with a coverslip using aqueous mounting medium.

xiii) Viral antigen can be visualised by the brown/ red stain, the colour depending on the chromagen used.

All the above test methods should be considered as a guide only; each test parameter will need to be optimised for each testing laboratory, as they will vary according to specific laboratory conditions.

4. Serological tests

In laboratories doing serological testing, particularly in outbreak situations, several strategies have been adopted to reduce the risk of exposure of laboratory personnel to HeV and NiV. Sera received from a suspected disease outbreak or from an endemic region should be gamma-irradiated (6 kilograys) or diluted 1/5 in phosphate buffered saline (PBS) containing 0.5% polysorbate 20 and 0.5% octylphenol ethoxylate and heat-inactivated at 56°C for 30 minutes prior to testing. The process used will be based on a risk assessment. Specimens for surveillance testing

and testing for animal movement certification may be considered a lesser biohazard than those for disease investigation during an outbreak. In some circumstances heat inactivation may be adopted as a sufficient precaution. However, there is value in having a standardised approach for all samples in managing a test, rather than be maintaining multiple test methods.

In Australia, the introduction of equine vaccination against Hendra virus has affected the possible range of purposes of testing of assays that detect antibody to the G protein. The test may be used to detect immune responses to vaccination, and detection of antibodies no longer necessarily indicates prior infection in situations where vaccine may have been used. The possibility of vaccination must be considered when interpreting serological test reactions.

4.1. Virus neutralisation tests

The virus neutralisation test (VNT) is accepted as the reference standard. The most commonly used is the microtitre assay, which must be performed under appropriate conditions of biological risk management. Test sera are incubated with either HeV or NiV in the wells of 96-well microtitre plates prior to the addition of Vero cells. Sera are screened starting at a 1/2 dilution although this may lead to problems with serum-induced cytotoxicity. Where sample quality is poor or sample volumes are small, as may be the case with flying-fox or microbat sera, an initial dilution of 1/5 may be appropriate. Cultures are read on day 3, and those sera that completely block development of CPE are designated as antibody positive. If cytotoxicity is a problem an immune plaque assay (Crameri *et al*., 2002) approach would have merit because the virus/serum mixtures are removed from the Vero cell monolayers after the adsorption period, thereby limiting their toxic effect. VNT results are considered positive if virus neutralisation is observed at any of the dilutions used in the test. If neutralising antibodies are present for both HeV and NiV, the higher titre >four-fold is considered the positive and if titres differed by <four-fold the serum is considered positive for an unspecified henipavirus. For laboratories that do not have appropriate biosecure facilities, a neutralisation test using a pseudotype vesicular stomatitis virus expressing green fluorescent protein has been described (Kaku *et al*., 2009).

4.2. Enzyme-linked immunosorbent assay

Henipavirus antigens derived from tissue culture for use in the enzyme-linked immunosorbent assay (ELISA) are irradiated with 6 kilograys prior to use, a treatment that has negligible effect on antigen titre. In the indirect ELISA developed in response to the initial outbreak at Hendra in 1994, antigen was derived from HeV-infected cells subjected to several cycles of freezing and thawing and treatment with 0.1% (w/v) sodium dodecyl sulphate. Now a recombinant expressed soluble form of the Hendra G protein (HeV-sG) (Bossart *et al*., 2005) is the preferred antigen and its use has enabled improvements in Hendra virus immunoassays including the development of a blocking ELISA for multiple species (horses, cats and dogs) (Di Rubbo *et al.,* 2019). An IgM antibody capture (MAC) ELISA for the detection of IgM HeV antibodies for use in horses has been recently described (McNabb *et al*., 2021). The HeV IgM MAC ELISA is intended to supplement other molecular and serology test results, with selective use, and is the only serology test which can provide an indication of recent infection.

In the national swine surveillance programme in Malaysia in 1999 an indirect ELISA format was used in which antigen was derived by non-ionic detergent treatment of NiV-infected cells. Subsequently, to control for high levels of nonspecific binding activity in some porcine antisera, a modified ELISA was developed based on the relative reactivity of sera with NiV antigen and a control antigen derived from uninfected Vero cells. For NiV, an ELISA using a recombinant nucleocapsid antigen has also been described (Yu *et al*., 2006), which is configured to detect either IgG or IgM.

The current approach is to test all ELISA reactive sera by VNT, with sera reacting in the VNT considered to be positive. Confirmatory VNT should be done under conditions where the risks of working with live virus are adequately managed and this may entail sending the samples to an internationally recognised laboratory with established procedures for such work.

4.2.1. Hendra sG I-ELISA method for use with horse serum – test procedure

A recombinant expressed soluble form of the Hendra G protein (Bossart *et al.,* 2005) has been used in the assay of choice. The Hendra soluble G indirect ELISA eliminated almost all false-positive results from the previously used HeV I-ELISA, which used a crude SDS (sodium dodecyl sulphate) viral preparation, with marginally decreased relative sensitivity (Colling *et al.,* 2018).

Assay robustness was evaluated in inter-laboratory and proficiency testing panels (Colling *et al.,* 2018). This assay is considered to be fit for purpose for serosurveillance and international movement of horses when virus neutralisation is used for follow-up testing of positive or inconclusive serum samples.

i) I-ELISA method

a) Coat a 96 well microtitre flat bottom hard ELISA plate with HeV sG recombinant protein diluted 1/3000 in PBS A coating buffer (50 µl/well equivalent to a coating concentration of 0.23 µg per ml) for 1 hour at 37°C on a plate shaker

b) Proceed to step c or seal plate with tape and store at 4°C (no longer than overnight).

c) Block plate by adding 50 µl/well of blocking buffer (5% skimmed milk powder [SMP]in PBS A) (no wash at this step).

d) Incubate for a further 30 minutes to 1 hour at 37°C on a plate shaker.

e) Wash plate 4× with PBS containing 0.05% polysorbate 20 (PBST) using plate washer.

f) Dilute test sera and controls 1/100 in ELISA diluent (1% SMP in PBST) and add 50 µl/well. Note: dilute samples 1/20 if they have been octylphenol ethoxylate/ polysorbate 20 treated.

g) Incubate for 1 hour at 37°C on a plate shaker, cover with plate sealer.

h) Wash plate 4× with PBST using plate washer.

i) Add anti-equine-horseradish peroxidase conjugate 1/5000 in ELISA diluent (1% SMP in PBST).

j) Incubate for 30 minutes at 37°C on a plate shaker, cover with plate sealer.

k) Wash plate 4× with PBST using plate washer

l) Add 50 µl/well TMB (tetramethylbenzidine) chromogen/substrate and incubate at room temperature for 7–10 minutes.

m) Stop reaction with 50 µl/well 1 M H_2SO_4.

n) Read optical density at 450 nm on plate reader.

Data transformation: Mean OD values are calculated for the test and control results. The mean OD for the negative control serum, OD C(–) is subtracted from all mean OD values. These are converted to a signal-to-positive ratio (S/P) of the median low positive serum (C+).

$S/P = (OD_{TEST} – OD_{NHS})/(\text{average } OD_{L\_POS} – OD_{NHS})$

ii) Acceptance criteria

The test is valid if all of the following criteria are met

OD C(+) – OD C(–)	0.30 to 1.0
OD C(–)	< 0.25
S/P C(++)	1.4 to 2.4

iii) Interpretation of results

Samples with S/P less than 0.25 are negative.

Samples with S/P between 0.25 and 0.4 are considered inconclusive and a VNT is required to clarify status. Samples with S/P between > 0.4 are considered positive and a VNT is required to confirm this result.

One limitation of this assay is that it does not distinguish between antibodies due to natural infection and those due to vaccination (due to soluble G being present in the current Hendra virus vaccine). Any positive result must be interpreted in the context of the horse's vaccination history. Also, this assay can only be used with horse serum due to the use of an anti-equine horseradish peroxidase conjugate in the ELISA.

4.2.2. Hendra B-ELISA method

A blocking Hendra virus ELISA has been validated for use for equine, feline and canine sera (Di Rubbo *et al.,* 2019).

i) B-ELISA method

a) Pre-treatment of sera with octylphenol ethoxylate/polysorbate 20 (1/5 dilution in PBS containing 0.5% [v/v] octylphenol ethoxylate and 0.5% [v/v] polysorbate 20).

b) Coat a 96 well microtitre flat bottom hard ELISA plates with 50 µl/well of Hendra virus soluble G antigen in PBS, equivalent to 4.4 ng. Incubate at 37°C for 1 hour on a plate shaker.

c) Block plates by adding 50 µl of ELISA diluent (1 × blocking buffer) directly to the wells without washing the plate. Place the plate on the plate shaker for 30 minutes at 37°C.

d) Wash the plates 3 × with PBST.

e) Dilute serum (30 µl of sera + 120 µl of blocking buffer) and add 50 µl per well. If using octylphenol ethoxylate/polysorbate 20 treated samples add 50 µl directly to wells. Place on plate shaker for 1 hour at 37°C.

f) Add monoclonal antibody (MAb) 1.2 diluted in blocking buffer 50 µl per well. Do not add MAb to the blank wells. Place on plate shaker for 1 hour at 37°C.

g) Wash the plates 3 × with PBST.

h) Add diluted conjugate (Jackson anti-mouse horseradish peroxidase) in blocking buffer 50 µl per well.

i) Wash the plates 3 × with PBST.

j) Add 50 µl of TMB chromogen/substrate. Colour development occurs in 7–10 minutes.

k) Add 50µl of stop solution (1 M sulphuric acid) to all wells.

l) Read using a plate reader at 450 nm within 5 minutes of stopping.

Results are expressed as percentage inhibition (PI) relative to the average OD of the negative control sera:

$PI = \{100 \times (1 - [\text{Test } OD_{AVG} / \text{NEG CONTROL } OD_{AVG}])\}$

ii) Interpretation of results

If MAb1.2 PI is less than 33% the test result is negative. If the PI is greater than or equal to 33% the test result is positive. Positive results should be confirmed by the VNT. Negative results are reported without the need for further testing.

This Hendra B-ELISA also does not distinguish between antibodies due to natural infection and those due to vaccination (due to soluble G being present in the current Hendra virus vaccine used in horses). Any positive result must be interpreted in the context of the animal's vaccination history.

The following procedure for the NiV ELISA has been developed at Australian Centre for Disease Preparedness (ACDP) for porcine sera and standardised after collaborative studies in the Veterinary Research Institute, Ipoh, Malaysia. Other ELISA protocols for henipavirus diagnostics in pigs have been published.

4.2.3. Nipah indirect ELISA (NiV I-ELISA) for use with pig serum

Detailed methodology for production and/or supply of irradiated NiV and uninfected Vero cell antigens are available from the WOAH Reference Laboratory.

i) Preparation of test sera

a) Preparation of blood samples prior to centrifugation should be done in a biological class II safety cabinet with appropriate personal protective equipment or a class III cabinet.

b) Dilute test serum 1/5 in PBS containing 0.5% (v/v) octylphenol ethoxylate and 0.5% (v/v) polysorbate 20 in the wells of a 96-well microtitre plate. Seal the microtitre plate. Laboratory personnel should wear gowns and gloves and spray both their hands and the sealed microtitre plate with suitable disinfectant before removing the microtitre plate from the biosafety cabinet to heat at 56°C for 30 minutes.

c) Mix 22.5 µl heat-inactivated serum with an equal volume of uninfected Vero cell antigen diluted 1/100 in PBS. Mix thoroughly and incubate at 18–22°C for 30 minutes.

d) Add 405 µl blocking solution (PBS containing 5% chicken serum and 5% SMP) to give a final serum dilution of 1/100 and incubate at 18–22°C for 30 minutes. Aliquots of 100 µl are added to two wells containing NiV antigen and two wells containing uninfected Vero cell control antigen as described in step ii) *ELISA method* below.

ii) ELISA method

a) Dilute Vero cell control and NiV antigens in PBS to ensure that control and virus antigen wells are coated in parallel and at a similar concentration of protein. Antigen is usually diluted 1/1000 to 1/4000, but a specific dilution factor must be determined for each batch of antigen. Add 50 µl virus and cell control antigen to the wells of a 96-well microtitre plate as follows: virus antigen in columns 1, 3, 5, 7, 9 and 11 and cell control antigen in columns 2, 4, 6, 8 10 and 12. Incubate at 37°C for 1 hour with shaking. Plates can be also incubated at 4°C overnight.

b) Wash ELISA plates three times with PBS containing 0.05% polysorbate 20 (PBST) (250 µl/well) and block with PBS containing 5% chicken serum and 5% SMP (100 µl/well) for 30 minutes at 37°C on a shaker.

c) Wash plates three times with PBST and add 100 µl of inactivated, absorbed sera from step i) *Preparation of test sera* above to each well. Add 100 µl PBS containing 5% chicken serum and 5% SMP to conjugate and substrate control wells. Incubate the plates without shaking for 1 hour at 37°C and wash three times with PBST.

d) Dilute protein A/G-horseradish peroxidase conjugate in PBST containing 1% (w/v) skim milk powder. The dilution factor is approximately 1/50,000. Mix well and add 100 µl protein A-conjugate to all wells except the substrate control wells. Add 100 µl PBST containing 1% SMP to the substrate control wells. Incubate the plates for 1 hour at 37°C without shaking and wash four times with PBST.

e) Prepare the chromogen/substrate (3,3',5,5'-tetramethylbenzidine; TMB) by dissolving one tablet (1 mg) in 10 ml of 0.05 M phosphate citrate buffer, pH 5.0, and add 2 µl of fresh 30% (v/v) H_2O_2. Add 100 µl of the TMB substrate to each well. Incubate for 10 minutes at 18–22°C and stop the test by adding 100 µl 1 M sulphuric acid to each well.

f) Read plates after blanking on a substrate control well. The optical density (OD) at 450 nm on NiV antigen and control Vero cell antigen are used to calculate an OD ratio for each serum (OD on NiV antigen/OD on Vero control antigen).

iii) Interpretation of results

Samples with NiV antigen OD value less than 0.20 are negative. Samples with NiV antigen OD value greater than 0.2 are assessed by OD ratio (antigen/control) value accordingly as:

a) an OD ratio >2.0 are considered positive

b) an OD ratio between 2.0 and 2.2 should be considered inconclusive

Inconclusive and positive sera should be tested by VNT to either confirm or clarify a result.

4.3. Bead-based assays

An advantage of using bead-based assays is that serum can be tested for both Hendra and Nipah virus antibodies in the same well using one experiment instead of multiple tests (ELISAs and VNTs) as validated by McNabb *et al.* (2014). The validated methods below are examples of such assays.

Two multiplexed bead-based serological assays have been developed using magnetic bead-based technology and incorporate identification of antibodies to both HeV or NiV in a single test (McNabb *et al*., 2014). Both assays measure antibodies to recombinant expressed soluble glycoprotein (sG) of HeV and NiV. One assay measures antibodies that bind directly to sG (binding assay) and the other assay measures the ability of antibodies to block the henipavirus receptor ephrinB2 binding to sG (blocking assay). The recombinant HeV or NiV sG proteins are first coupled to individually identifiable magnetic beads. The coupled beads are then mixed with test sera. For the binding assay, bound sera are then detected using a biotinylated protein A/G secondary conjugate and Streptavidin-phycoerythrin (S-PE). For the blocking assay, sera must compete with biotinylated ephrinB2 for binding to the sG and S-PE is again used to quantify the reaction. The beads are then interrogated by lasers in a bead-based multiplexed immunoassay system and the results recorded as the median fluorescent intensity (MFI) of 100 beads. Similar to the approach taken with ELISA, any suspect positive sera are then tested by VNT.

4.3.1. Bead-coupling procedure

i) Bead activation

a) Bring the bead activation buffer (0.1 M NaH_2PO_4, pH6.2) to room temperature prior to use.

NOTE: Be careful to protect the beads from light as they photobleach (cover tubes with foil where possible).

b) Select the magnetic carboxylated beads supplied as 1.25×10^7 beads/ml for the protein coupling reaction for the relevant virus (HeV or NiV). Vortex the beads for 30 seconds at medium speed, then sonicate the beads by bath sonication for ~30–60 seconds. It is important that the beads are completely resuspended as single monodisperse particles.

c) Transfer 300 µl of magnetic carboxylated beads (3.75×10^6 beads) into 2 ml screw capped microtubes. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

d) Wash beads by adding 300µl of PBST to the tubes and vortexing. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet. Repeat.

e) Add 600 µl of bead activation buffer to the tubes and vortex. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet. Repeat.

f) Add 240 µl of bead activation buffer to the tubes, cover with foil and shake for 3 minutes.

g) Prepare crosslinkers (1-ethyl-3-[3-dimethylamino-propyl] carbodiimide [EDC] = a carboxy- and amine-reactive zero-length crosslinker and S-NHS = sulfo-N-hydroxysulfosuccinimide) in bead activation buffer immediately prior to use to a concentration of 50 mg/ml (20 µl buffer/mg powder). Add 30 µl of the freshly made 50 mg/ml EDC into the tubes, closely followed by 30 µl of the freshly made 50 mg/ml S-NHS into the tubes. NOTE: Discard unused portion and make fresh each time.

h) Cover the tubes with aluminium foil and shake the beads at room temperature for 20 minutes.

i) While beads are incubating, prepare sG proteins. Use 90 μg each of HeV sG & NiV sG and use PBS (do not use PBST, as it blocks carboxy groups) to bring proteins up to a final volume of 300 μl.

j) After incubation, the beads are now activated and ready for coupling. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

ii) Protein coupling

a) Wash beads by adding 300 μl of PBS to the tubes and vortexing (do not use PBST as it blocks carboxy groups). Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

b) Add all of the 300 μl of prepared protein, above, to the activated beads.

c) Cover the tubes with aluminium foil and shake the beads moderately at room temperature for 2 hours.

d) The protein is now coupled to the beads. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

e) Wash the beads twice with 300 μl of PBST as described above. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

f) Resuspend the coupled beads in 1.8 ml bead storage buffer (10 ml PBS, 1% bovine serum albumen [BSA], 0.05% sodium azide and 1 protease inhibitor tablet and store at 4°C.

NOTES: Check reactivity of sG with henipavirus sera before use. Use 1 μl of coupled beads per well for henipavirus binding and blocking serological assays (this procedure couples enough beads to test around 1800 sera). Coupled beads are able to be stored at 4°C for at least 1 year and maintain reactivity.

4.3.2. Henipavirus binding assay procedure

i) Test method

a) Select previously coupled HeV and NiV sG beads. Vortex the beads for 30 seconds at maximum speed, then sonicate the beads by bath sonication for ~30–60 seconds.

b) Dilute beads in blocker (2% skim milk in PBST) at an appropriate concentration for the number of sera to be tested (1 μl of each bead set/well).

c) Add 100 μl of diluted beads to appropriate wells of a 96-well flat bottom plate.

d) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

e) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

f) Wash twice with PBST or alternatively, use automated magnetic plate washer.

g) Add 100 μl of control and test sera diluted 1/100 in PBST to the wells (bat sera dilute 1/50).

NOTE: All sera should be heat-inactivated for 35 minutes at 56°C before testing.

h) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

i) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

j) Wash twice with PBST or alternatively, use automated magnetic plate washer.

k) Dilute biotinylated protein A 1/500 (2 ug/ml) and biotinylated protein G 1/250 (2 μg/ml) in the same tube in PBST and add 100 μl to the wells.

l) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

m) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

n) Wash twice with PBST or alternatively, use automated magnetic plate washer.

o) Add 100 μl of Streptavidin R-PE diluted 1/1000 (1 ug/ml) in PBST to the wells.

p) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

q) Read plate using an appropriate bead-based multiplexed immunoassay system and software.

ii) Interpretation of results

The results can be interpreted from the raw mean fluorescence intensity (MFI) values or can be transformed into a percentage relative to the MFI for the positive control (%P) using the following formula:

(MFI test serum/MFI positive control) × 100

A sample giving an MFI >1500 or %P >5 should be first retested in the binding assay. If the sample is still positive, it should be tested further by VNT for confirmation.

4.3.3. Henipavirus blocking assay procedure

i) Test method

a) Select previously coupled HeV and NiV sG beads. Vortex the beads for 30 seconds at max speed, then sonicate the beads by bath sonication for ~30–60 seconds.

b) Dilute beads in blocker (2% skim milk in PBST) at an appropriate concentration for the number of sera to be tested (1 μl of each bead set/well).

c) Add 100 μl of diluted beads to appropriate wells of a 96 well TC flat-bottom plate.

d) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

e) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

f) Wash twice with PBST. Or, alternatively, use automated magnetic plate washer.

g) Add 100 μl of control and test sera diluted 1/50 in PBST to the wells (bat sera dilute 1/25).

NOTE: All sera should be heat-inactivated for 35 minutes at 56°C before testing.

h) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

i) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

j) Wash twice with PBST or alternatively, use automated magnetic plate washer.

k) Dilute biotinylated ephrinB2 1/1000 (50 ng/ml) in PBST and add 100 μl to the wells.

l) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

m) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

n) Wash twice with PBST or alternatively, use automated magnetic plate washer.

o) Add 100 µl of streptavidin R-PE diluted 1/1000 (1 ug/ml) in PBST to the wells.

p) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

q) Read plate using an appropriate bead-based multiplexed immunoassay system and software.

ii) Interpretation of results

For the blocking assay, the raw MFI readings are converted into a percentage inhibition (%I) using the following formula: (1 - [MFI test serum/MFI negative serum]) × 100

A sample giving a %I >15 should be first retested in the blocking assay. If the sample is still positive, it should be tested further by VNT for confirmation.

4.4. DIVA

A HeV DIVA assay is used at the Australian Centre for Disease Preparedness to help distinguish between horses that are infected with Hendra virus and horses that have been vaccinated with the Hendra soluble G vaccine. It is currently undergoing validation (please contact the WOAH Reference Laboratory for details).

C. REQUIREMENTS FOR VACCINES

1. Background

The original outbreak of NiV in Malaysia and Singapore was linked to transmission of the virus from pigs to humans, and all of the human infections with Hendra virus in Australia have been linked to contact with sick horses. Development of veterinary vaccines against henipaviruses is important both to protect susceptible domestic animal species (i.e. porcine, equine, feline, and canine) and to reduce transmission from domestic animals to humans. This was the rationale for development of the vaccine for HeV which is currently available for use in horses in Australia. There is no vaccine approved for the prevention of HeV in humans.

2. Soluble G henipavirus vaccine

A preliminary study using HeV in ferrets (Pallister *et al*., 2011) provided strong evidence that a HeV soluble G (HeVsG) glycoprotein subunit–based vaccine could prevent disease in animals exposed to an otherwise lethal dose of HeV. The henipavirus surface-expressed G glycoprotein has the critical role of initiating infection by binding to receptors on host cells, and antibodies directed against this protein can neutralise virus. A Hendra virus horse vaccine has been formulated using HeVsG and a proprietary adjuvant by the vaccine manufacturer. The vaccine, released in Australia in November 2012, is only available for administration by registered veterinarians. For primary immunisation two doses of vaccine should be administered 3–6 weeks apart in horses four months of age or above, followed by a third vaccine 6 months after the second dose. For continued effect, a booster dose every 12 months is recommended by the manufacturer.

3. Experimental vaccines

Recently WHO included Nipah virus as one of 11 prioritised pathogens most likely to cause severe outbreaks in the near future. Alongside this has been the development of an R&D Blueprint for Action to Prevent Epidemics, which establishes a platform for R&D preparedness that is intended to accelerate research and product development in advance of and during epidemics (WHO, 2019). At least 13 Nipah virus vaccine candidates have been confirmed to be under development in preclinical stages (Gouglas *et al.,* 2019). Candidates have focused on the use of NiV glycoprotein (G) and/or fusion protein (F) as immunogens in various platforms, including DNA vaccines, subunit vaccines, non-replicating vectors, as well as replicating vectors. A vaccine based on NiV-B G protein in a replication-

deficient simian adenovirus vector in Syrian hamsters (ChAdOx1 NiVB) has shown promising results (Van Doremalen *et al.*, 2019). A prime-only as well as a prime-boost regime protected Syrian hamsters against challenge with a lethal dose of NiV-B and NiV-M.

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* *

NB: There is a WOAH Reference Laboratory for Hendra and Nipah virus diseases
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Hendra and Nipah virus diseases

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.1.16.

PARATUBERCULOSIS (JOHNE'S DISEASE)

SUMMARY

Description of the disease: *Paratuberculosis (Johne's disease) is a chronic enteritis of ruminants caused by* Mycobacterium avium *subsp.* paratuberculosis *(MAP).*

Diagnosis of paratuberculosis is made on clinical grounds confirmed by the demonstration of MAP *in the faeces by microscopy, culture, or by the use of DNA probes and the polymerase chain reaction (PCR). Diagnosis is made at necropsy by the finding of the pathognomonic lesions of the disease in the intestines, either grossly with the demonstration of typical acid-fast organisms in impression smears of the lesions or histologically, and by isolation of* MAP *in culture.*

Detection of the agent: *The diagnosis of paratuberculosis is divided into two parts: the diagnosis of clinical disease and the detection of subclinical infection. The latter is essential for control of the disease at the farm, national or international level.*

The detection of subclinical infection depends on the detection of specific antibodies by serology, culture of MAP *from faeces or tissues collected at necropsy, PCR, or the demonstration of cell-mediated responses. The choice of test depends on the circumstances and the degree of sensitivity and specificity required at individual animal or herd level.*

Cultures of MAP *may be obtained from faeces or tissues, after treatment to eliminate contaminants, by inoculation into artificial media with and without the specific growth factor – mycobactin – that is essential for the growth of* MAP.

Serological tests: *Control of paratuberculosis is difficult because of the prolonged course of infection, the predominantly subclinical nature of the disease and lack of tests for accurate detection of subclinically infected animals.*

The serological test commonly used for paratuberculosis in cattle is absorbed enzyme-linked immunosorbent assay (ELISA). Agar gel immunodiffusion remains a valuable test for the detection of paratuberculosis in sheep. Sensitivity and specificity are often determined by reference to results of faecal culture, which itself has unknown sensitivity in subclinically infected cattle. When used to confirm diagnosis of paratuberculosis in cows with typical clinical signs ELISA performs very well.

Tests for cell-mediated immunity: *Interferon-gamma release assay and delayed type hypersensitivity methods (skin test) have been used, but interpretation in both cases is difficult.*

Requirements for vaccines and diagnostic biologicals: *Vaccines for paratuberculosis may be live attenuated or killed bacteria either incorporated with an adjuvant or lyophilised and adjuvanted on reconstitution. Bacterial counting is difficult and bacterial content of vaccines may be based on weight, while vaccine potency may be judged by batch tests for sensitising ability in guinea-pigs.*

Vaccine safety or abnormal toxicity may also be tested in guinea-pigs.

For diagnostic skin tests, Johnin and avian tuberculin are purified protein derivatives (PPD) of a heat-treated culture of MAP *or* M. avium*, respectively. Johnin is standardised for content of PPD by chemical assay and its biological activity is identified in guinea-pigs sensitised with* MAP. *Avian tuberculin activity is determined in guinea-pigs sensitised with* M. avium *by comparison with a reference preparation calibrated in international units.*

A. INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (*MAP*) is an organism first observed by Johne & Frothingham in 1895. *MAP* causes paratuberculosis or Johne's disease, an intestinal granulomatous infection (Thorel *et al*., 1990). Paratuberculosis is found most often among domestic ruminants (cattle, sheep, goats, camelids and buffaloes) as well as wild ruminants (cervids) and has a global distribution. The disease has also been reported in horses, donkeys, pigs, rabbits, stoats, foxes and weasels (Greig *et al*., 1999). Under natural conditions, the disease in cattle spreads by ingestion of *MAP* from the contaminated environment. The disease persists after the introduction of infected animals. Infection can be spread vertically to the fetus (Larson & Kopecky, 1970) and semen can be infected with the organism (Sweeney *et al*., 1995). The primary source of infection in calves is faeces from infected cows or milk that is contaminated with the faeces of diseased cattle. Isolates from sheep have different characteristics from those in cattle, but the laboratory detection and diagnostic methods are the same.

The identification of *MAP* is based on its mycobactin requirement and on its association with clinical signs and defined laboratory findings, such as culture and PCR results. Mycobactin dependence has long been used as a taxonomic characteristic for *MAP* because most mycobacteria are able to make mycobactin for themselves. *MAP, M. silvaticum* and some primary isolates of *M. avium* lack this capacity, however, and require mycobactin to grow in the laboratory. Thus, the mycobactin requirement is not confined to *MAP;* this characteristic exists to various degrees within the *M. avium* group.

Clinical signs of paratuberculosis are a slowly progressive wasting and diarrhoea, which is intermittent at first, becoming progressively more severe until it is constantly present in bovines. It is also reported as a significant clinical sign in farmed deer. Diarrhoea is less common in small ruminants.

Early lesions occur in the walls of the small intestine and the draining mesenteric lymph nodes, and infection is confined to these sites at this stage. As the disease progresses, gross lesions occur in the ileum, jejunum, terminal small intestine, caecum and colon, and in the mesenteric lymph nodes. *MAP* is present in the lesions and, terminally, throughout the body. The intestinal lesions are responsible for a protein leak and a protein malabsorption syndrome, which lead to muscle wasting. Clinical signs usually first appear in young adulthood, but the disease can occur in animals at any age over 1–2 years and in dairy cattle is most frequently reported in the 3- to 5-year old age group.

Within a few weeks of infection, a phase of multiplication of *MAP* begins in the walls of the small intestine. Depending on the natural resistance of the individual, this infection is eliminated or the animal remains infected as a healthy carrier. The proportion of animals in these categories is unknown. A later phase of multiplication of the organisms in a proportion of carriers leads to the extension of lesions, interference with gut metabolism and clinical signs of disease. Subclinical carriers excrete variable numbers of *MAP* in the faeces. In most cases larger numbers of organisms are excreted as clinical disease develops.

Cell-mediated immune responses (CMI) are detectable early in the infection and remain present in a proportion of the subclinically infected carriers, but as the disease progresses, CMI wanes and may be absent in clinical cases. Serum antibodies are detectable later than CMI. They may also be present in animals that have recovered from infection. Serum antibodies are present more constantly and are of higher titre as lesions become more extensive, reflecting the amount of antigen present. In sheep, there may be a serological response that is more likely to be detected in multibacillary than in the paucibacillary form of the disease.

Other mycobacterial diseases and infections, including mammalian and avian tuberculosis, stimulate CMI and the presence of serum antibodies. It follows therefore that these diseases need to be differentiated from paratuberculosis, both clinically and by the use of specific diagnostic tests. Exposure to environmental saprophytic mycobacteria may also sensitise livestock, resulting in nonspecific CMI reactions.

Animals vaccinated against paratuberculosis with whole cell vaccines develop both CMI and serum antibodies. Vaccination is an aid to the prevention of clinical disease, but does not necessarily prevent infection. It also interferes with programmes for the diagnosis and control of bovine tuberculosis. Thus, if it is necessary to attempt a diagnosis of infection in vaccinates, it is advisable to use tests detecting the antigen *MAP* in samples of faeces or tissues.

In individual animals, especially from a farm in which the disease has not previously been diagnosed, a tentative clinical diagnosis must be confirmed by laboratory tests. However, a definitive diagnosis may be warranted on clinical grounds alone if the clinical signs are typical and the disease is known to be present in the herd. Confirmation

of paratuberculosis depends on the finding of either gross lesions with the demonstration of typical acid-fast organisms in impression smears or microscopic pathognomonic lesions and the isolation in culture of *MAP*.

MAP organisms and potentially infected specimens should be handled at an appropriate biosafety and containment level determined by biorisk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of paratuberculosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Histopathology*	+	–	+	+++	–	–
Faecal ZN staining	–	–	–	+	–	–
Culture	–	+	+	+++	+	–
PCR	+++	+++	+	++	+	–
Detection of immune response[(b)]						
AGID**	++	+	+	++	+++	+++
ELISA	+++	+++	+++	+	+++	+++
IFN-γ release assay	–	–	+	–	–	+++
DTH	–	–	+	–	–	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose;
* = post-mortem use only; ** = appropriate for the use in sheep and goats.
ZN = Ziehl–Neelsen; PCR = polymerase chain reaction; AGID = agar gel immunofiffusion; ELISA = enzyme-linked immunosorbent assay; IFN-γ = gamma interferon; DTH = delayed-type hypersensitivity.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.
[(b)]A combination of the listed serological tests is recommended.

To diagnose the presence of paratuberculosis in an individual clinically suspect animal, a number of laboratory tests can be used including: faecal smears, faecal and tissue culture, DNA probes using faeces or tissues, serology, necropsy and histology (Table 1).

Herd tests to detect subclinical infection are carried out to determine the prevalence of the infection, usually so that control measures can be instituted. As no test is 100% sensitive or specific, control of the disease by the disposal of positive reactors depends on repeated tests at 6-month or yearly intervals over a number of years and the elimination of reactors to serological tests or faecal shedders; the removal of offspring from female reactors is also considered to be prudent. Even these procedures are not always successful without changes in hygiene and livestock management to reduce the transmission of infection within a herd. Similar test strategies with repeated tests at the herd level can also be applied within control programmes to estimate herd-level probabilities of freedom from infection, and thus to identify low risk herds for safer trade.

1. Detection of the agent

1.1. Necropsy

Paratuberculosis cannot be diagnosed on superficial examination of the intestines for signs of thickening. The intestines should be opened from the duodenum to the rectum to expose the mucosa. There is not always a close correlation between the severity of clinical signs and the extent of intestinal lesions. The mucosa, especially of the terminal ileum, is inspected for pathognomonic thickening and corrugation. Early lesions are seen by holding the intestine up to the light, when discrete plaques can be visualised. Mucosal hyperaemia, erosions and petechiation have been observed in deer with paratuberculosis. The earliest lesions are thickening and cording of lymphatics. The mesenteric lymph nodes are usually enlarged and oedematous. Caseous and/or calcified lesions in mesenteric lymph nodes are often seen in goats and to a lesser extent in sheep (Fodstad & Gunnarsson, 1979). Smears from the affected mucosa and cut surfaces of lymph nodes can be stained by Ziehl–Neelsen's method and examined microscopically for acid-fast organisms that have the morphological characteristics of *MAP*. However, acid-fast organisms are not present in all cases. Diagnosis is therefore best confirmed by the collection of multiple intestinal wall and mesenteric lymph node samples into fixative (10% formol saline) for subsequent histology. Both haematoxylin-and-eosin-stained sections and Ziehl–Neelsen-stained sections should be examined. The typical lesions of paratuberculosis consist of infiltration of the intestinal mucosa, submucosa, Peyer's patches and the cortex of the mesenteric lymph nodes with large macrophages, also known as epithelioid cells, and multinucleate giant cells, in both of which clumps or singly disposed acid-fast bacilli are usually, but not invariably, found.

1.2. Bacteriology (microscopy)

Ziehl–Neelsen-stained smears of faeces or intestinal mucosa are examined microscopically. A presumptive diagnosis of paratuberculosis can be made if clumps (three or more organisms) of small (0.5–1.5 μm), strongly acid-fast bacilli are found. The presence of single acid-fast bacilli in the absence of clumps indicates an inconclusive result. The disadvantages of this test are that it does not differentiate among other mycobacterial species and only a small proportion of cases can be confirmed on microscopic examination of a single faecal sample.

1.3. Bacteriology (culture)

The isolation of *MAP* from an animal provides the definitive diagnosis of infection with the organism. Although culture is technically difficult and time-consuming to carry out, it is the only test that does not produce false-positive results (100% specificity). However, due to the potential pass-through phenomenon, it is theoretically possible that faecal culture testing of non-infected animals on contaminated premises can lead to false-positive reactions (Nielsen & Toft, 2008).

The faecal culture is widely considered to be the gold standard for the diagnosis of paratuberculosis in live animals (affected animals). Actually, the faecal culture is able to detect most animals in advanced stages of the disease, but identifies only a few animals in early stages of infection (Nielsen & Toft, 2008) according to the conditions, sensitivity of faecal culture is 70% for affected cattle, 74% for infectious cattle and 23–29% for infected cattle. The culture of bovine, ovine and caprine tissues for *MAP* is more sensitive than histopathological examination (Fodstad & Gunnarsson, 1979; Koh *et al.,* 1988; Whittington *et al.,* 1999).

There are several culture methods, which vary with respect to media and sample processing protocols. The cultivation of *MAP* is always performed using special media supplemented with mycobactin J, which is available commercially.

MAP organisms are vastly outnumbered by other bacteria or fungi in faecal and intestinal tissue specimens. The successful isolation of *MAP* from such samples depends on efficient inactivation of these undesirable organisms. The optimal method of decontamination must have the least inhibitory effect on growth of *MAP*. Routine decontamination protocols were shown to decrease the number of *MAP* organisms isolated per sample by about 2.7 $\log_{10}$ and 3.1 $\log_{10}$ for faeces and tissues, respectively.

There are two basic methods of sample decontamination: the method using oxalic acid and NaOH for decontamination and Löwenstein–Jensen (LJ) medium for growth, and the method using hexadecylpyridinium chloride (HPC) for decontamination in combination with solid media such as

Herrolds's egg yolk medium (HEYM) or Middlebrook 7H10 and liquid media such as Middlebrook 7H9 or commercial equivalents for growth. Although it has been published that HEYM supports growth of bovine isolates of *MAP* significantly better than LJ, recent studies have shown that certain strains grow better on LJ or Middlebrook media. The advantage of 7H10 medium is that it better supports the growth of ovine strains compared with HEYM. Liquid media have been reported to be more sensitive than solid media for both ovine and bovine strains.

Primary colonies of *MAP* on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation. Sheep strains, including the uncommon, bright yellow pigmented types, grow less well than cattle strains on commonly used media such as HEYM or LJ, and primary cultures should not be discarded as negative without prolonged incubation. The solid medium Middlebrook 7H10 supplemented with egg yolk and mycobactin is excellent for the cultivation of ovine strains of *MAP* (Whittington *et al*., 1999).

Primary colonies of the cattle strain of *MAP* on HEYM are very small, convex (hemispherical), soft, non-mucoid and initially colourless and translucent. Colony size is initially pinpoint. It may remain at 0.25–1 mm, and tend to remain small when colonies are numerous on a slope. Colony margins are round and even, and their surfaces are smooth and glistening. The colonies become bigger more raised, opaque, off-white cream to buff or beige coloured as incubation continues. Older isolated colonies may reach 2 mm. The colonial morphology changes with age from smooth to rough, and from hemispherical to mammilate.

On modified 7H10 medium, colonies of the cattle strain are less convex than those on HEYM, especially in aged cultures. They are pinpoint to approximately 1 mm in diameter and, being buff coloured, are only slightly lighter than the media. Compared with colonies of cattle strains on HEYM, those on 7H10 are more difficult to detect. Colonies of the sheep strain of *MAP* on modified 7H10 are convex, soft, moist, glistening, off-white to buff, and very similar to the colour of the media. Colonies are typically between pinpoint and 0.5 mm, but can reach 1 mm, and rarely 1.5 mm if few colonies occur on a slope.

Saprophytic mycobacteria may have a similar appearance on either medium but are often evident after 5–7 days.

For identification of *MAP,* small inoculum of suspect colonies should be subcultured on the same medium with and without mycobactin, to demonstrate mycobactin dependency. Mycobactin is present in the cell wall of the organism, and heavy inoculum may contain enough mycobactin to support the growth of *MAP* on medium that contains no mycobactin. In addition, the PCR confirmation (targeting IS*900* or F57) should be used to confirm the identification of *MAP* isolates.

1.3.1. Media

Examples of suitable media are:

i) Herrold's egg yolk medium with mycobactin

For 1 litre of medium: 9 g peptone; 4.5 g sodium chloride; 2.7 g beef extract; 27 ml glycerol; 4.1 g sodium pyruvate; 15.3 g agar; 2 mg mycobactin; 870 ml distilled water; six egg yolks (120 ml); and 5.1 ml of a 2% aqueous solution of malachite green. Measure the first six ingredients and dissolve by heating in distilled water. Adjust the pH of the liquid medium to 6.9–7.0 using 4% NaOH, and test to ensure the pH of the solid phase is 7.2–7.3. Add the mycobactin dissolved in 4 ml ethyl alcohol. Autoclave at 121°C for 25 minutes. Cool to 56°C and aseptically add six sterile egg yolks[1] and sterile malachite green solution. Blend gently and dispense into sterile tubes.

It is permissible to add 50 mg chloramphenicol, 100,000 U penicillin and 50 mg amphotericin B.

1 Use fresh eggs not more than 2 days old from a flock that is not receiving antibiotics. With a brush, scrub the eggs with water containing a detergent. Rinse with water and place the eggs in 70° alcohol for 30 minutes. Dry by inserting between two sterile towels. With sterile rat-tooth forceps, crack one end of the eggshell, making a hole of approximately 10 mm, and remove the egg white with the forceps and gravity. Make the hole larger and break the yolk. Mix the egg yolk by twirling the forceps, and remove the yolk sac. Pour the mixed egg yolk into media.

ii) Modified Dubos's medium

For 1 litre of medium: 2.5 g casamino acids; 0.3 g asparagine; 2.5 g anhydrous disodium hydrogen phosphate; 1 g potassium dihydrogen phosphate; 1.5 g sodium citrate; 0.6 g crystalline magnesium sulphate; 25 ml glycerol; 50 ml of a 1% solution of Tween 80; and 15 g agar. Dissolve each salt in distilled water with minimum heat and make up to 800 ml. Add mycobactin in alcoholic solution at 0.05% (2 mg dissolved in 4 ml ethyl alcohol), heat the medium to 100°C by free-steaming, and then sterilise by autoclaving at 115°C for 15 minutes. Cool to 56°C in a water bath, add antibiotics (100,000 U penicillin; 50 mg chloramphenicol; and 50 mg amphotericin B) and serum (200 ml of bovine serum sterilised by filtering through a Seitz 'EX' pad and inactivated by heat at 56°C for 1 hour). The medium is kept thoroughly mixed and then dispensed into sterile tubes. An advantage of this medium is that it is transparent, which facilitates the early detection of colonies.

iii) Modified Middlebrook 7H10 and 7H9

Middlebrook media with added Mycobactin and various commercially available supplements can be used. Further advice on formulation can be obtained from the WOAH Reference Laboratories.

iv) Löwenstein–Jensen medium with or without mycobactin

1.3.2. Sample preparation

i) Processing tissue specimens

Chemical preservatives should not be used. The tissues can be frozen at –70°C.

To avoid contamination, the faeces should be rinsed from portions of intestinal tract before shipment to the laboratory.

a) Digestion/sedimentation method for decontamination of tissues

Approximately 4 g of mucosa from the ileocaecal valve or 4 g of mesenteric node are placed in a sterile blender jar containing 50 ml of trypsin (2.5%). The mixture is adjusted to neutrality using 4% NaOH and pH paper, and stirred for 30 minutes at room temperature on a magnetic mixer. The digested mixture is filtered through gauze. The filtrate is centrifuged at approximately 2000–3000 ***g*** for 30 minutes. The supernatant fluid is poured off and discarded. The sediment is resuspended in 20 ml of 0.75% HPC and allowed to stand undisturbed for 18 hours at room temperature. The particles that settle to the bottom of the tube are to be used as the inoculum and are removed by pipette without disturbing the supernatant fluid. Alternatively, other methods of decontamination can be used, such as treatment with 5% oxalic acid.

b) Double incubation method for decontamination of tissues

About 2 g of tissue sample (trimmed of fat) is finely chopped using a sterile scalpel blade or scissors and homogenised in a stomacher for 1 minute in 25 ml 0.75% HPC. Allow the sample to stand so that foam dissipates and larger pieces of tissue settle. Pour tissue homogenate into a centrifuge tube taking care to avoid carry over of fat or large tissue pieces. Allow to settle for 30 minutes then take 10 ml of the suspension from just above the sediment to a 30 ml tube and incubate for 3 hours at 37°C. Centrifuge for 30 minutes at 900 ***g***, discard supernatant fluid and resuspend pellet in 1 ml antibiotic cocktail containing 100 μg of each of vancomycin, amphotericin and nalidixic acid (VAN). Incubate overnight at 37°C. Use the suspension to inoculate media as described below.

c) Inoculation of culture media and incubation

Approximately 0.1 ml of inoculum is transferred to each of three slants of Herrold's medium containing mycobactin and to one slant of Herrold's medium without mycobactin. The inoculum is distributed evenly over the surface of the slants. The tubes are allowed to remain in a slanted position at 37°C for approximately 1 week with screw caps loose. The tubes are returned to a vertical position when the free moisture has evaporated from the slants. The lids are tightened and the tubes are placed in baskets in an incubator at 37°C.

The egg in Herrold's medium contributes sufficient phospholipids to neutralise the bactericidal activity of residual HPC in the inoculum. The other media (Modified Dubos and Middlebrook) do not have this property. Other treatments can be used for sample decontamination, for example oxalic acid at 5%.

HPC is relatively ineffective in controlling the growth of contaminating fungi. Amphotericin B (fungizone) was found to control effectively fungal overgrowth of inoculated media. Fungizone may be incorporated in the Herrold's medium at a final concentration of 50 μg per ml of medium. Due to loss of antifungal activity, storage of Herrold's medium containing fungizone should be limited to 1 month at 4°C.

The slants are incubated for at least 4 months and observed weekly from the sixth week onwards.

ii) Processing faecal specimens

No chemical preservative is used. The faecal specimens can be frozen at –70°C.

a) Suspension and decontamination of faeces

1 g of faeces is transferred to a 50 ml tube containing 20 ml of sterile distilled water. The mixture is shaken for 30 minutes at room temperature. The larger particles are allowed to settle for 30 minutes. The uppermost 5 ml of faeces suspension is transferred to a 50 ml tube containing 20 ml of 0.95% HPC. The tube is inverted several times to assure uniform distribution and allowed to stand undisturbed for 18 hours at room temperature.

b) Inoculation of culture media

0.1 ml of the undisturbed sediment is transferred to each of four slants of Herrold's medium, three with mycobactin and one without mycobactin. A smear may be made from the sediment and stained by the Ziehl–Neelsen method.

c) Incubation and observation of slants

The same as for tissue specimens.

Variations in the above methods have been described (Collins *et al*., 1990; Merkal *et al*., 1968). The sensitivity of culture may be enhanced using liquid media and with centrifugation rather than sedimentation techniques. The double incubation method described by Whitlock *et al*. (1991) assists with decontamination of the inoculum and offers higher sensitivity than the sedimentation or filtration protocols (Eamens *et al*., 2000). The double incubation method involves mixing 2 g faeces with 15 ml saline or water followed by sedimentation for 30 minutes and transferring (avoiding fibrous matter) the top 5 ml of the suspension to 25 ml of 0.9% HPC in half-strength brain–heart infusion. After incubating at 37°C for 16–24 hours, the mixture is centrifuged at 900 ***g*** for 30 minutes (room temperature), the supernatant is discarded and pellet resuspended in 1 ml VAN. The mixture is incubated for 24–72 hours at 37°C and used to inoculate media as described above.

1.4. DNA probes and polymerase chain reaction

DNA probes are being developed that offer a means of detecting *MAP* in diagnostic samples and of rapidly identifying bacterial isolates (Ellingson *et al*., 1998). They have been used to distinguish between *MAP* and other mycobacteria.

McFadden *et al.* have identified a sequence (McFadden *et al*., 1987), termed IS*900*, which is an insertion sequence specific for *MAP* (Vary *et al.,* 1990). It has been reported that a small number of isolates other than *MAP* have produced amplified products the same size as expected from *MAP*. A restriction enzyme digest may be applied to positive IS*900* products to confirm that their sequence is consistent with *MAP*.

The identifications of new DNA sequences considered to be unique to *MAP* (ISMav2, f57, and ISMap02 sequences), offer additional tools for rapid identification of this organism using the polymerase chain reaction (PCR) technology (Stabel & Bannantine, 2005; Strommenger *et al*., 2001; Vansnick *et al*., 2004). The restriction enzyme analysis of IS*1311*, an insertion sequence common to *M. avium* subsp. *avium* and

MAP can be used to distinguish between these species and for typing of ovine, bovine and bison strains of *MAP* (Sevilla *et al*., 2005; Whittington *et al*., 1998).

In recent years, real-time PCR methods have been extensively developed to detect *MAP* from different specimens (blood, milk, faeces, tissues and environmental samples). The technique is rapid and offers hope for detection of fastidious and slow growing microorganisms, such as *MAP*. However, this molecular tool is greatly influenced by the quality of nucleic acid samples. Therefore, a DNA extraction method that provides a high quality DNA sample and a maximum bacterial DNA recovery is a critical step to use real-rime PCR (Parka *et al*., 2014).

PCR assays have now improved to the point where they play an important role in the diagnosis of disease (Leite *et al.,* 2013).

Commercial diagnostic PCR tests for the detection of *MAP* in milk and faecal samples are available but users should consider the interpretation of acquired data and fitness for purpose before adopting such methods.

2. Serological tests

The serological test commonly used for paratuberculosis in cattle is enzyme-linked immunosorbent assay (ELISA). The complement fixation test (CFT) and agar gel immunodiffusion (AGID) show poorer sensitivity and specificity and are no longer recommended in cattle. However AGID remains a valuable test for the detection of paratuberculosis in sheep. There is no international reference serum.

2.1. Enzyme-linked immunosorbent assay

The ELISA is, at present, the most sensitive and specific test for serum antibodies to *MAP* in cattle. Its sensitivity is comparable with that of the CFT in clinical cases, but is greater than that of the CFT in subclinically infected carriers. The specificity of the ELISA is increased by *M. phlei* absorption of sera. The absorbed ELISA, designed by Yokomizo *et al*. (1983; 1985) and modified by Milner *et al*. (1988), was developed into a commercial kit by Cox *et al*. (1991).

The ELISA detects about 30–40% cattle identified as infected by culture of faeces on solid media (Whitlock *et al*., 2000). Similarly to the culture methods, the sensitivity of the ELISA depends on the level of *MAP* shedding in faeces and the age of animals. A large study performed in Australia showed that the actual sensitivity of the ELISA in 2-, 3- and 4-year-old cows was 1.2%, 8.9% and 11.6%, respectively, but remained between 20 and 30% in older age-groups (Jubb *et al*., 2004). The overall actual sensitivity for all age-groups was calculated to be about 15% (Jubb *et al*., 2004; Whitlock *et al*., 2000). In cattle, the sensitivities of ELISA are in the range 7–94%, and the specificities of ELISA are in the range 40–100% (Nielsen & Toft, 2008).

In small ruminants the commercially available ELISA had a specificity of 98.2–99.5% (95% confidence intervals [CI]) and detected 35–54% (95% CI) of animals with histological evidence of infection (Hope *et al*., 2000). In another study, the estimated specificity of an in-house ELISA was 99% and its sensitivity measured against histological results was 21.9% (Sergeant *et al*., 2003). In small ruminants, the sensitivities of ELISA are in the range 16–100%, and the specificities of ELISA are in the range 79–100% (Nielsen & Toft, 2008).

The absorbed ELISA combines the sensitivity of ELISA with the added specificity of an absorption step. Sera to be tested are diluted with buffer containing soluble *M. phlei* antigen prior to testing in an indirect ELISA. This procedure eliminates nonspecific cross-reacting antibodies. In early versions, sera were absorbed with whole *M. phlei*, which were removed by centrifugation prior to testing.

A microtitre plate format has been developed in which *MAP* antigen is coated on to 96-well plates. Samples are diluted in sample diluent containing *M. phlei* to remove cross-reacting antibodies. On incubation of the diluted sample in the coated well, antibody specific to *MAP* forms a complex with the coated antigens. After washing away unbound materials from the wells, horseradish peroxidase (HRPO)-labelled anti-bovine immunoglobulin is added. This reacts with immunoglobulins bound to the solid-phase antigen. The rate of conversion of substrate is proportional to the amount of bound

immunoglobulin. Subsequent colour, measured spectrophotometrically (at the wavelength appropriate to the chromogen used) is proportional to the amount of antibody present in the test sample.

Several absorbed ELISA kits are commercially available. The method and test materials needed, the interpretation of the results and calculations are fully described in the instructions accompanying the commercial kit. It has been reported that several commercially available ELISAs have similar sensitivities and specificities. Some commercial kits offer an option of testing milk samples. The ELISA on bovine and caprine milk has been found to have specificity similar to that of the serum ELISA, but less sensitive than the blood test (Hendrick *et al*., 2005; Salgado *et al*., 2005). In cattle, the sensitivities of milk ELISA are in the range 21–61%, and the specificities of milk ELISA are in the range 83–100% (Nielsen & Toft, 2008).

2.2. Complement fixation test

The CFT has been the standard test used for cattle for many years. The CFT works well on clinically suspect animals, but does not have sufficient specificity to enable its use in the general population for control purposes. Thus, the CFT is not recommended for control purposes nor for individual animal testing prior to international movement. A variety of CFT procedures are used internationally. There are no international pattern sera with standardised complement fixation units for use as a reference. An example of a microtitre method for performing the CFT is given.

2.1.1. Test procedure

i) The antigen is an aqueous extract of bacteria from which lipid has been removed (strain *M. paratuberculosis* 316F). *Mycobacterium avium* D9 may also be used.

ii) All sera are inactivated in the water bath at 60°C for 30 minutes and diluted at 1/4, 1/8 and 1/16. A positive control serum and a negative control serum should be included on each plate. The following controls are also prepared: antigen control, complement control and haemolytic system control.

iii) Reconstituted, freeze-dried complement is diluted to contain six times H_{50} (50% haemolysing dose) as calculated by titration against the antigen.

iv) Sheep erythrocytes, 2.5%, are sensitised with 2 units of H_{100} haemolysin.

v) All dilutions and reagents are prepared in calcium/magnesium veronal buffer; 25 µl volumes of each reagent are used in 96-well round-bottom microtitration plates.

vi) Primary incubation is at 4°C overnight and secondary incubation is at 37°C for 30 minutes.

vii) *Reading and interpreting the results:* Plates may be left to settle or centrifuged and read as follows: 4+ = 100% fixation, 3+ = 75% fixation, 2+ = 50% fixation, 1+ = 25% fixation and 0 = complete haemolysis. The titre of test sera is given as the reciprocal of the highest dilution of serum giving 50% fixation. A reaction of 2+ at 1/8 is regarded as positive. Results should be interpreted in relation to clinical signs and other laboratory findings.

2.3. Agar gel immunodiffusion test

The AGID test is useful for the confirmation of the disease in clinically suspect cattle, sheep and goats. It has been reported that in small ruminants in New Zealand and Australia the AGID offers slightly higher sensitivity and specificity than that obtained by the ELISAs (Gwozdz *et al*., 2000; Hope *et al*., 2000; Sergeant *et al*., 2003). The reported specificity and sensitivity of the AGID measured against histological results were 99–100% (95%CI) and 38–56% (95% CI), respectively (Hope *et al*., 2000).

The antigen employed is a crude protoplasmic extract of laboratory strain *M. avium* 18 (formally *M. paratuberculosis* 18) prepared by disruption of cells in a hydraulic press cell fractionator. Disrupted cells are centrifuged at 40,000 ***g*** for 2 hours to remove cell wall debris, and the supernatant fraction is retained and lyophilised. This antigen is resuspended in water at a concentration of 10 mg/ml.

Agarose is dissolved in barbital buffer, pH 8.6, containing sodium azide, to give a final agarose concentration of 0.75%. Agarose may be poured into Petri dishes or on to glass slides. Wells are cut in a hexagonal pattern. Wells are 4 mm in diameter, 4 mm apart, and the agar should be 3–4 mm deep.

Antigen is added to centre wells. Test, positive and negative control sera are added to alternate peripheral wells.

Plates are incubated in a humid chamber at room temperature. Gels are examined for precipitation lines after 24 and 48 hours' incubation. The appearance of one or more clearly definable precipitation line(s), showing identity with that of a control positive serum, before or at 48 hours, constitutes a positive test result. Absence of any precipitation lines is recorded as a negative test result. Nonspecific lines may occur.

Several variations of the method are in use.

3. Tests for cell-mediated immunity

The detection of a systemic cell-mediated response precedes detectable antibody production. Animals that are minimally infected frequently fail to react on serological testing but may react positively to tests that measure CMI. In infected populations, a much higher number of animals is expected to react in tests for CMI compared with antibody tests, as CMI is indicative of exposure while antibodies indicate progress of infection.

3.1. Interferon-gamma release assay

The assay is based on the release of gamma interferon (IFN-γ) from sensitised lymphocytes during an 18–36-hour incubation period with specific antigen (avian purified protein derivative [PPD] tuberculin, bovine PPD tuberculin or johnin[2]). The quantitative detection of bovine IFN-γ is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine IFN-γ. A commercial diagnostic test based on the detection of IFN-γ has been developed for the diagnosis of bovine tuberculosis. The method and test materials needed are fully described in the instructions accompanying the commercial kit. This test has not been validated by the manufacturer for the diagnosis of paratuberculosis. As such, results derived from this assay are frequently difficult to interpret because there is no agreement with respect to the interpretation criteria and types and amounts of antigens used to stimulate blood lymphocytes. In cattle the reported specificity of the test varied from 94% to 67% and the sensitivity varied from 13% to 85%, depending on the interpretation criteria (Kalis *et al*., 2003; Nielsen &Toft, 2008).

Several ELISA kits are commercially available for quantitative detection of IFN-γ on bovine, ovine and caprine plasmas.

3.2. Delayed-type hypersensitivity

The skin test for delayed-type hypersensitivity (DTH) is a measure of cell-mediated immunity, but has limited value. The test is carried out by the intradermal inoculation of 0.1 ml of antigen into a clipped or shaven site, usually on the side of the middle third of the neck. In the past, avian PPD tuberculin or johnin was used for this purpose as it was believed that avian tuberculin and johnin are of comparable sensitivity and specificity. The skin thickness is measured with calipers before and 72 hours after inoculation. Increases in skin thickness of over 2 mm should be regarded as indicating the presence of DTH. It should be noted that positive reactions in deer may take the form of diffuse plaques rather than discrete circumscribed swellings, thus making reading of the test more difficult. The presence of any swelling should be regarded as positive in this species. However, sensitisation to the *M. avium* complex is widespread in animals, and neither avian tuberculin nor johnin are highly specific. Furthermore the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria. In a study in which johnin was used to test cattle, the skin test specificity was 88.8% at the cut-off value of ≥2 mm, 91.3% at the cut-off value of ≥3 mm and 93.5% at the cut-off value of ≥4 mm (Kalis *et al*., 2003). The effect of these cut-off values on the sensitivity has not been determined. The performance of this test may also be significantly affected by minor antigenic differences that occur in different batches of antigen (Kalis *et al*., 2003). Further research is required to increase the value of the skin test.

2 Johnin can be obtained from ID-Lelystad, The Netherlands

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

C1. Vaccines

1. Background

Vaccination may interfere with eradication programmes based on immunological testing and elimination of animals identified as infected and can interfere with the interpretation of DTH skin tests for bovine tuberculosis. All commercial vaccines now available are whole-cell-based vaccines. Vaccination of cattle or small ruminants is not allowed in many countries mainly due to the fear of giving rise to false positive results in the skin test for bovine tuberculosis.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Quality criteria

Purity tests should be carried out on seed cultures and final harvest by stained smears.

2.1.2. Validation as a vaccine strain

Seed strains should be of a prevalent type, which may be checked by biotyping or genetic analysis. They should have been demonstrated to be innocuous when administered by the recommended route of vaccination to intended target species.

2.2. Method of manufacture

2.2.1. Procedure

For vaccine batches, the organisms may be grown on a liquid synthetic medium, such as Reid's synthetic medium. The organisms grow as a pellicle on the liquid surface. To ensure a good surface area, it is convenient to use vessels such as conical flasks containing one-third of their nominal volume of liquid medium. These flasks may be seeded directly from potato slant cultures, but with some strains, one or more passages on liquid medium may be necessary to ensure adequate pellicle growth for the final, vaccine batch passage. Such passaging should usually take place at 2-week intervals as longer periods may result in over-maturation and sinking of the pellicle. Incubation is at 37°C.

To prepare the vaccine, the pellicle growth from 2-week-old cultures of each strain to be included may be separated from the liquid medium by decantation, filtration and pressing between filter paper pads. The moist *MAP* culture is blended with an adjuvant, such as liquid paraffin, olive oil and pumice.

2.2.2. In-process controls

Adequate growth of culture and cultural purity need to be checked. Presence of contaminating organisms may be detected by conventional sterility tests on harvests. Tests for pathogenic mycobacteria are carried out by injection of moist culture, taken prior to blending with adjuvant and diluted tenfold in saline, into two guinea-pigs, each receiving 1 ml. These are observed for 8 weeks, killed humanely, and examined for any abnormal lesions.

2.2.3. Final product batch test

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9. The vaccine organism will not normally grow to a detectable level in conventional sterility tests.

ii) Identity

Identity of culture is performed by Ziehl–Neelsen staining and PCR (see Section B.1.4).

iii) Safety

These tests are normally performed in laboratory animals, although multidose tests in target animals would also be satisfactory. A typical laboratory animal test would be as follows. Each of two guinea-pigs is inoculated, subcutaneously, with an acceptable batch of vaccine at a fraction of the cattle dose previously determined to give a nodule but no overt necrosis at the injection site. Animals are observed for 8 weeks, killed humanely and examined for any abnormal lesions.

iv) Batch potency

As protection tests appear to be impractical, a test of sensitising ability may be used. This may then be related to bacterial content based on weight. A typical test would be as follows: guinea-pigs are sensitised by intramuscular injection of 0.5 ml of a 100-fold dilution in liquid paraffin of the vaccine under test. Skin tests are performed 6 weeks after sensitisation using intradermal inoculations of 0.2 ml of at least three serial dilutions of an *MAP* antigen, such as johnin PPD, the dilutions being chosen to give expected skin reactions of from 8 mm to 25 mm diameter. Each guinea-pig receives several dilutions per flank, their distribution being chosen by a Latin square design. After 24–48 hours, skin reactions are measured. A reference preparation for tests of this type has not yet been fully established. Avian tuberculin PPD of known international unitage may be used as a skin test antigen in tests of this type to ensure that the vaccine is capable of producing adequate sensitisation (corresponding to the vaccination).

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

A preservative is normally included for vaccine in multidose containers.

2.3.2. Safety requirements

i) Target and non-target animal safety

See Section C1.2.2.3.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Live vaccines are not available.

iii) Precautions (hazards)

The vaccine causes some side-effects, nodule formation and sensitisation of animals to the tuberculin test (Gwozdz *et al*., 2000). In humans, accidental injection of vaccine has resulted in chronic inflammatory reactions requiring surgical treatment.

2.3.3. Efficacy requirements

The vaccine should be used as part of a control programme and will not on its own provide complete protection against disease caused by *MAP*.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

No DIVA vaccine yet available

2.3.5. Duration of immunity

After vaccination at the age of 14–30 days, the vaccination effect is expressed as the reduction in the rate of excretors among vaccinated animals as compared with nonvaccinated bovines.

C2. Johnin

1. Background

Johnin PPD is a preparation of the heat-treated products of growth and lysis of *MAP.* Avian tuberculin PPD is a preparation of heat-treated products of growth and lysis of *M. avium* subsp. *avium* D4ER or TB 56. Details of avian tuberculin PPD are in Chapter 3.3.6 *Avian tuberculosis*. These two preparations are used, by intradermal injection, to reveal DTH as a means of identifying animals infected or sensitised with *MAP.*

Guidelines for the production of veterinary biologicals are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for Johnin

2.1. Characteristics of the seed

2.1.1. Quality criteria

Cultures should be checked by staining smears for the presence of contaminating organisms.

To test for lack of sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test, are each injected intradermally on each of three occasions at 5-day intervals, with 0.01 mg of the preparation under test in a volume of 0.1 ml. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same johnin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–48 hours later.

2.1.2. Validation as a strain

Strains of *MAP* used to prepare seed cultures should be identified by biotyping or genetic tests. They should be shown to be free from contaminating organisms.

2.2. Method of manufacture

2.2.1. Procedure

Johnin for skin test diagnosis is a PPD prepared from one or more strains of *MAP*. It may be prepared by the following method.

MAP strains are grown as a pellicle on liquid Reid's medium. Production cultures are usually inoculated from liquid seeding cultures rather than directly from seed on solid medium (Reid's synthetic medium). Production cultures are incubated at 37°C for 10 weeks.

At the end of the incubation period, the culture medium has a pH of about 5 and little or no johnin will be obtained unless the pH is raised, using sodium hydroxide, to about 7.3 before steaming. After thorough mixing, the cultures are free steamed for 3 hours. The bulk of the killed organisms is removed by coarse filtration and the filtrate is clarified by further filtration. Protein in the filtrate is precipitated chemically with 40% trichloroacetic acid, washed and redissolved (alkaline solvent). The product is sterilised by filtration. An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Glycerol

(not more than 10% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile glass containers, which are then sealed.

2.2.2. In-process controls

After final filtration the sterility of each filtrate of the PPD solution is checked.

Sterile filtrates are tested for protein content by a Kjeldahl method (British Pharmacopoeia [Veterinary], 1985). The protein content is adjusted to give between 0.475 and 0.525 mg/ml of protein in the final product. The pH is adjusted to the range 6.5–7.5.

2.2.3. Final product batch test

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9. The organism will not normally grow to a detectable level in conventional sterility tests.

ii) Identity

Identity of culture is performed by Ziehl–Neelsen staining and PCR (see Section B.1.4).

iii) Safety

Two guinea-pigs should each be injected subcutaneously with 0.5 ml of the johnin under test. No significant local or systemic lesions should be seen within 7 days (British Pharmacopoeia [Veterinary], 1985).

Tests on johnin for living mycobacteria may be performed either on the material immediately before it is dispensed into final containers or on samples taken from final containers themselves. A sample of at least 10 ml should be taken, and this should be injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, say 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and post-mortem examinations are carried out. Any macroscopic lesions are examined microscopically and culturally.

iv) Batch potency

The potency of johnin is currently determined by chemical assay for protein using a Kjeldahl method. A PPD content of 0.5 ± 0.025 mg/ml of final product is recommended (British Pharmacopoeia [Veterinary], 1985).

2.3. Requirements for regulatory approval

For johnin, the phenol used is no more than 0.5% (w/v). The concentration of the preservative in the final product and its persistence through shelf life should be checked.

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NB: There are WOAH Reference Laboratories for paratuberculosis
(please consult the WOAH Web site at:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for paratuberculosis

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.1.17.

Q FEVER

SUMMARY

Definition of the disease: *Query* (*Q*) *fever* (*or Coxiellosis*) *is a zoonosis that occurs in most countries. Humans generally acquire infection through air-borne transmission from animal reservoirs, especially from domestic ruminants, but other domestic and wildlife animals* (*pets, rabbits, birds, etc.*) *can be involved. The causal agent is the obligate intracellular bacterium,* Coxiella burnetii*, which displays different morphological forms in its developmental cycle. Some forms can survive extracellularly and even accumulate in the environment. All manipulations with potentially infected or contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis*

Description of the disease: *In humans, the disease exhibits a large polymorphism. Q fever occurs either as an acute form or a severe chronic form following an early infection that may go unnoticed. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy* (*for 2 years or more*)*, coupled with serological monitoring. In Australia, a vaccine is available for professionally exposed population groups.*

In domestic ruminants, Q fever is mostly associated with sporadic abortions or outbreaks of abortions and dead or weak offspring, followed by recovery without complications. Moreover, data suggest that Q fever plays a role in infertility or problems such as metritis in cattle. Coxiella burnetii *infection persists for several years, and is probably lifelong. Sheep, goats and cows are mainly subclinical carriers, but can shed bacteria in various secretions and excreta.*

Identification of the agent: *For laboratory diagnosis in the context of serial abortions and/or stillbirths, samples can be taken from the placenta, vaginal discharges and tissues of aborted fetuses* (*spleen, liver, lung or stomach content*)*. For investigation of bacterial shedding, samples can be taken from vagina, milk and colostrum.*

As an obligate intracellular bacterium, Coxiella burnetii *can be isolated by inoculation of specimens into conventional cell cultures, embryonated chicken yolk sacs or laboratory animals. Inoculation of laboratory animals* (*guinea-pig, mouse, hamster*) *is helpful in cases requiring isolation from tissues, faeces, milk or environmental samples contaminated with various microorganisms.*

The bacteria can be visualised in stained tissue or vaginal mucus smears using a microscope with an oil-immersion objective lens. Because it is acid resistant, the bacteria can be stained by several methods: Stamp, modified Ziehl–Neelsen, Gimenez, Giemsa and modified Koster. Because of lack of specificity, a positive finding is only presumptive evidence of Q fever and confirmatory tests should be carried out.

Demonstration of the agent by immunohistochemical staining, by in-situ *hybridisation or by polymerase chain reaction* (*PCR*) *is more specific and sensitive than classical staining methods. No specific antibodies for immunochemistry are commercially available, but PCR kits are proposed for ruminants and can be used easily in suitably equipped laboratories. PCR is considered to be a useful and reliable test for screening large numbers and various types of samples. Currently, PCR has become the tool of choice for Q fever diagnosis.*

Two PCR-based typing methods are becoming widely used, MLVA (*multi-locus variable number of tandem repeats analysis*) *and multispacer sequence typing* (*MST*)*, permitting the typing of* C. burnetii *without the need for isolation of the organism. Moreover, SNP genotyping* (*single nucleotide polymorphism*) *has been recently described.*

Serological tests: *A number of tests can be used, particularly the indirect immunofluorescence (IFA) test, the enzyme-linked immunosorbent assay (ELISA), and the complement fixation test (CFT). The presence of specific IgG antibodies provides evidence of a recent* C. burnetii *infection or a past exposure. ELISAs are preferred for practical reasons and for their higher sensitivity.*

Serological antigens are based on the two major antigenic forms of C. burnetii: *phase I, obtained from spleens after inoculation of laboratory animals, and phase II, obtained by repeated passages in embryonated eggs or in cell cultures. Currently available commercial tests allow the detection of phase II or of both phases II and I anti-*C. burnetii *antibodies.*

Requirements for vaccines: *Several inactivated vaccines against Q fever have been developed, but only vaccines containing or prepared from phase I* C. burnetii *should be considered protective. An inactivated phase I vaccine is commercially available. Repeated annual vaccination, particularly of young animals, is recommended in at-risk areas.*

A. INTRODUCTION

1. Definition of the disease and transmission routes

Q fever (or Coxiellosis) is widely distributed throughout the world with the exception of New Zealand. The causal agent, *Coxiella burnetii,* is present in virtually all animal kingdoms, including arthropods, but the disease affects mostly humans, cattle, sheep and goats (Lang, 1990). Domestic ruminants are considered the main reservoirs of *C. burnetii*, but cats, dogs, rabbits, birds, etc., have also been reported to be implicated in human disease/infection. There is clear epidemiological and experimental evidence that the infection is principally transmitted by inhalation of desiccated aerosol particles, and through exposure in the vicinity of infected animals, their reproductive tissues or other animal products, like wool (ECDC, 2010). Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, but no good evidence has shown significant transmission to humans by food. Q fever also seems very rarely transmissible from person to person, although exposure during childbirth, through sexual transmission or blood transfusion is possible. In animals, vertical transmission and sexual transmission could occur but their importance is not known. Finally, arthropods, principally ticks, may be involved in Q fever transmission. The risk of transmission seems to be linked to wildlife animals. It could be associated with bites as well as with contaminated dust from dried excrement.

2. Description of the causal pathogen

The aetiological agent, *Coxiella burnetii*, is a Gram-negative obligate intracellular bacterium, adapted to thrive within the phagolysosome of the phagocyte. It has been historically classified in the *Rickettsiaceae* family. However, phylogenetic investigations, based mainly on 16s rRNA sequence analysis, have shown that the *Coxiella* genus is distant from the *Rickettsia* genus of the *alpha* subdivision of *Proteobacteria* (Drancourt & Raoult, 2005). *Coxiella burnetii* has been placed in the *Coxiellaceae* family in the order *Legionellales* of the *gamma* subdivision of *Proteobacteria*. The complete genome sequencing of *C. burnetii* has been achieved and confirms its systematic position (Seshadri *et al.,* 2003). In general, the genomes of *C. burnetii* isolates from a wide range of biologically and geographically diverse sources are highly conserved, but notable polymorphism occurs such as rearrangement of syntenic blocks (Beare *et al.*, 2009). This genomic plasticity might contribute to different phenotypes and is of great interest for genotyping methods (Massung *et al.*, 2012; Sidi-Boumedine & Rousset, 2011). Unlike rickettsiae, *C. burnetii* produces a small, dense, highly resistant spore-like form (Heinzen *et al.,* 1999; Minnick & Raghavan, 2012). This ability has been attributed to the existence of *C. burnetii* developmental cycle variants described from *in-vitro* studies: large-cell variants (LCV), small-cell variants (SCV), and small dense cells (SDC) measuring 0.2 µm wide and between 0.5 and 2 µm long or 0.4 to 0.7 µm diameter (Heinzen *et al.,* 1999; Minnick & Raghavan, 2012). The SDC and SCV represent the small morphological variants of the bacteria likely to survive extracellularly as infectious particles, a trait that is important for persistence in the environment and transmission (ECDC, 2010; Kersh *et al.* 2010).

Another essential characteristic is that *C. burnetii* has two antigenic forms: the pathogenic phase I, isolated from infected animals or humans, and the attenuated phase II, obtained by repeated *in-ovo* or *in-vitro* passages. An LPS (lipopolysaccharide) change occurs during serial passages: phase I cells, with full-length LPS O-chains, change to intermediate phases with decreasing LPS O-chain lengths and then to phase II, with truncated LPS. Thus, the long phase I LPS contains the phase II part. The latter has been described as a major immunogenic determinant. Currently available commercial tests allow the detection of at least the anti-*C. burnetii* phase II antibodies, which appear to be

present whatever the infection stage or form. In contrast, vaccination is effective with a phase I vaccine but not with a phase II vaccine (O'Neil *et al.*, 2013).

3. Description of the disease in humans

Q fever is a zoonosis. In humans, the infection can manifest as an acute, chronic or subclinical form (Anderson *et al.*, 2013; ECDC, 2010). Diagnosis and the treatment is often delayed because of the various and nonspecific clinical expressions. The acute forms commonly range from a self-limiting flu-like syndrome to pneumonia or granulomatous hepatitis that may require hospitalisation. The main clinical manifestations of chronic Q fever are endocarditis, valvular, vascular or aneurismal infections, hepatitis, pneumonia or chronic fatigue syndrome. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy (for 2 years or more), coupled with serological monitoring. In the absence of any appropriate antibiotic treatment, complications of the chronic form may be severe to fatal. Moreover, *C. burnetii* infection of pregnant women can provoke placentitis and lead to premature birth, growth restriction, spontaneous abortion or fetal death. Overall, the chronic disease is more likely to develop in individuals with high risk factors (e.g. immuno-compromised or valvulopathies). The infection is endemic in many areas leading to sporadic cases or explosive epidemics. Its incidence is probably greater than reported. Q fever affects all ages but is mostly reported in those aged 30–60 years. Awareness for Q fever is increased during human outbreaks, which are generally temporary and rarely comprise more than 300 acute cases. However, the largest community outbreaks ever reported emerged in 2007 in the Netherlands. In subsequent years, the peak incidence from February to September has increased and the geographical area has expanded progressively. The country reported more than 4000 human cases with a hospitalisation rate of 20%, and it is expected to result in more cases of chronic Q fever among risk groups in the coming years (ECDC, 2010). The losses caused by this epidemic have been estimated to be approximately 307 million euros (van Asseldonk *et al*., 2013).

4. Description of the disease in animals

In cows, ewes and goats, Q fever has been associated mostly with late abortion and reproductive disorders such as premature birth, dead or weak offspring (Lang, 1990). Moreover, *C. burnetii* might be associated with metritis and infertility in cattle. Given the lack of specificity of these latter signs, it is not recommended to rely on them for clinical diagnosis of Q fever. Domestic ruminants are mainly subclinical carriers but can shed bacteria in various secretions and excreta. In the environment, *C. burnetii* can survive for variable periods and can spread. The levels of bacterial contamination in the environment have been tackled using quantitative PCR (polymerase chain reaction) for detection of *C. burnetii* DNA, but a rapid test assessing viability is required to evaluate the infectious risk in the environment (Kersh *et al.,* 2010). For now, the lack of knowledge of shedding patterns among ruminants has made the determination of Q fever status difficult. Concomitant shedding into the milk, the faeces and the vaginal mucus may be rare (Guatteo *et al.*, 2007; Rousset *et al.*, 2009). The vaginal shedding at the day of kidding may be the most frequent (Arricau-Bouvery *et al.*, 2005). In herds or flocks experiencing abortion problems caused by *C. burnetii*, most animals may be shedding massive numbers of bacteria whether they have aborted or not. The global quantities are thus clearly higher than in subclinically infected herds/flocks. At the parturitions following an abortion storm, higher bacterial discharges were measured among the primiparous compared with the other females (de Cremoux *et al.*, 2012; Guatteo *et al.*, 2008). Moreover, the shedding may persist for several months, following either an intermittent or a continuous kinetic pattern. Animals with continuous shedding patterns might be heavy shedders. These latter animals seem mostly to exhibit a highly-seropositive serological profile (Guatteo *et al.*, 2007). Importantly, shedding and serological responses are associated at the group level but not at the individual level.

5. Differential diagnosis in ruminants

Diagnosis of Q fever in ruminants, including differentiating it from other causes of abortion, traditionally has been made on the basis of microscopy on clinical samples, coupled with positive serological results (Lang, 1990). At present, no gold standard technique is available, but direct detection and quantification by PCR and serological ELISA (enzyme-linked immunosorbent assay) should be considered as the methods of choice for clinical diagnosis (Niemczuk *et al.*, 2014; Sidi-Boumedine *et al.*, 2010). Proposals have been elaborated for the development of harmonised monitoring and reporting schemes for Q fever, so as to enable comparisons over time and between countries (Sidi-Boumedine *et al.*, 2010). Q fever diagnostic tests are also required for epidemiological surveys of at risk and suspected flocks in limited areas (following recent outbreaks in humans or animals), or for exchanges between herds or flocks. Thus, efforts are encouraged both for the validation of the methods for each purpose given (see Table 1), and for development of reference materials for quality control,

proficiency and harmonisation purposes (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

6. Zoonotic risk and biosafety requirements

Concerns about the risks posed by Q fever have been raised in Europe, where the European Commission requested scientific advice and risk assessment for humans as well as animals (ECDC, 2010). The main conclusions were that the necessary actions to stop an outbreak must be carried out by health authorities together with veterinary authorities at the national and the local levels. The overall impact of *C. burnetii* infection on public health is limited but there is a need for a better surveillance system. In human epidemic situations, active surveillance of acute Q fever is the best strategy for avoiding chronic cases. Measures for the control of animal Q fever should be implemented, particularly for domestic ruminants. Only a combination of measures is expected to be effective. Among these options, preventive vaccination, manure management, changes to farm characteristics, wool-shearing management, a segregated kidding area, removal of risk material, visitor ban, control of other animal reservoirs and ticks could be used. Moreover, the culling of pregnant animals, a temporary breeding ban, identifying and culling shedding herds or flocks and controlling animal movements may have a role in the face of human outbreaks.

Because of its ability to cause incapacitating disease in large groups of people, its resistance in the environment as a pseudo-spore and its natural spread as an aerosol, *C. burnetii* is currently considered a potential agent of bioterrorism and is classified by the Centers for Disease Control and Prevention as a group B biological agent (Drancourt & Raoult, 2005; Kersh *et al.,* 2010). Regarding biosafety and biosecurity, *C. burnetii* is extremely hazardous to humans. Q fever is thus a recognised occupational zoonosis. All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Precautions must be taken with both phase I and phase II *C. burnetii*. Even if the phase II bacteria are considered avirulent, phase I bacteria may be present in a phase II preparation. In particular, it is advised to wear full coverage protective clothing and a class 3 filtering face piece (FFP3) respiratory protection and to handle infectious and potentially infectious material with two pairs of gloves, inside a biological safety cabinet (BSC). Centrifugation of infected materials must be carried out in closed containers placed in sealed safety cups, or in rotors that are unloaded in a BSC. The use of needles, syringes, and other sharp objects should be strictly limited. After all manipulations where there is a known or potential exposure to aerosols of viable *C. burnetii*, showers must be taken when leaving the laboratory. Sporicidal disinfectants are recommended. An appropriate serological survey would help when following up the evolution of immune status of the laboratory personnel. In some countries, vaccination is practised for occupationally exposed people, such as abattoir workers, veterinarians and laboratory personnel. Phase I vaccines are effective, but vaccination is contraindicated for individuals who had seroconverted or had been exposed to *C. burnetii* prior to immunisation.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of Q fever and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
PCR	+++	–	+++	+++	++	+[(a)]
Culture	+	–	+	–	+	–
Staining	+	–	+	+	+	–
Genotyping	–	–	–	–	++	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
ELISA	+++	–	+++	++	+++	+++
IFA	++	–	++	++	++	++
CFT	–	–	–	++	+	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
IFA = indirect immunofluorescence assay; CFT = complement fixation test.
[(a)]Confirmation of immune status should be accompanied by tests for the absence of vaginal shedding of the organism

Clearly, a confirmed positive identification of *C. burnetii* from an individual animal would support a diagnosis. However as a general principle, the methods for the diagnosis of Q fever allow only an interpretation at the population level and not at the individual level. Moreover, laboratory test results should be interpreted in the context of herd or flock history (abortions, vaccination, movement and introduction, etc.).

Coxiella burnetii can be demonstrated in various ways, depending on the type of sample and the purpose of investigations (Sidi-Boumedine *et al.*, 2010). The ability to detect and quantify *C. burnetii* DNA by real-time PCR has dramatically enhanced diagnostic and study approaches. Individual vaginal, milk or colostrum samples or milk from the tank can be taken for investigation of bacterial shedding. However, detection of shedders is still difficult to achieve as the shedding dynamics are not well known (de Cremoux *et al.*, 2012; Guatteo *et al.*, 2007; Rousset *et al.*, 2009). Indeed, the PCR cannot be relied on to determine the infection status because of the variability of shedding by animals (different shedding routes, potentially intermittent shedding). Serological analyses may be carried out using ELISA, indirect immunofluorescence assay (IFA) or complement fixation test (CFT). Several published works showed that the relative sensitivity is lowest for the CFT, but conversely it has a high specificity for the high levels of anti-*C. burnetii* antibodies generated in a Q fever aborted herd or flock (Emery *et al.,* 2014; Horigan *et al.*, 2011; Kittelberger *et al.,* 2009; Niemczuk *et al.*, 2014; Rousset *et al.,* 2007; 2009). IFA has the disadvantage of being less reproducible between operators, and therefore between laboratories. Although the ELISA methods are, not fully validated and harmonised, they are robust and can be automated and are recommended for routine serological testing of animals for Q fever.

A serological survey is a good way to evaluate prevalence. The presence of specific anti-*C. burnetii* antibodies provides evidence of a recent infection as well as a past exposure. Serological assays are suitable for screening herds or flocks, but interpretation at the individual animal level is not possible. Indeed, a significant proportion of animals shedding *C. burnetii* bacteria, and even some Q fever aborted animals, are found to be seronegative (de Cremoux *et al.*, 2012; Guatteo *et al.*, 2007; Rousset *et al.*, 2007, 2009). Sampling should target a representative number of animals (in particular from different age categories). Sampling strategy should take into account the possibility of a low prevalence if no prevalence data are available in the studied area. Alternatively, testing bulk tank milk (BTM) or pooled individual samples (i.e. vaginal swabs or milk samples) can be used for prevalence estimation, but must be assessed in relationship to the intra-herd or intra-flock shedding prevalence. For example, PCR analyses of BTM have been performed every 2 months since 2009 in the Netherlands to monitor a herd or flock with proven clinical status.

The herd or flock status can be assessed serologically by ELISA investigation of all animals (or a significant sample). However, some discordant results can be observed using different ELISA kits (Horigan *et al.*, 2011). One option is to use at least three kits to determine the status of a serum. Available serological methods do not, unfortunately, distinguish between infected and vaccinated ruminants. Analysis by PCR in BTM or individual samples (vaginal swabs, preferably at the time of parturition) is required and may need to be repeated if the purpose is to determine free status. Individual animals can only be assessed as free if the herd or flock is free and has no serological or clinical history of Q fever. It is difficult to ensure that the status of the animal has not changed over time because transmission is by air.

PCR is the most reliable tool for the diagnosis of infectious abortions (Sidi-Boumedine *et al.*, 2010). For laboratory diagnosis in the context of serial abortions and stillbirths, samples should be collected from aborted fetuses, placenta and vaginal discharges soon after abortion or parturition. Early detection of a Q fever storm of abortions in a herd or flock and implementation of the correct measures are essential to the handling of both farm-based and environmental route of infection. The confirmation of clinical cases should always include a differential investigation of major abortive agents and target at least two aborted animals. The interpretation of results is possible only at the group level. A positive case is a herd or flock with clinical signs (abortion and/or stillbirth) for which the presence of the agent has been confirmed. If possible, vaginal swabs at the day of abortion (or taken less than 8 days after) should be collected in order to limit the number of false-negative PCR results. Effectively, the vaginal bacterial load may decrease progressively after abortion or parturition. In the placenta, at least three cotyledons should be tested for *Coxiella* as colonisation can be heterogeneous. Bacterial quantification is helpful on vaginal or placental swabs, as high levels are more likely to be associated with clinical cases. The fetal organs may provide useful samples, but negative results can be questionable. Bacteria are likely to spread to different organs (spleen, lung, liver, stomach contents, etc.) depending on the progression of the infection, so that the absence in one organ cannot exclude its presence somewhere else.

When difficulties in interpretation of diagnostic results are encountered, an association with a positive serological result at the herd or flock level is useful. ELISA, IFA as well as CFT methods may be used for testing clinical cases but it is essential to define the test characteristics (sensitivity, specificity, accuracy around the cut-off, and reproducibility) under local conditions. Serological cut-off values used to diagnose Q fever are given by kit suppliers. Interpretation of the results requires samples from at least six ewes or goats and ten cows (with priority to those that have aborted).

Determination of the immune status of populations post-vaccination should be based on the more sensitive tests (ELISA or IFA); if possible, it should be linked to PCR testing of vaginal swabs collected at parturition. If the infection pressure is high, vaccination may only limit the magnitude of infection and shedding without inducing solid protection. The combination of seroconversion with the absence of vaginal shedding, at the following parturition, is indicative of immune protected status.

1. Identification of the agent

1.1. Isolation of the agent

For specific laboratory investigations, it may be necessary to isolate the agent. Where microscopic examination has revealed large numbers of *C. burnetii* combined with a low contamination rate with other bacteria, direct isolation by inoculation of embryonated chicken eggs or cell culture is possible. To achieve isolation, a concentration above 10^5 bacteria per ml is recommended.

i) Embryonated chicken eggs

A portion of placenta is homogenised in phosphate-buffered saline (PBS) containing antibiotics (streptomycin 100–200 μg/ml and penicillin or gentamicin 50–100 μg/ml). After low-speed centrifugation, dilutions of the supernatant fluid are inoculated into 6- to 7-day-old embryonated chicken eggs via the yolk sac. Eggs are preferably from specific pathogen free (SPF) hens. Embryos that die during the first 5 days after inoculation are discarded. The yolk sacs are harvested after 10–15 days of incubation. Stained smears of the yolk sac wall are examined to ensure the absence of bacterial contamination and to determine the presence of *C. burnetii*. PCR analysis can also be used to detect the presence of *C. burnetii* and to monitor the process of isolation. Further passages may be required to obtain an isolate in pure culture.

ii) Cell culture

A cell microculture system from a commercially available method used for virus culture, the shell vial cell culture, has been adapted for isolating strict or facultative intracellular bacteria, including *C. burnetii*. Such a method was described for *C. burnetii* in 1990 (Raoult *et al.,* 1990). Suspensions of samples are inoculated into human embryonic lung (HEL) fibroblasts grown on a 1 cm^2 cover-slip within a shell vial. Various cell lines may be used to allow the observation of characteristic vacuoles of *C. burnetii* multiplication. Centrifugation for 1 hour at 700 ***g*** enhances the attachment and penetration of bacteria into the cells. Three shell vials are used for the same sample, and by day 3, 10 and 21, the cytopathic effect (CPE) – *C. burnetii* characteristic vacuoles in cells – are examined using an inverted microscope. After 10 days, detection of growing

C. burnetii within the cells is achieved directly on the cover-slip inside a shell vial by a direct immunofluorescence assay with polyclonal anti-*C. burnetii* antibodies and an appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC). Cells of the remaining shell vial are harvested and transferred in a 25 cm^2 culture flask. Incubation can be conducted for 3 months, with a culture medium change once a week (trypsinisation is not used). The infection can be monitored by microscopy of Gimenez-stained cells cyto-centrifuged from the culture supernatant and by PCR analysis of the culture supernatant. When the CPE observations and Gimenez staining or PCR results are positive, a passage in a 75 cm^2 culture flask is performed. Culture supernatant is then inoculated on confluent layers of Vero cells or L929 mouse fibroblasts in a 150 cm^2 culture flask in order to establish a *C. burnetii* isolate. This method was developed for humans but could be adapted for animals.

iii) Laboratory animals

With heavily multi-contaminated samples, such as placentas, vaginal discharges, faeces, or milk, the inoculation of laboratory animals may be necessary as a filtration system. Experimentally infected rodents must be housed in appropriate biosafety and containment conditions, determined by biorisk analysis (see Chapter 1.1.4). Mice and guinea-pigs are the most appropriate laboratory animals for this purpose (Scott *et al.,* 1987). Following intraperitoneal inoculation with a dose of 0.5 ml per animal, body temperature and antibody status can be monitored. This method should be performed in conjunction with serological tests on other guinea-pigs or mice that have been inoculated with the same samples. Sera are collected 21 days after inoculation. A positive result confirms a diagnosis of *C. burnetii* infection. If pyrexia develops, the animal is killed and the spleen is removed for isolation of the agent by inoculation into embryonated chicken eggs or in cell cultures. Microscopic examination of *C. burnetii* can be done using impressions and staining of the collected spleens. Alternatively, the process can be simplified by performing PCR for detection of *C. burnetii* DNA (see below) on spleens.

1.2. Staining

In the case of an abortion having a suspected infectious origin, smears of placental cotyledon are prepared on microscope slides. Spleen, lung, liver and abomasal contents of the aborted fetus or vaginal discharge may be used in the same manner. These could be stained according to several methods: Stamp, Gimenez, Macchiavello, Giemsa and modified Koster (Gimenez, 1964; Quinn *et al.,* 1994). The first three techniques give the best results. These methods are close to the modified Ziehl–Neelsen method involving basic fuchsin to stain bacteria. For example, the Stamp staining method is performed with 0.4% basic fuchsin solution, followed by rapid decolouration with 0.5% acetic acid solution, and counterstaining with 1% methylene blue or malachite green solution. The smears are examined microscopically with an oil-immersion objective lens (×500 or more). The Stamp method is preferred in veterinary diagnostic laboratories while the Gimenez method is widespread for monitoring infected cultural cells in research laboratories. Gimenez is fastest because an acidic solution is not included for differentiation. *Coxiella burnetii* are characterised by a very large number of thin, pink-stained coccobacillary bacteria against a blue or green background. They may sometimes be difficult to detect because of their small size, but this is compensated for by their large numbers; often inclusions within the host cells appear as red masses against the blue or green background. The staining method is rapid. The limit of detection is high (>10^5 bacteria/ml) and appropriate to the clinical diagnostic purpose as high levels of bacteria are present in samples found positive. Attention must be taken in the interpretation of the results as, microscopically, *C. burnetii* can be confused with *Chlamydia abortus* or *Brucella* spp. However, using the same staining procedure, *Chlamydia* have sharper outlines, are round, small and may resemble globules. *Brucella* spp. are larger (0.6–1.5 μm long × 0.5–0.7 μm wide), more clearly defined and stain more intensely. Control positive slides of *C. burnetii*, *Chlamydia abortus* and *Brucella* must be used for comparison. Diagnosis of clinical cases made on the basis of microscopy, coupled with positive serological results, is usually adequate for routine purposes. When biological staining is inconclusive, one of the other specific methods may be used as a confirmatory test. PCR methods are preferred.

1.3. Specific detection methods

Detection of *C. burnetii* in samples can also be achieved by specific immunodetection (capture ELISA, immunohistochemistry), *in-situ* hybridisation or DNA amplification (Jensen *et al.*, 2007; Thiele *et al.,*

1992). Immunohistology may be used with paraffin-embedded tissues or on acetone-fixed smears (Raoult *et al.,* 1994). The method is an indirect immunofluorescence or immunoperoxidase assay using specific polyclonal *C. burnetii* antibodies produced in laboratory animals (rabbit or guinea-pig). An anti-species (rabbit or guinea-pig) anti-IgG conjugate, labelled with FITC or peroxidase, is then used to visualise the bacteria. Control positive slides of *C. burnetii* antigen should be available for comparison. No specific antibodies for immunochemistry are commercially available.

Fluorescent *in-situ* hybridisation (FISH) using specific oligonucleotide probes targeting 16s rRNA may be used on paraffin embedded tissues, especially placenta samples (Jensen *et al.,* 2007).

PCR methods have been used successfully to detect *C. burnetii* DNA in cell cultures and biological samples. To ensure the safety of laboratory personnel, biological samples can be inactivated prior to carrying out the PCR by heating at 90°C for 30–60 minutes, depending on the nature of the samples, their size or their weight. The inactivation process must be checked and validated under local conditions, before use. The PCR technique can be performed in suitably equipped laboratories using primers derived from various targets, such as multicopy insertion sequence IS1111 (accession number M80806), the most largely employed (Berri *et al.,* 2000). The use of these primers for the amplification of this sequence allows the sensitivity of the test to be increased due to the presence of several copies in the *Coxiella* genomes. The other target genes reported to be used in the PCR for specific *C. burnetii* identification are: superoxide dismutase (sodB) gene (accession number M74242); com1 encoding a 27 kDa outer membrane protein (accession number AB004712); heat shock operon encoding two heat shock proteins (htpA and htpB) (accession number M20482); isocitrate dehydrogenase (icd) (accession number AF069035); and macrophage infectivity potentiator protein (cbmip) (accession number U14170). Some primer and probe sequences can be obtained on the web site of the French national reference centre for human Q fever[1].

The real-time PCR provides an additional means of detection and quantification (Klee *et al.,* 2006; Stemmler & Meyer, 2002). As with the conventional PCR, various target genes are used: for example IS1111; IS30; com1; and icd. To quantify the bacteria in biological samples using the real-time PCR, it is recommended to amplify a unique and specific sequence. Indeed, recent data show that the number of the insertion sequence (IS1111) varied widely (between 7 and 110) depending on the isolate (Klee *et al.,* 2006). Whereas the use of this sequence could increase the sensitivity of the test, it may not be accurate for quantification when different strains are involved. It is nevertheless sufficiently informative and accurate for high quantities of bacteria (i.e. $>10^4$ per vaginal swab) for abortive diagnosis (Sidi-Boumedine *et al.*, 2010). Regarding complex matrices, the DNA eluates should be evaluated for their ability to inhibit a PCR by adding an internal DNA control (such as a GAPDH sequence target) or an external control.

Ready-to-use kits are commercially available and can detect the bacteria in various sample types. Specific quantitative methods based on PCR kits have been validated for diagnosis of abortions according to a French standard for real-time PCR validation (Rousset *et al.,* 2012). An external reference material of quantified bacteria is available from the French national reference laboratory either for method validation or for a control chart to routinely monitor quality of the assays.

1.4. Genotyping methods

Q fever epidemiology is complex as represented by its wide host range, its capacity to persist in the environment and its multifactorial air-borne transmission. Although characterisation of isolates seems necessary for understanding the varying epidemiology of Q fever in different geographical areas, assessment of discriminatory typing methods for molecular epidemiology are in progress (Massung *et al.*, 2012; Sidi-Boumedine & Rousset, 2011). These tools are very useful for epidemiological investigation, particularly to clarify links regarding source of infection, for better understanding the epidemiological emerging factors, elucidating human outbreaks, and to a lesser extent for evaluating control measures.

Several typing methods have been used for the characterisation of *C. burnetii* strains, such as restriction endonuclease of genomic DNA, PFGE (pulsed-field gel electrophoresis), and sequence

1 At: http://ifr48.timone.univ-mrs.fr/Fiches/Fievre_Q.html#toc22

and/or PCR-RFLP (restriction fragment length polymorphism) analysis of icd, com1 and mucZ genes. More recently, two PCR-based typing methods have been described, MLVA (multi-locus variable number of tandem repeats analysis) and multispacer sequence typing (MST) that permit the typing of *C. burnetii* without the need for isolation of the organism. Research continues on the development of new tools, such as single nucleotide polymorphism (SNP), and the comparison of their discriminatory capabilities and informative value.

To date, MLVA and MST are considered to be the most discriminating methods for *C. burnetii*, allowing the identification of up to 36 distinct genotypes. Moreover, databases have been established http://mlva.u-psud.fr/MLVAnet/ and http://ifr48.timone.univ-mrs.fr, respectively for MLVA and MST. The availability of such databases allows interlaboratory comparisons to be made easily and this will lead to a better understanding of the propagation of the *C. burnetii* isolates or to identify new emerging strains. Furthermore, their use in the characterisation of field samples or isolates is increasing and efforts to produce a standardised scheme for MLVA, based on common decisions for allele calling and marker panels to be used, should be encouraged so that they can be made available in the near future (Massung *et al.*, 2012; Sidi-Boumedine & Rousset, 2011).

2. Serological tests

2.1. Enzyme-linked immunosorbent assay (ELISA)

This technique has a high sensitivity and a good specificity according to comparative evaluations between methods (Emery *et al.*, 2014; Horigan *et al.*, 2011; Kittelberger *et al.,* 2009; Niemczuk *et al.*, 2014; Rousset *et al.,* 2007; 2009). It is easy to perform in laboratories that have the necessary equipment (a spectrophotometer) and reagents. The ELISA is preferred to IFA and CFT, particularly for veterinary diagnosis, because it is convenient for large-scale screening and the most robust. Ready-to-use kits are commercially available and can detect mixtures of anti-phase I and II antibodies. The quality control for some ELISA kits was recently improved using an external reference material, available from the French national reference laboratory, showing the standardisation between kit batches.

Coxiella burnetii ELISA antigen is prepared by growth of standard strains in either embryonated hens' eggs or in cell culture, as described below under IFA. Wells of the microplate are coated with *C. burnetii* whole-cell inactivated antigen. Diluted serum samples are added to the wells and react to antigens bound to the solid support. Unbound material is removed by washing after a suitable incubation period. Conjugate (horseradish-peroxidase-labelled anti-ruminant Ig) reacts with specific antibodies bound to the antigen. Unreacted conjugate is removed by washing after a suitable incubation period. Enzyme substrate is added. The rate of conversion of substrate is proportional to the amount of bound antibodies. The reaction is terminated after a suitable time and the amount of colour development is measured spectrophotometrically.

2.1.1. Materials and reagents

Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with *C. burnetii* antigen; microplate reader (spectrophotometer; 405 and/or 450 and/or 492 nm filters); 37°C humidified incubator; 8-and 12-channel pipettes with disposable plastic tips; microplate shaker (optional).

Positive and negative control sera; conjugate (ruminant anti-immunoglobulin or protein A/G labelled with peroxidase); tenfold concentration of diluent (PBS–Tween); distilled water; substrate or chromogen (TMB [tetramethylbenzidine], ABTS [2,2'-azino-bis-(3-ethylbenzo-thiazoline-6-sulphonic acid)] for peroxidase); hydrogen peroxide.

2.1.2. Test procedure

i) Dilute the serum samples, including control sera, to the appropriate dilution (1/100 or 1/400 depending on the kit used) and distribute 0.1 ml per well in duplicate. Control sera are positive and negative sera provided by the manufacturer and an internal positive reference serum from the laboratory in order to compare the titres between different tests.

ii) Cover the plate with a lid and incubate at room temperature for 30–90 minutes. Empty out the contents and wash three times in washing solution at room temperature.

iii) Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well).

iv) Cover each plate and incubate as in step ii. Wash again three times.

v) Add 0.1 ml of freshly prepared chromogen substrate solution to each well (for example: TMB in 0.1 M acetic acid and 30% H_2O_2 solution [0.2 µl/ml]; or 0.25 mM ABTS in citrate phosphate buffer, pH 5.0, and 30% H_2O_2 solution [0.1 µl/ml]).

vi) Shake the plate; incubate according to the manufacturer recommendations, stop the reaction by adding stopping solution to each well, e.g. 0.05 ml 2 M sulphuric acid for TMB or 10% sodium dodecyl sulphate for ABTS.

vii) Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 450 nm (TMB). The absorbance values will be used to calculate the results.

2.1.3. Interpretation of the results

For commercial kits, interpretations and values are provided with the kit.

For example: calculate the mean absorbance (Ab) of the sample serum and of the positive (Ab_{pos}) and negative (Ab_{neg}) control sera, and for each serum, calculate the percentage

$$\frac{Ab - Ab_{neg}}{Ab_{pos} - Ab_{neg}} \times 100$$

Interpret the results as follows:
Ab <30% negative serum
Ab > 30% positive serum

Prepare a control chart and estimate the measurement uncertainty around the cut-off in order to interpret results close to the cut-off.

2.2. Indirect immunofluorescence assay (IFA)

In human medicine, the IFA adapted as a micro-immunofluorescence technique is the current method for the serodiagnosis of Q fever (Tissot-Dupont *et al.,* 1994). The procedure can be adapted to perform an immunoperoxidase assay. Briefly, both phase I and phase II *C. burnetii* antigens are used; phase II antigen is obtained by growing *C. burnetii* Nine Mile reference strain in cell culture, while phase I antigen is obtained from the spleens of laboratory animals. Antigen is diluted, dropped onto the wells of a glass microscope slide, allowed to dry, and fixed with acetone. The two forms of the infection in humans, acute and chronic, have different serological profiles: during acute Q fever, IgG antibodies are elevated against phase II only whereas during chronic Q fever, high levels of IgG antibodies to both phase I and II of the bacteria are observed (Tissot-Dupont *et al.,* 1994). In addition, antigen-spot slide wells may be purchased from a supplier providing the phase II form, or the phase I and II forms of *C. burnetii*. These can be adapted by replacing the human conjugate by a conjugate adapted to the animal species. Nevertheless, the interpretation as acute or chronic forms has not been validated for ruminants.

2.2.1. Antigen preparation

An example of *C. burnetii* preparation for IFA serological diagnostic based on phase II and phase I antigens is given below, but other modified protocols are used around the world. Significant amounts of *C. burnetii* (>10^{10} bacteria) can be obtained in 2–5 weeks in embryonated eggs or cell cultures. An infection in mice can require 7–14 days. Purification of bacteria from host material includes differential centrifugations and takes 1 or 2 days.

Phase II *C. burnetii* Nine Mile are grown in confluent layers of Vero or L929 cells in 150 cm^2 culture flasks at 35°C under 5% CO_2 with minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 4% fetal bovine serum. The infection is monitored by microscopic examination of intracellular vacuoles or by Gimenez-stained cells collected from the supernatants of the flasks. Recent specific real-time quantitative PCR has been extremely valuable in routine monitoring. When a heavy *C. burnetii* infection is seen, the supernatants of 15 flasks are individually pelleted by centrifugation

(5000 ***g***, 15 minutes) resuspended in 1 ml of PBS with 0.1% formaldehyde and incubated for 24 hours at 4°C. After pooling, the remaining cells are broken by sonication. Cellular debris is removed by two successive centrifugation steps (100 ***g***, 10 minutes each). The 15 ml suspension is then centrifuged through 20 ml of PBS with 25% sucrose (6000 ***g***, 30 minutes, without a break). The resulting pellet is washed three times in PBS (6000 ***g***, 10 minutes), resuspended in the smallest possible volume of sterile distilled water, and adjusted to 2 mg/ml by UV spectroscopy. An antibacterial preservative, such as sodium azide at a final dilution of 0.1% or thiomersal at 0.01%, is added. Antigen prepared in this manner is frozen at –20°C.

To obtain phase I antigen, mice are inoculated with *C. burnetii* grown in cells (mainly in phase II). The spleens are removed 9 days after infection. Each one is ground in 7.5 ml MEM, and inoculated into three 75 cm^2 culture flasks containing L929 or Vero cell monolayers (2.5 ml per flask). Amplification of phase I *C. burnetii* is conducted for 4 weeks, with a culture medium change once a week. The infected cells are then harvested and the bacteria are purified as described above (mainly in phase I).

Antigen production can also be performed by culture of *C. burnetii* in SPF embryonated eggs. At 6–7 days of age, the microorganism is inoculated into the yolk sac of the embryonated eggs, which are harvested after 10–15 days of incubation. Infected yolk sacs have a characteristic straw-yellow colour and white spot patches. Uninfected yolk sacs are orange in colour and have a viscous consistency. Any embryos that die before 5 days of incubation are discarded. The strain used for egg inoculation is a 1/100 homogenate of yolk sac in PBS containing penicillin (500 International Units/ml) and streptomycin (0.5 mg/ml). The yolk sacs are pooled and homogenised with three parts PBS. The suspension is inactivated with 1.6% formaldehyde for 24 hours at 37°C. The lipid supernatant fluid is discarded. The suspension is then centrifuged at moderate speed (~500 ***g***) for 30 minutes. After removal of the supernatant fluid, more PBS is added and centrifugation is repeated. The final suspension is diluted with PBS. Sodium azide or thiomersal is added as an antibacterial preservative. The abundance of *C. burnetii* and the absence of bacterial contaminants in homogenates of yolk sacs suspended in PBS are verified by microscopic examination of a smear on a microscope slide, stained by Stamp's method. In order to obtain phase I antigen, *C. burnetii* recovered from spleen material of infected laboratory animals can be propagated, as ground spleen extracts are subsequently transferred in the yolk sacs, given that the amount of phase I cells is still high until the sixth egg passage.

Titration of antigen with at least three different known sera (with high, moderate and low titres, respectively) is sufficient to determine the appropriate dilution for further immunofluorescence tests.

2.2.2. Materials and reagents

Microscope equipped for fluorescence, humidified incubator, washing basin.

Slides suitable for the antigen are necessary. The latter may be either prepared in the laboratory or purchased from a supplier (see above). The method described is adapted from the BioMérieux kit, and is given as an example. Ready-to-use slides contain 12 wells per slide, each of 7 mm diameter, coated with phase II antigen obtained from culture on Vero cells and can be stored at 4°C or –20°C.

Concentrated fluorescent conjugate, to be diluted when required with PBS + 1% Evans blue at the dilution recommended by the manufacturer.

PBS, buffered glycerine, Evans blue dye 1% solution.

2.2.3. Test procedure

Twofold dilutions of the serum under test are placed on immunofluorescence slides with wells previously coated with one or two antigens. If specific antibodies are present, they are bound by the antigen on the slide. The complex is then detected by examination with a fluorescence microscope following the addition of the fluorescent conjugate recognising the species-specific immunoglobulins.

i) Dilute the sera serially from 1/40 to 1/640 in PBS.

ii) Allow the previously antigen-coated slides to warm to room temperature. Do not touch the wells.

iii) Add 20 µl of each serum dilution to the wells. Add negative and positive control sera. To one well, add 20 µl of PBS to serve as antigen control.

iv) Incubate in a humid chamber for 30 minutes at 37°C. Wash the slide twice with PBS for 10 minutes each. Rinse with distilled water and air dry.

v) Add to the wells, including the controls, 20 µl of the conjugate directed against the appropriate species (e.g. FITC-labelled rabbit anti-goat or anti-sheep IgG[H+L]), freshly diluted in PBS + Evans blue. Incubate in a humid chamber for 30 minutes at 37°C. Rinse with distilled water and air-dry. Add a few drops of buffered glycerine and cover with a cover-slip. Examine under a fluorescence microscope at magnification ×400 or more.

2.2.4. Interpretation of the results

A positive reaction will consist of small brilliant points against a dark background. Verify that the conjugate by itself and the negative control serum give a negative result (absence of small brilliant points). Nonspecific fluorescence usually takes the form of spots of irregular shape. The positive control must give the known titre with ± one dilution.

2.3. Complement fixation test (CFT)

As mentioned above the CFT is considered less sensitive than ELISA or IFA and its use for veterinary diagnosis has declined.

This cold fixation micromethod of the type developed by Kolmer is performed with 96-well U-bottomed microtitre plates. The test detects complement-fixing antibodies present in the serum. This method uses antigen in phase I and II mixture prepared from human or Nine Mile strain.

The reaction is done in two stages. Antigen and complement-fixing antibodies are first mixed, and incubated overnight at 4°C. The next day sheep erythrocytes, sensitised by the anti-sheep erythrocyte serum, are added. Fixation of the complement by the antigen/antibody complex during the first step does not permit lysis of erythrocytes; in contrast, if there are no complement-fixing antibodies, the complement induces the lysis of the sensitised erythrocytes. Then the haemolysis rate is inversely proportional to the level of specific antibodies present in the sample serum.

2.3.1. Reagents

Veronal/calcium/magnesium buffer (VB), pH 7.2.

The haemolytic system: a mixture of equal parts of a 2% suspension of sheep erythrocytes in VB; and haemolytic serum diluted to a specified titre in VB.

Complement: commercial freeze-dried preparation or fresh guinea-pig serum.

Antigen: use commercial antigens at the titre recommended by the manufacturer if the antigen titration is performed with this method.

Positive and negative control sera.

2.3.2. Pretitrations

i) Dilute the sheep erythrocytes to a final concentration of 2% in VB.

ii) Titrate the haemolytic serum on a microplate: 25 µl of complement at a known haemolytic concentration (e.g. 1/30); 25 µl of increasing dilutions of haemolytic serum + 2% sheep erythrocytes. Include controls without complement. Incubate for 30 minutes at 37°C. Establish the dilution equivalent to 2 haemolytic units.

iii) Dilute the antigen as recommended by the manufacturer. The antigen may also be titrated: make increasing dilutions of antigen (25 µl horizontally) and a positive serum of known titre (25 µl, vertically). Add 25 µl of the suspension of sensitised erythrocytes and incubate for 30 minutes at 37°C. The antigen titre is the highest dilution producing a positive reaction with the highest serum dilution. Verify the absence of anticomplementary activity of the antigen at different dilutions.

iv) Titrate the complement on a microplate: serially dilute the complement or guinea-pig serum in VB, for example from 1/15 to 1/200. To each well containing 25 µl of this dilution, add 25 µl of antigen and 25 µl of the haemolytic system. Incubate for 30 minutes at 37°C and establish the dilution equivalent to 2 haemolytic units of complement.

2.3.3. Test procedure

i) Make twofold dilutions of inactivated sample sera from 1/10 to 1/320 in six wells and in four additional wells at dilutions from 1/10 to 1/80 to detect anticomplementary activity (25 µl per well).

ii) Add 25 µl of diluted antigen or 25 µl of VB to control serum wells.

iii) Add 25 µl diluted complement to all wells. Cover the plate with plastic adhesive film and incubate for 18 hours at 4°C.

iv) Remove the plates from the refrigerator, allow them to reach room temperature, and add 25 µl of freshly prepared haemolytic system. Incubate at 37°C for 30 minutes. Centrifuge the plates at 500 ***g*** for 5 minutes at 4°C. Examine the controls and read the results.

2.3.4. Interpretation of the results

Titres between 1/10 and 1/40 are characteristic of a latent infection. Titres of 1/80 or above in one or more sera from a group of from five to ten animals reveal an active phase of the infection.

C. REQUIREMENTS FOR VACCINES

C1. Inactivated vaccine

1. Background

1.1. Rationale and intended use of the product

For the control of *C. burnetii* infection in humans and animals, ruminants can be vaccinated using inactivated *C. burnetii* vaccines. The aim of this vaccination is to reduce shedding and the risk of abortion. To date only inactivated vaccines are available against *C. burnetii*. The inactivated vaccines that are available on the market are derived from *C. burnetii* strains in phase I (Nine Mile) or phase II stages, however it has been scientifically demonstrated that the protective antigen of *C. burnetii* is the full-length phase I LPS (Arricau-Bouvery *et al.,* 2005; Okimoto *et al.,* 2004; Ormsbee *et al.,* 1964; To *et al.,* 1998; Williams *et al.,* 1992). To generate an appropriate immune response while minimising the safety hazards, efficient vaccine production should be targeted at vaccines containing phase I antigen (Elliott *et al*., 2015; Zhang *et al*., 2013).

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The *C. burnetii* vaccine strain must be well characterised, of known origin, pure and composed exclusively of the selected phase. See Chapter 1.1.8 *Principles of veterinary vaccine production* for guidelines on master seeds.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Coxiella burnetii seeds must be pure culture and free from extraneous bacteria and fungi (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*). Production must be by the seed-lot system (see chapter 1.1.8). The seed production method is the same as for the antigen production method up to the inactivation step. The seeds are either lyophilised or are stored below –40°C. The seeds are tested for live titre, identity and purity (see chapter 1.1.9).

2.1.3. Validation as a vaccine strain

Suitability as a vaccine strain is demonstrated in efficacy and safety trials.

2.2. Method of manufacture

2.2.1. Procedure

As the current production strains are derived from virulent field isolates, the propagation of live bacteria should be carried out using appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). The personnel involved in this work must be well-trained and should be vaccinated against Q fever.

The production process includes culturing *C. burnetii* on specific pathogen free (SPF) embryonated chicken egg yolk sac membrane. After 5–9 days of incubation, the eggs are placed to cool and the yolk sacs are harvested. The harvest is homogenised, diluted in buffer and then inactivated with appropriate inactivating agent (e.g. formaldehyde) to ensure that no live organism will survive. The inactivated antigen is subjected to 5–10 × concentration, followed by combined chemical extraction and centrifugation steps to decrease the egg-derived ballast material from the matrix. This will result in a more purified antigen and avoid post-vaccinal reactions in the target species when vaccinating with the final product.

The concentrated purified antigen is diluted and formulated to the established protective dose. The vaccine formulation is based on antigen quantification (e.g. by weight or, ELISA) and may contain thiomersal as preservative.

Alternative Q fever antigen production processes are under development in some laboratories using cell cultures or cell growth in axenic media (Lockhart *et al*., 2013; Omsland *et al*, 2009). Studies of mimetic peptide antigen development have also been published (Peng *et al*., 2012).

2.2.2. Requirements for ingredients

See chapter 1.1.8.

2.2.3. In-process controls

As with all inactivated vaccines, the live titre is determined prior to inactivation so as to guarantee that it is below the maximum value for which the inactivation procedure was validated. The titre can be determined by egg inoculation or by quantitative PCR. The microbiological purity of the cultures is determined at each stage of production prior to inactivation. The success of inactivation must be determined by the cultivation test in appropriately sensitive media (e.g. embryonated eggs or cell culture).

As differentiation of phase I from phase II antigens is essential, analytical tools should be established from the start of the vaccine development process. See also Section A.2 of this chapter. These tools should include both in-process and final product quality control tests. For Phase I vaccines, both differentiation and quantification of phase I antigen are highly important. Differentiation of phase I from phase II antigens can be determined at the cellular level by either PCR or ELISA, and at the purified antigen or vaccine level by immunochemical methods (e.g. dot-blot, ELISA, IFA) using phase-specific antisera. The simplest qualitative method for this purpose is cross-checking the sample in dot-blot with both phase I-specific and phase II-specific antisera. Phase I antisera contain specific antibodies to both phase I and phase II antigens while phase II antisera contain only phase II-specific antibodies. Hence, bacteria in the phase I stage will give a double positive result in dot-blot, while phase II bacteria will be positive only with phase II antisera. PCR and immunochemical methods may also serve as identification tools.

The quantification of the purified antigen for vaccine formulation can be determined by weight, optical density or ELISA. The best choice for antigen quantification is ELISA because of its high sensitivity and specificity. As such assays are not readily available commercially, they must be developed and validated by the vaccine manufacturers.

2.2.4. Final product batch tests

i) Sterility/purity

Sterility tests are done on finished product. Each lot must pass sterility requirements, for example those detailed in the European Pharmacopoeia monograph 2.6.1 (see also chapter 1.1.9.).

ii) Identity

The identity of the antigens in inactivated products is ensured through the seed-lot concept and good manufacturing controls. The identity and phase type should be validated at various points throughout production e.g. by ELISA or PCR.

iii) Safety

Safety tests in target animals are not required in many regions for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for relevant regulatory approval, and on animals of the youngest recommended vaccination age.

iv) Batch potency

The efficacy of Q fever veterinary vaccines is demonstrated by vaccination and a subsequent challenge in target species using a heterologous strain during mid-pregnancy. For obvious safety reasons, these trials must be performed in animal facilities using appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4. The two main parameters to be assessed are a significant decrease in abortion rate and a decrease in bacterial shedding. The applied batch potency tests are correlated to the guaranteed minimum protection dose and shelf life of the vaccine. *In-vitro* potency methods are preferred to *in-vivo* tests. Tests are preferred that can both quantify the potency and provide specific identification at the same time (e.g. ELISA).

v) Formaldehyde content

Vaccines inactivated with formaldehyde are tested for residual formaldehyde.

2.3. Requirements for regulatory approval

The following section is based on the requirements for inactivated *C. burnetti* vaccines in the European Union. Other countries may have slightly different requirements.

2.3.1. Manufacturing process

The manufacturer must demonstrate that the production method preserves the protective antigenicity and that the procedure used to inactivate the bacteria is sufficient for complete inactivation. The inactivation process must be demonstrated on the highest possible antigen titre. The sensitivity of the inactivation test must be demonstrated on the antigen matrix in such a way that the test could detect antigen titre below the minimum infectious dose. The inactivation must be demonstrated on each production batch.

2.3.2. Safety requirements

i) Target and non-target animal safety

The safety of the product must be demonstrated during the development phase of the vaccine through normal dose, overdose and repeated dose applications on target animals as well as in extended field studies at the minimal age of vaccination and on pregnant animals. All safety laboratory tests are carried out on the recommended youngest age for primary vaccination. This age is considered to be the most sensitive category to any signs of vaccine intolerance.

Laboratory tests are performed in controlled environments while field studies under conditions of normal use with limited untreated or placebo control animals. The safety of

the vaccine is demonstrated on the evaluation of signs of local reactions and general signs during 21 days after the first vaccination

Impact on reproductive performance is evaluated on field study data by comparing the calving data of animals vaccinated during the different stages of pregnancy by *Coxiella* vaccine and placebo. No statistical difference should be demonstrated on statistically relevant numbers of animals between placebo and *Coxiella* vaccinated animals.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Not applicable.

iii) Precautions (hazards)

It is common to see a palpable reaction of some centimetres in diameter at the injection site. The reaction reduces and disappears without further treatment within a few days. It is common to observe a slight increase in rectal temperature up to 4 days post-vaccination.

2.3.3. Efficacy requirements

The efficacy of the vaccines must be demonstrated in the target species. The product lot used in the challenge study must represent the final industrial production process with the highest allowable passage number derived from the master seed. The efficacy of the product is demonstrated by a statistically significant difference in abortion and shedding data between vaccinated and control groups. For example in the case of small ruminants, the following protocol can be applied: seronegative animals are vaccinated closest to the minimal allowable time to artificial insemination (e.g. 3 weeks before). The animals are challenged subcutaneously in mid-pregnancy by heterologous challenge strain calibrated in mice and goats. The specific antibody levels are monitored by ELISA from the sera during gestation and from the milk after parturition to demonstrate the establishment of protective immunity. Shedding is monitored weekly after challenge from faeces samples during pregnancy, and from vaginal mucus, milk and via placenta in the case of abortion, and by quantitative PCR post-parturition. The abortion rate of the vaccinated and non-vaccinated groups is monitored. The vaccinated group should show significant reduction in shedding and abortion compared with the non-vaccinated control group.

Efficacy claims can also be supported by extended field studies in the presence of natural challenge by statistical analysis of shedding and abortion data using analytical methods similar to those in laboratory studies. Guatteo *et al.* (2008) successfully demonstrated that non-pregnant vaccinated cattle had five times lower probability of becoming a shedder than non-vaccinated animals.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable to this disease.

2.3.5. Duration of immunity

Duration of immunity can be demonstrated by serological data, via challenge or by fertility data in field studies.

2.3.6. Stability

Vaccine stability is validated by vaccine release tests performed at periodic intervals during the intended shelf-life of the product. For the demonstration of stability at least three representative product lots should be used.

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NB: There are WOAH Reference Laboratories for Q fever (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Q fever

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2015 (DIAGNOSTIC SECTION) AND 2018 (VACCINE SECTION).

CHAPTER 3.1.18.

RABIES (INFECTION WITH RABIES VIRUS AND OTHER LYSSAVIRUSES)

SUMMARY

Rabies is a major zoonosis for which diagnostic techniques have been standardised inter-nationally. As there are neither gross pathognomonic lesions nor specific and constant clinical signs for rabies, confirmatory diagnosis can only be made in the laboratory. Laboratory techniques are preferably undertaken on central nervous system (CNS) tissue removed from the cranium (for example brain stem, Ammon's horn, thalamus, cerebral cortex, cerebellum and medulla oblongata). A composite of CNS samples should be tested and the brain stem is the most important component of the sample. Laboratories should follow appropriate biosafety and containment procedures as determined by biorisk analysis.

Detection and identification of the agent: *Agent detection is preferably undertaken using primary diagnostic tests such as the direct fluorescent antibody (DFA) test, the direct rapid immunohistochemistry test (dRIT), or pan-lyssavirus reverse-transcription polymerase chain reaction (RT-PCR) assays. DFA test, dRIT, and RT-PCR provide a reliable diagnosis in 98–100% of cases for all lyssavirus strains if an appropriate conjugate or primer/probe is used. For a large number of samples, conventional and real-time RT-PCR can provide rapid results in specially equipped laboratories.*

Histological techniques such as Seller's staining (Negri bodies) are no longer recommended for diagnosis.

In cases of inconclusive results from primary diagnostic tests (DFA test, dRIT, or pan-Lyssavirus RT-PCR), further confirmatory tests (molecular tests, cell culture or mouse inoculation tests) on the same sample or repeat primary diagnostic tests on other samples are recommended. Wherever possible, virus isolation in cell culture should replace mouse inoculation tests.

Characterisation of the agent can be carried out in specialised laboratories using monoclonal antibodies, partial and full genome sequencing followed by phylogenetic analysis. Such techniques can distinguish between field and vaccine strains, and identify the geographical origin of the field strains. These very sensitive tests should be conducted and interpreted by well trained expert personnel.

Serological tests: *Virus neutralisation (VN) and enzyme-linked immunosorbent assays (ELISA) are suitable tests for monitoring the antibody response of vaccinated animals in the framework of rabies control. For the purposes of measuring antibody responses to vaccination prior to international animal movement or trade, only VN methods (fluorescent antibody virus neutralisation test and rapid fluorescent focus inhibition test) are acceptable. Serological tests should not be used for primary diagnosis.*

Requirements for vaccines: *For rabies vaccination in animals, inactivated virus (for companion animals and livestock), live attenuated virus (for wildlife and free-roaming dogs), and recombinant vaccines (for wildlife, cats and dogs) are used. Certain vaccines can be categorised in more than one of these groups.*

Vaccine manufacturers should make known the characteristics of the product and undertake necessary experiments satisfying minimum requirements established at national and international levels. Before vaccines can receive relevant regulatory approval, the duration of immunity resulting from their use should be determined in vaccinated animals of the target species. Vaccines should confer protective immunity for at least 1 year.

The potency, efficacy and safety of vaccines are established and controlled using tests formulated by recognised pharmacopoeia.

A. INTRODUCTION

Rabies is caused by neurotropic viruses of the genus *Lyssavirus* in the family *Rhabdoviridae* of the order *Mononegavirales* (Kuhn *et al.,* 2021; Walker *et al.,* 2022), and is transmissible to all mammals. Rabies causes 60,000 human fatalities annually, approximately 1 death every 10 minutes. As the viruses are transmissible to humans, all suspect infected human material must be handled under the appropriate safety conditions specified by the World Health Organization (WHO, 2018). Laboratories working with lyssaviruses or suspect animal material must comply with national biocontainment and biosafety regulations as well as following appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Rabies virus (RABV) represents the taxonomic prototype species "Rabies lyssavirus" in the Lyssavirus genus, which includes other genetic and antigenically-related lyssavirus species (ICTV, 2017)[1]. RABV is found worldwide, and is responsible for the overwhelming majority of reported animal and human rabies cases. Other lyssaviruses appear to have more restricted geographical and host range, with the majority having been isolated from bats with limited public and animal health implications. However, all lyssaviruses tested cause clinical disease indistinguishable from RABV.

The lyssaviruses have been divided into at least three phylogroups with distinct pathogenicity and immunogenicity (Kuzmin *et al*., 2010). RABV vaccines may not provide adequate cross-protection against all genetically divergent lyssaviruses. Little or no cross-protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis was observed against lyssaviruses of phylogroups 2 and 3 (Badrane *et al*., 2001; Brookes *et al*., 2005; Hanlon *et al*., 2005). WHO recommends the preventive immunisation of all staff handling infected or suspect materials (WHO, 2013).

As no clinical sign or gross post-mortem lesion can be considered pathognomonic in domestic or wild animals, the diagnosis of rabies has to rely on laboratory testing. Serological testing is not used for ante-mortem diagnosis because of late seroconversion and the high mortality rate of host species, but is very useful for assessing sero-conversion following vaccination and for epidemiological studies.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of rabies and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
DFA (antigen detection)	+++	–	+++	+++	+++	–
dRIT (antigen detection)	+++	–	+++	+++	+++	–
RTCIT (virus isolation)	–	–	+++	+++	+++	–

1 https://ictv.global/report/chapter/rhabdoviridae/rhabdoviridae/lyssavirus

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
MIT (virus isolation)	–	–	+	+	+	–
Conventional RT-PCR (RNA detection)	+++	–	+++	+++	+++	–
Real-time RT-PCR (RNA detection)	+++	–	+++	+++	+++	–
Detection of immune response						
VN	–	+++	+++	–	–	+++
ELISA	–	–	+++	–	–	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
DFA = direct fluorescent antibody test; dRIT = direct rapid immunohistochemistry test; RTCIT = rabies tissue culture infection test; RT-PCR = reverse-transcription polymerase chain reaction; MIT = mouse inoculation test; VN = virus neutralisation; ELISA = enzyme-linked immunosorbent assay.

1. Detection and identification of the agent

Clinical observation may only lead to a suspicion of rabies because signs of the disease are not characteristic and may vary greatly from one animal to another. The only way to undertake a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests.

As RABV is rapidly inactivated, refrigerated diagnostic specimens should be sent to the laboratory by the fastest means available. Shipment conditions must be considered to be part of the 'rabies diagnostic chain' and should follow international guidelines.

Several laboratory techniques may be used that vary in their efficiency, specificity and reliability. In animals, they are classically applied to brain tissue, but they can also be applied with variable sensitivity and specificity to other organs (e.g. salivary glands). In the brain, RABV antigen is particularly abundant in the thalamus, pons and medulla. It is recommended that a pool of brain tissues, including the brain stem, should be collected and tested (Bingham & van der Merwe, 2002). The most widely used test for rabies diagnosis is the direct fluorescent antibody (DFA) test.

1.1. Collection of brain samples

Precautions should be taken when handling central nervous system tissues from suspected rabies cases. Protective personal equipment (such as gloves, face shield, mask) should always be worn and precautions must be taken to prevent aerosols. Cutting tools, scissors and scalpels, should be used with care to prevent injury and contamination.

Ideally, the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected, preferably brain stem, Ammon's horn, thalamus, cerebral cortex, cerebellum and medulla oblongata. Alternatively, methods of collecting some brain samples without opening the skull can also be applied; these methods are described in Sections B.1.1.1 *Occipital foramen route for brain sampling* and B.1.1.2 *Retro-orbital route for brain sampling*.

1.1.1. Occipital foramen route for brain sampling

A sample containing portions of medulla oblongata, base of the cerebellum, Ammon's horn region and cerebral cortex can be obtained by introduction of an approximately 5 mm in diameter sturdy plastic cylinder (e.g. 1–2 ml truncated syringe, artificial insemination sheath, 2 ml disposable plastic pipette with tip removed, or similar) into the occipital foramen in the direction of the eye (Barrat & Blancou, 1988). Alternatively, a scalpel and thumb forceps can be used to harvest a complete cross-section of brain stem accessed through the foramen magnum, followed by retrieval of portions of cerebellum using a plastic pipette (Patrick *et al.,* 2019; or see Kansas State Veterinary Laboratory for a video of the sampling technique).

1.1.2. Retro-orbital route for brain sampling

In this technique (Montano Hirose *et al*., 1991), a trocar is used to make a hole in the posterior wall of the eye socket, and an appropriate biopsy needle is then introduced through this hole. The sampled parts of the brain are the same as in the former technique, but they are taken in the opposite direction.

1.2. Shipment of samples

Suspect material should be transported by road according to the regulations given in the International Carriage of Dangerous Goods by Road (ADR). For international air transport, Dangerous Goods Regulations of the International Air Transport Association (IATA) should be followed. These regulations are summarised in Chapter 1.1.3 *Transport of biological material*.

If refrigerated/frozen shipment of samples is not possible, other preservation techniques may be used. The choice of the preservative is dependent on the tests to be used for diagnosis:

i) Formalin-fixed specimens

Formalin fixation (10% [w/v] solution in phosphate buffered saline [PBS]) allows testing with DFA test, immunohistochemistry, conventional and real-time RT-PCR, however modifications may be required and tests can be less sensitive compared with using fresh specimens (Warner *et al*., 1997). Formalin fixation inactivates the virus thus preventing virus isolation.

ii) Glycerol/ phosphate buffered saline (PBS)

For transportation of specimens, infectivity may be extended for several days if diagnostic specimens are kept in a mixture of 50% glycerol in PBS. Glycerol/PBS slows bacterial action and therefore protects against the chemical and biological effects of putrefaction. Due to the fact that RABV is thermo-labile, this method does not prevent a decline in the viral load in the specimen. Under routine transport conditions in regions with high temperatures (above 30°C), this protection may only be effective for a matter of several days. Therefore, whenever possible specimens in glycerol/PBS should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used to test these specimens.

iii) Preservation for molecular techniques

For molecular techniques, lysis buffers for nucleic acid extraction and RNA preservation buffers impregnated onto filter paper can be used (Picard-Meyer *et al*., 2007). These buffers preserve RABV RNA and allow transport of specimens at ambient temperature without specific biohazard precautions for detection of viral RNA and further genetic characterisation of RABV strains.

1.3. Laboratory tests

1.3.1. Immunochemical identification of rabies virus antigen

i) Direct fluorescent antibody (DFA) test

The most widely used test for rabies diagnosis is the DFA, which is recommended by both WHO and WOAH. This test is used directly on a brain impression smear. It is also used to confirm the presence of RABV antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis. The DFA test is highly sensitive and specific (between 96% and

99%), and gives reliable results on fresh specimens in less than 2 hours. Sensitivity depends on the specimen, the degree of autolysis (McElhinney *et al*., 2014) and the sample type (Barrat & Aubert, 1995).

Impression smears should be prepared from a composite sample of brain tissue, that includes the brain stem and the cerebellum. If the cerebellum is not available, a cross-section of the Ammon's horns may be used. The smears are fixed in 100% high-grade cold acetone (–20°C) for at least 20 minutes or heat-fixed by passing the slide 2–3 times through a flame. They are subsequently air dried and then stained with specific FITC (fluorescein isothiocyanate)-labelled polyclonal or monoclonal anti-rabies antibody conjugate, diluted to working dilution and sufficient to cover the whole smear, for 30 minutes at 37°C in a humid chamber. DFA test slides should then be examined for specific fluorescence using a fluorescence microscope and filter appropriate for the wavelength (490 nm and re-emits at 510 nm). Aggregates of nucleocapsid protein are identified by specific fluorescence of bound conjugate. It is recommended that two independent trained operators read each DFA test slide. Conserved antigenic sites on the nucleocapsid proteins permit identification of all lyssaviruses with modern commercial preparations of polyclonal anti-rabies antibody conjugates used for diagnostic tests on brain tissue, while monoclonal anti-rabies antibody conjugates may have limited sensitivity regarding different lyssaviruses. Fluorescent antibody conjugates, in particular if made locally, should be fully validated for specificity and sensitivity before use.

The DFA test may be applied to glycerol-preserved specimens after a washing step. If the specimen has been preserved in a formalin solution, the DFA test may be used only after the specimen has been treated with a proteolytic enzyme (Warner *et al*., 1997). However, the DFA test on formalin-fixed and digested samples is always less reliable and more cumbersome than when performed on fresh tissue (Barrat, 1992).

In cases of inconclusive results from DFA test, or in all cases of human exposure, further tests on the same sample or repeat DFA test on other samples are recommended. This is particularly important where sample autolysis is confirmed or suspected.

a) Test protocol

1) Label four microscope slides: two control slides for impression smears of control brain tissue (one positive and one negative) and two slides for duplicate testing of the appropriate brain regions.

2) Prepare impression smears of control brain tissue (one positive and one negative), as well test samples, by inverting the microscope slide onto the tissue placed on blotting paper. Remove excess tissue by blotting the slide onto clear blotting paper. Work with one sample at a time, using fresh blotting paper for each, and process the positive control last.

3) Allow the slides to air dry.

4) Within the biosafety cabinet place all slides into a Coplin jar containing cold acetone (–20°C) for at least 20 minutes.

5) Remove the slides from the Coplin jar and allow to air dry.

6) Prepare FITC-labelled anti-rabies conjugate as directed by the manufacturer.

7) Add conjugate at working dilution to the positive and negative control smears and to all smears of test samples, in sufficient quantity to cover the whole of the smears.

8) Place the slides in a 37°C incubator in a humid chamber for 30 minutes (45 minutes maximum).

9) Remove the slides and wash in 0.1 M PBS 7.2 for 5 minutes in a Coplin jar, then for a further 5 minutes with fresh PBS.

10) Allow the slides to air dry.

11) Mount cover slips on the slides using 50% glycerol/50% PBS solution.

b) Results

The slides are read on a fluorescence microscope capable of excitation at 488 nm (FITC), using an excitation filter with narrow passband windows in the blue spectrum (475–490 nm). The latter filter reduces breakthrough wavelength excitation. Each impression is observed for rabies-specific fluorescence (indicating the presence of viral antigen) at a magnification of 200× or greater. Specific fluorescence is denoted by bright 'apple' green fluorescence generally in the peri-nuclear area of cells, or longer 'string-like' neurons. Dull green or red/green auto-fluorescent granules should not be counted as positive antigen. Always read the positive control slide first.

Read the sample slide(s). Examine the tissue samples carefully, if necessary keep returning to the positive control for comparison.

A second operator should examine all slides and the diagnoses of both operators should be the same.

ii) Direct rapid immunohistochemistry test (dRIT)

The dRIT can be used as an alternative to DFA in routine rabies diagnosis as it has similar sensitivity and specificity (Lembo *et al*., 2006). The principle is similar to the DFA except that the dRIT uses streptavidin–biotin peroxidase staining (Coetzer *et al*., 2014; Madhusudana *et al*., 2012; Rupprecht *et al*., 2014). This test can be used in laboratories that do not have access to a fluorescence microscope. Primary antibodies should be fully validated for specificity and sensitivity before use, taking into consideration regional diversity of lyssaviruses.

a) Test protocol

1) Make touch impressions of suspect CNS tissues (including brainstem) on labelled glass microscope slides (always include standard positive and negative controls).

2) Air-dry slides for ~ 5 minutes at room temperature.

3) Immerse slides in 10% buffered formalin at room temperature for 10 minutes in a Coplin jar or other suitable container.

4) Dip-rinse slides several times to wash off any excess fixative in wash buffer (PBS plus 1% Tween 80 – TPBS).

5) Immerse slides in 3% hydrogen peroxide for 10 minutes.

6) Remove excess hydrogen peroxide by dip-rinsing slides in TPBS. Transfer slides to another TPBS rinse. Work with one slide at a time (leave the remaining slides immersed within the TPBS), remove slide, shake off excess buffer, and blot excess buffer from slide edges surrounding the tissue impression.

7) Add enough primary antibody conjugate (e.g. biotinylated anti-nucleoprotein poly- or monoclonal antibodies) to cover the impression. Incubate for 10 minutes in a "humidity chamber". This may be accomplished by placing slides on a moistened paper towel and covering with the plastic top of a cell culture plate or another simple cover.

8) After incubation shake off excess conjugate. Dip-rinse slides with TPBS. Shake off excess TPBS and blot buffer from slide edges surrounding the impression.

9) Treat each slide with streptavidin–peroxidase complex, adding enough of this reagent to the slide to cover the impression. Incubate in the humidity chamber at room temperature for 10 minutes. After incubation, shake off excess.

10) Dip-rinse slides with TPBS. Shake off excess buffer and blot excess buffer from slide edges surrounding the impression).

11) Incubate slides with amino-ethylcarbizole (AEC) substrate (note, other suitable chromogens may be used). To prepare the AEC stock solution: dissolve one 20 mg tablet of 3-amino 9-ethyl carbizole in 5 ml of N,N, dimethyl formamide in a glass vial or jar. The AEC stock solution should be stored at 4°C for ~ 1–2 months.

To prepare the AEC working dilution: add 7 ml of acetate buffer to a 15 ml centrifuge tube using a 10 ml plastic pipette. Add 0.5 ml of AEC stock solution (above) using a 1 ml glass or Pyrex pipette. Add 75 µl of 3% hydrogen peroxide. Filter through a nylon filter (0.45 µm) into a separate 15 ml tube. Once prepared, this mixture is only stable for 2–3 hours, so should be made just prior to use. Add enough of this reagent to the slide to cover the impression, and incubate in the humidity chamber at room temperature for 10 minutes. After incubation, shake off excess substrate.

12) Dip-rinse slides in distilled water.

13) Counterstain with diluted haematoxylin for 2 minutes.

14) Immediately dip-rinse stain from slides with deionised/distilled water. Make a second dip-rinse of slides with fresh deionised/distilled water to ensure removal of excess stain.

15) Transfer slides to fresh distilled water. Working with one slide at a time, shake off excess deionised/distilled water, blot excess from slide edges surrounding the impression, apply water-soluble mounting medium and cover-slip. Do not allow slides to air-dry prior to cover-slipping. If multiple slides are stained, they may stay in the deionised/distilled water rinse before cover-slipping.

16) View slides by light microscopy, using a 20× objective to scan the field thoroughly, and a 40× objective for higher power inspection. Lyssavirus antigens appear as reddish, intra-cytoplasmic inclusions against a blue neuronal background, using AEC and haematoxylin counterstain.

1.3.2. Virus isolation

These tests detect viable (replicating) RABV from specimens using cell cultures or laboratory animals. They should be used as confirmatory tests if the DFA test, dRIT, other antigen detection tests or RT-PCR give inconclusive results. Wherever possible, virus isolation in cell culture should replace the mouse inoculation test (MIT). Cell culture tests are as sensitive as MIT (Robardet *et al.,* 2011; Rudd & Trimarchi, 1989), but are less expensive, give more rapid results and avoid the use of animals. For virus isolation from samples during routine rabies diagnostics, the use of cell culture techniques for isolating RABV should always take precedence over the use of the MIT.

i) Rabies tissue culture infection test (RTCIT)

Neuroblastoma cells e.g. N2a, CCL-131 in the American Type Culture Collection (ATCC) are highly susceptible to infection with lyssaviruses. The cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS), incubated at 37°C with 0.5% or 5% CO_2 depending on the culture vessel. Cell culture tests may be undertaken in multi-well plastic plates, multi-chambered glass slides, or on glass cover-slips. Additional passages could be considered, including use of T25 flasks, to increase sensitivity; usually three consecutive passages should be conducted to confirm a negative result. Cytotoxicity is a commonly reported factor limiting test robustness. Techniques proposed to reduce cytotoxicity include adding antibiotics, reducing the incubation time before changing media (to as short as 35 minutes) and dilution of samples. Cell culture tests and their variations should be fully validated before use.

a) Protocol for a 96-well plate

1) 100 µl of clarified brain homogenate (20% w/v in PBS, 0.1 M, pH 7.4) is added to 200 µl of a 2×10^5 cells/ml suspension of 2- to 3-day old cells, freshly prepared from a sub-confluent flask in four wells of a 96-well plate.

2) After 24 hours incubation at 5% CO_2 and 37°C, the supernatant from each well is removed and 200 µl of fresh medium is added to each well.

3) After a further 72 hours incubation the supernatant is removed by pipette and kept for onward passage if required.

4) The cells are fixed with 80% acetone and stained at 37°C for 30 minutes with fluorescent antibody according to manufacturers' recommendations.

Variations include reduced incubation time before changing media to reduce cell toxicity, the use of cell permeability agents (e.g. DEAE-dextran), and further passages. Up to three passages may be considered to increase sensitivity.

b) Protocol for use in 8-chamber slides

1) 50 µl of clarified brain homogenate (20% in a grinding substrate made of PBS, 0.1 M, pH 7.4 with heat-inactivated fetal calf serum) is added to 400 µl of a 10^5 cells/ml suspension, freshly prepared from a sub-confluent flask.

2) After 24 hours incubation at 5% CO_2 and 37°C, the supernatant from each chamber is removed and 400 µl of fresh medium is added to each chamber.

3) After a further 24 hours (or more) of incubation the supernatant is removed, the chamber structure removed, the cell layer dried and fixed with pure high grade 80% cold acetone.

4) The fixed cell layer is then stained with fluorescent antibody at 37°C for 30 minutes according to manufacturers' recommendations.

c) Alternative protocol

1) 500 µl of clarified brain homogenate (20% [w/v] in growth medium [90% DMEM, 1.0% fetal calf serum (FBS) and 2% antibiotics] and centrifuged at approximately 700 ***g*** for 10 minutes) is mixed with 500 µl of 2×10^6 cells/ml freshly prepared from a sub-confluent flask in DEAE-dextran working solution (0.2 ml Dextran-stock solution in 25ml DMEM Dextran-stock solution is 0.50 g DEAE-dextran dissolved in 100 ml PBS [Hanks], sterile filtered).

2) After incubation for 30 minutes at 5% CO_2 and 37°C (agitate cell suspension carefully twice or three times during the incubation period), the cell suspension is gently centrifuged and the cell pellet is resuspended in 10 ml of fresh DMEM.

3) 8 ml of the cell suspension is put in a tissue culture flask (T25) and 2 ml onto a 6- or 24-well plate or petri dish (35/10 mm) to monitor the infection by DFA.

4) After a further 3–4 days incubation, the supernatant is removed by pipette from the monitor plate and discarded, while the T25 flask remains untouched.

5) The monitor plate is fixed in 80% acetone, then stained with fluorescent antibody according to laboratory procedures and observed under an inverse fluorescence microscope.

6) If the monitor plate is negative, cells are trypsinised from the T25 flask, split in a ratio of 1:2 up to 1:4 in fresh DMEM and the cell suspension put into a tissue culture flask (8 ml) and onto a 6- or 24-well plate or petri dish (2 ml).

7) Steps 4–6 are repeated. Three consecutive passages are conducted to confirm a negative result.

Whilst protocols (a) and (b) above only allow consecutive passaging of the supernatant, passaging in protocol (c) is based on splitting of the potentially infected cell monolayer thereby facilitating virus isolation in samples of low viral load.

ii) Mouse inoculation test (MIT)

Three-to-ten mice, 3- to 4-weeks old (12–14 g), or a litter of 2-day-old new-born mice, are anesthetised and inoculated intracerebrally. The inoculum (0.01 ml for new-born mice or up to 0.03 ml for older mice) is the clarified supernatant of a 10–20% (w/v) homogenate of brain tissue including brainstem (e.g. cortex, Ammon's horn, thalamus, medulla oblongata) in an isotonic buffered solution containing antibiotics. Mice should be anaesthetised for inoculation. The mice are observed daily for 28 days, and every dead mouse is examined for rabies using the DFA or dRIT test. For faster results in new-born mice, it is possible to check one mouse on days 5, 7, 9 and 11 post-inoculation. Any deaths occurring during the first 4 days are regarded as nonspecific (due to stress/bacterial infection etc.).

Once a validated and reliable cell culture unit exists in the laboratory, consideration should be given to replace the mouse inoculation test with cell culture whenever possible as it avoids the use of live animals, is less expensive and gives more rapid results. However, advantages of MIT are that when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes and the assay can be easily and practicably applied in situations where skills and facilities for other tests (e.g. cell culture) are not available.

1.3.3. Rapid immunochromatographic tests (lateral flow devices)

Commercial rapid immunochromatographic tests, also referred to as lateral flow devices (LFDs), for viral antigen detection are available. Some studies have found that when used under strict adherence to manufacturers' instructions, various available LFDs have shown highly variable results, some with inadequate or even complete lack of diagnostic sensitivity; test kits have also demonstrated inconsistency between batches and based on the type of diagnostic samples used (Eggerbauer *et al.,* 2016; Klein *et al.,* 2020). Field studies showed high sensitivity and specificity for some of those tests, particularly when the manufacturers' instructions were modified, with reported sensitivities compared with DFA ranging between 93% and 100% in field samples (Lechenne *et al.,* 2016; Markotter *et al.,* 2009; Nishizono *et al.,* 2008; Servat *et al.,* 2012). However, further improvements in sensitivity, consistency and validation using appropriate diagnostic samples are still required before LFDs can be recommended by WOAH. If data support the use of LFDs for routine surveillance and establishment of infection, a manufacturer could apply for inclusion of the assay on the WOAH Register of diagnostic kits[2].

Depending on the product-specific sensitivity and specificity, additional confirmation of results should be undertaken using other primary test methods (DFA, RTCIT, RT-PCR). Therefore, standard techniques should be either established or reinforced in parallel for the use of an LFD as a screening tool at a central laboratory. Nevertheless, positive LFD results should be a strong indication for bite victims to seek post-exposure prophylaxis.

1.3.4. Histological identification of characteristic cell lesions

Negri bodies correspond to the aggregation of viral proteins, but the classical staining techniques detect only an affinity of these structures for acidophilic stains. Techniques that stain sections of paraffin embedded brain tissues (e.g. Mann's technique) are time consuming, less sensitive and more expensive than DFA and dRIT. Seller's method on unfixed tissue smears has a very low sensitivity is only suitable for perfectly fresh specimens. These methods are no longer recommended for routine diagnosis.

1.3.5. Reverse-transcription polymerase chain reaction (RT-PCR)

RT-PCR assays are sensitive tools for the detection of lyssavirus-derived ribonucleic acid (RNA) within suspect specimens with the advantage that they do not require the presence of live virus.

RT-PCR assays that target the 3' proximal viral gene are considered the most sensitive as the replication cycle of lyssaviruses dictates that the N gene coding viral nucleoprotein is transcribed in the greatest abundance with a transcriptional gradient occurring for downstream genes.

RT-PCR assays should meet the WOAH Standards for validation (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*) and should be able to detect a broad spectrum of globally circulating RABV strains. Different methods for RNA extraction (manual, commercial conventional column or magnetic bead-based rapid RNA extraction methods) are available and can differ in sensitivity.

RT-PCR assays that have been evaluated in accordance with WOAH Standards (Chapter 1.1.6), have shown similar sensitivity and specificity to the DFA or DRIT, and can ideally detect all known Lyssaviruses and be used as an alternative to DFA or DRIT for routine rabies diagnosis. RT-PCR assays used as primary diagnostics should be conducted from a composite sample of brain tissue that includes brain stem and cerebellum, as stated in Section B.1.1. RT-PCR assays that have

2 https://www.woah.org/en/what-we-offer/veterinary-products/diagnostic-kits/the-register-of-diagnostic-kits/

reduced sensitivity and specificity or are not able to detect all Lyssaviruses should be considered for confirmatory diagnostics when primary diagnostic assays are indeterminate.

Two reverse-transcriptase polymerase chain reaction (RT-PCR) methods for the detection of lyssavirus RNA in clinical samples are described. The first is a conventional (gel-based) pan-lyssavirus hemi-nested RT-PCR assay (hnRT-PCR). The second is a real-time pan-lyssavirus RT-PCR assay (based on a fluorescent DNA stain). The principal advantages of the hnRT-PCR assay include the applicability to laboratories that only have conventional PCR apparatus and the ability to obtain genetic data from the generated amplicons. The advantages of the fluorescent DNA stain-based assay include increased sensitivity over the conventional assay and a significantly reduced turnaround time. Both assays are based on a one-step approach, which reduces the risks of contamination and during manipulation. The fluorescent DNA stain-based assay is approximately one log more sensitive than the hemi-nested conventional RT-PCR.

There are numerous alternative rabies RT-PCR methodologies that are fit for purpose (for example Freuling *et al*., 2014; Fischer *et al*., 2013; Hayman *et al*., 2011; Suin *et al*., 2014; Wadhwa *et al*., 2017; Wakeley *et al*., 2005). Alternative assays may target different genes with different primers and could also be considered for use where suitable validation data have been obtained (see chapter 1.1.6).

i) Conventional RT-PCR techniques

The use of conventional RT-PCR is of benefit to laboratories that lack real-time RT-PCR platforms or that wish to obtain partial gene sequence data. Further equipment is required to enable electrophoretic resolution of amplicons on a gel. Prior to testing, RNA is extracted from suspect biological samples using a validated method of nucleic acid extraction. The one-step RT-PCR assay combines reverse transcription and first round PCR in a single tube, using a reverse transcriptase to generate a DNA copy of any viral RNA present that then acts as the template for the DNA polymerase to amplify the cDNA template exponentially. It has the added advantage of reducing the turnaround time of the assay. The assay described below is an example of a fully validated, reproducible conventional RT-PCR with a high sensitivity and specificity (modified from Heaton *et al.,* 1997). An optional second round amplification (hemi-nested [hn] PCR) is possible to increase the sensitivity and/or to confirm the specificity of the first round PCR product.

a) Primer sequences

1) JW12 Primer 5'-ATG-TAA-CAC-CYC-TAC-AAT-G-3'

   7.5 pmol/µl (first round)

   3.5 pmol/µl (second round)

2) JW6UNI Primer 5'-CAR-TTV-GCR-CAC-ATY-TTR-TG-3'

   7.5 pmol/µl (first round)

3) JW10UNI Primer 5'-GTC-ATY-ARW-GTR-TGR-TGY-TC-3'

   3.5 pmol/µl (second round)

b) Test procedure

Each test must contain positive (PC) and negative (NC) control tissue samples, as well as a no template control (NTC), which are run alongside the test samples.

First round one step (OS) RT-PCR (JW6UNI/12)

In clean room:

1) Wipe bench with an appropriate disinfectant prior to use or prepare PCR workstation. To prepare, open doors of the PCR workstation and wipe the cabinet surface with an appropriate disinfectant. Place an ice bucket and suitable pipette and tips within the station and close the doors. Switch on UV light for 10 minutes.

2) Obtain the required test reagents. Ensure the enzyme mix is kept on ice. The remaining reagents can be thawed at room temperature.

3) Put the required number of 0.2 ml tubes in a rack and label the tubes clearly with sample identification, denoting that this is the first round reaction. Include PC, NC and NTC.

4) Prepare a JW6UNI/JW12 reaction master mix as detailed below:

First round JW6UNI/12 master mix

Reagent	Volume per reaction (µl)
Molecular grade water	29.0
5× buffer	10.0
dNTPs (10 mM)	2.0
JW12 (7.5 pmol/µl)	3.0
JW6UNI (7.5 pmol/µl)	3.0
Enzyme mix	2.0
Total	49

Keep all reagents on ice, thaw and vortex before using. Allow for pipetting variation by preparing a volume of master mix at least one reaction greater than required.

5) Vortex the prepared master mix thoroughly, centrifuge and dispense 49 µl into each of the 0.2 ml tubes. Close the lids.

6) Transfer the sealed tubes to the ice/cool block in the template room on a tray. Once a tray has been removed it must not be returned to the clean room without decontamination using an appropriate disinfectant

In template room – addition of template

1) Wipe bench with an appropriate disinfectant prior to use.

2) Thaw samples and control RNA (positive and negative controls) on ice.

3) Add 1 µl of test RNA (where possible at concentration of 1µg/µl for extracted samples) below the surface of its allocated master mix tube and mix gently. Discard the tip directly into disinfectant after use. Repeat this process until all samples and controls have been added to their allocated tubes.

4) Press the lids down by hand and seal firmly.

5) Transfer the sealed tubes to the PCR machine and cycle as detailed below: In house validation of cycling parameters is essential to ensure optimisation for local PCR machines.

Hemi-nested RT-PCR first round cycling parameters:

Temperature	Time	Cycles
50°C	30 minutes	1
95°C	15 minutes	1
94°C 45°C 50°C 72°C	30 seconds 45 seconds 15 seconds 1 minute	45
72°C	7 minutes	1
4°C	∞	n/a

Once complete the resulting amplicons can be visualised on a 1.5–2% agarose gel using standard gel electrophoresis techniques and suitable marker DNA ladders to ensure the appropriate size amplicon has been generated in the positive control samples (for comparison with samples on test). A suitable DNA-detection chemical should be added to the gel and a UV light box used to visualise the products.

Second round OS RT-PCR (JW10/12)

Where no amplicon is generated on the first round reaction, a second round, hemi-nested reaction should be performed.

In clean room:

1) Prepare PCR workstation as described in Section B.1.3.**5**.i.**b**.1.

2) Obtain the required reagents. Ensure enzyme mix is kept on ice. The remaining reagents can be thawed at room temperature.

3) Put the required number of 0.2 ml tubes in a rack and label the tubes clearly with sample identification and denote that this is the second round reaction by labelling with '10/12', or '2'. Label the PCR negative as '–2' or 'NC2' and the no template control as 'NTC2'. This additional negative control must be included in every second round PCR experiment to confirm the master mix is not contaminated.

4) Prepare a JW10UNI/JW12 reaction master mix as detailed below:

Second round JW10UNI/12 master mix

Reagent	Volume per reaction (µl)
Molecular grade water	22.0
High fidelity Taq polymerase (2×)	25.0
JW12 (3.5 pmol/µl)	1.0
JW10UNI (3.5 pmol/µl)	1.0
Total	49

5) Thaw and vortex all reagents before using. Allow for pipetting variation by preparing a volume of master mix at least one reaction greater than required.

6) Vortex the prepared mastermix thoroughly, centrifuge at 700 ***g*** and dispense 49 µl into each of the 0.2 ml tubes. Seal the tubes.

7) Transfer the sealed tubes to the template room on a disposable tray. Once a tray has been removed it must not be returned to the clean room without appropriate decontamination.

In template room – addition of template:

1) In order to reduce cross contamination, the template may be added within a PCR workstation.

2) To prepare, open the doors of the PCR workstation and wipe the cabinet surface with an appropriate disinfectant. Place an ice bucket, suitable pipette and tips within the station and close the doors. Switch on UV light for 10 minutes.

3) Add 1 µl of undiluted first round PCR product below the surface of the prepared second round master mix to minimise aerosols and mix gently. Discard the tip directly into an appropriate disinfectant after use. Ensure the lid of the PCR tube is sealed firmly. Repeat this step until all first round PCR products and the second round PC, NC and NTC have been added to allocated Second Round master mix tubes. Change gloves regularly and at suitable points to avoid cross-contamination.

4) If using the PCR workstation, remove samples and supplies and switch on the UV for 10 minutes.

5) Run the PCR machine using the following second round cycling parameters. In-house validation of cycling parameters is essential to ensure optimisation for local PCR machines.

Temperature	Time	Cycles
95°C	15 minutes	1
94°C 45°C 50°C 72°C	30 seconds 10 seconds 15 seconds 1 minute	35
72°C	7 minutes	1
4°C	∞	n/a

Analyse the amplification reactions by electrophoresis on agarose gels using an appropriate DNA ladder.

ii) Real-time RT-PCR techniques

Where capabilities allow, real-time PCR platforms enable a more rapid evaluation of the presence or absence of lyssavirus RNA in suspect samples. The procedure detailed here is an example that uses the same forward primer as the conventional RT-PCR assay described in Heaton *et al*., 1997. The use of a universal one-step RT-PCR kit that uses a fluorescent DNA stain for the detection of *Lyssavirus* species from clinical specimens has been demonstrated to be both highly sensitive and specific for lyssavirus RNA. Furthermore, by using a fluorescent DNA stain as the detection system it is able to detect all lyssaviruses based on the pan-lyssavirus primer specificity. This method includes a separate RT-PCR assay containing a fluorescent DNA stain for amplification of the internal housekeeping control, mRNA for beta-actin as a template control for RNA extraction.

a) Primer sequences

1) Pan-*lyssavirus*-specific primers (synthesised to 0.05 μmol, HPLC purified) and diluted to 20 pmol/μl:

   a) JW12 RT/PCR primer 5'-ATG-TAA-CAC-CYC-TAC-AAT-G-3'

   b) N165-146 PCR primer 5'-GCA-GGG-TAY-TTR-TAC-TCA-TA-3'

2) Multispecies Beta Actin primers (synthesised to 0.05 μmole, HPLC purified) and diluted to 20pmol/μl:

   a) BatRat Beta-actin intronic primer 5'-CGA-TGA-AGA-TCA-AGA-TCA-TTG-3'

   b) BatRat Beta-actin reverse primer 5'- AAG-CAT-TTG-CGG-TGG-AC-3'

b) Test reliability

Instrumentation and equipment are monitored for satisfactory performance and calibrated once a year. Include a calibrated RABV RNA PC on every test run and an internal Beta-actin test may be used as an extraction control. A NC and NTC is included on every test run to confirm the absence of contamination. All test samples should be run at least in duplicate.

c) Test procedure

Gloves and a laboratory coat must be worn at all times.

In clean room/UV cabinet:

1) Prepare PCR workstation as described in Section B.1.3.**5**.i.**b**.1.

2) Obtain the required reagents. Ensure the enzyme mix is kept on ice, the remaining reagents can be thawed at room temperature.

3) Put the required number of 0.2 ml tubes in a rack and label the tubes clearly with sample identification. Include tubes for PC, NC and NTC.

4) Prepare a reaction master mix as below and keep all reagents on ice. Allow for pipetting variation by preparing at least two extra reaction mixes

Reagent	Volume per reaction (µl)
Molecular grade water	7.55
Universal reaction mix containing a fluorescent DNA stain (2×)	10.0
JW12 (20 pmol/µl)	0.6
N165-146 (20 pmol/µl)	1.0
RT enzyme mix	0.25
Total	19.4

5) Prepare a reaction master mix for the β-actin mRNA which assesses the quality in samples extracted from solid tissue. The assay for β-actin must be positive in order to have confidence that RNA was isolated from the starting material.

Reagent	Volume per reaction (µl)
Molecular grade water	7.55
Universal reaction mix containing a fluorescent DNA stain (2X)	10.0
Intronic (20 pmol/µl)	0.6
Reverse (20 pmol/µl)	1.0
RT enzyme mix	0.25
Total	19.4

6) Vortex the prepared master mixes and aliquot 19 µl into each of the relevant wells of a 96 well plate or 8-well strips.

In template room/UV cabinet – addition of template

1) Prepare PCR workstation as described in Section B.1.3.**5**.i.**b**.1.

2) Thaw samples and control RNA on ice.

3) Add 2 µl of test RNA (where possible at concentration of 0.5–1 µg/µl for extracted samples) below the surface of its allocated master mix tube and mix gently. Discard the tip directly into disinfectant after use. Repeat this process until all samples and controls have been added to their allocated tubes.

4) Press the lids down by hand and seal firmly.

5) Transfer the PCR plate/strips to the real-time machine for thermal cycling.

Setting up of real-time thermal cycler

1) Load the samples into the machine, ensuring that they are orientated the correct way. Ensure that all the lids are firmly sealed, and then close the machine's plate cover and door. Set up the run parameters according to the manufacturer's instructions.

2) Set up the thermal profile as follows:

Temperature	Time	Cycles
50°C	10 minutes	1
95°C	5 minutes	1

Temperature	Time	Cycles
95°C 60°C	10 seconds 30 seconds	40
95°C 55°C 55-95°C	1 minutes 1 minute 10 seconds	80

Thermal profiles may be subject to optimisation depending on PCR machines used.

3) In case of an inconclusive assay, an agarose gel can be run to confirm the presence/absence of an amplicon and its approximate size (100 bp).

1.4. Other identification tests

The tests above describe methods to accurately diagnose rabies and to isolate and identify the virus. Characterisation of the virus can provide useful epidemiological information and should be undertaken in specialised laboratories (such as WOAH, WHO or FAO Reference Laboratories). These techniques would include the use of monoclonal antibodies, as well as partial and full genome sequencing followed by phylogenetic analysis. These characterisations enable a distinction to be made between vaccine virus and a field strain of virus, and possibly identify the geographical origin of the latter.

2. Serological tests

The main application of serology for rabies is to determine responses to vaccination in domestic animals, particularly in connection with international travel, or for monitoring mass vaccination campaigns in dogs and other wildlife reservoir species. The measurement of rabies antibodies has typically involved virus neutralisation (VN) tests to detect RABV neutralising antibodies. ELISAs are now also recognised as acceptable tests to detect binding antibodies. A strong but not strict correlation in levels is observed between these two different antibody detection methods. Depending on the nature of the ELISA, there can be variable sensitivity and specificity. In contrast to the ELISA, poor quality sera can cause cytotoxicity in VN tests, which could lead to false-positive results. Depending on the intended purpose, both tests are useful for detecting responses to vaccination if appropriate cut-offs are used. However, ELISAs are currently not applicable to international movement of animals or trade.

Serological surveys have also been used to provide information on infection dynamics of lyssaviruses in bats although standardisation of serological tests for bats is still needed.

2.1. Virus neutralisation test in cell culture: fluorescent antibody virus neutralisation test (FAVN)

The principle of the FAVN test (Cliquet *et al*., 1998) is the *in-vitro* neutralisation of a constant amount of RABV ('challenge virus standard' [CVS-11] strain adapted to cell culture) before inoculating BHK-21 cells susceptible to RABV.

The serum titre is the dilution at which 100% of the virus is neutralised in 50% of the wells. This titre is expressed in IU/ml by comparing it with the neutralising dilution of the WOAH serum of dog origin under the same experimental conditions. The WHO standard[3] for rabies immunoglobulin [human] No. 2, or an internal control calibrated against the international control may also be used to calculate the IU/ml titre of test sera.

Generally, the minimum measurable neutralising VN antibody titre considered to represent a reasonable level of seroconversion is 0.5 IU per ml. The same measure is used in dogs and cats to confirm an adequate response to vaccination prior to international travel. However, within the framework of monitoring mass vaccination campaigns, a single cut-off level of seropositivity may not be universally applicable among different species (Moore *et al.,* 2017).

3 Available from: National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom (UK).

This microplate method uses 96-well plates, and is an adaptation of the technique of Smith *et al*. (1973). The FAVN test and the rapid fluorescent focus inhibition test (RFFIT) give equivalent results (Cliquet *et al*., 1998).

2.1.1. Viruses and WOAH standard serum

Virus: CVS-11 (previously ATCC reference VR 959) strain, which is available from the ATCC or the WOAH Reference Laboratory for rabies, Nancy, France[4]. Vials are stored at –80°C;

WOAH Standard Serum of dog origin[5] stored at +4°C and diluted to 0.5 IU/ml with sterile deionised or distilled water. This control serum may be used to calibrate an additional internal control that is used for regular FAVN testing.

Negative control serum: A pool of sera from naïve dogs stored at –20°C.

2.1.2. CVS production

i) Cell growth: the BHK-21 C13 cells (ATCC CCL-10) maintained in Dulbecco's modified Eagle's medium (DMEM) or Glasgow modified Eagle's medium (GMEM) with 10% heat-inactivated FCS and antibiotics, used to produce the CVS virus (ATCC VR 959 CVS-11) are trypsinised during the rapid growth phase, i.e. cells are in the exponential phase of their kinetic growth. If the confluence of the layer is complete, a new passage should be made. The cells in the cell suspension should not be aggregated; 2×10^7 cells are needed for a 75 cm^2 cell culture flask. Cells are collected within a volume of 20–30 ml in cell culture medium with 10% heat-inactivated FCS.

ii) Infection of cells: the multiplicity of infection (number of infective particles per cell) is adjusted to between 0.1 and 0.5. The glass bottle containing the virus/cell suspension is incubated for 60 minutes at 35.5–37°C. The contents of the bottle are gently stirred every 10–15 minutes.

iii) Virus growth: the virus/cell suspension is then centrifuged at 800–1000 ***g*** for 15 minutes and the cell pellet is resuspended in cell culture medium mixed with 10% heat-inactivated FCS. Virus is harvested 2 days later.

iv) Harvest and storage: the supernatant is centrifuged at 800–1000 ***g*** for 15 minutes at 4°C. If several flasks have been used, the different centrifuged supernatants are mixed and then aliquoted and frozen at –80°C. The infective titre of the harvest is established at least 3 days after freezing.

2.1.3. Titration of virus in $TCID_{50}$ (50% tissue culture infective dose)

This titration method uses BHK-21 C13 cells (ATCC CCL-10) in microtitre plates.

i) The day before titration, a cell suspension containing 10^5 cells/ml is prepared in cell culture medium containing 10% heat-inactivated FCS, and is distributed, 200 µl per well, into 96-well microtitre plates. The plates are then incubated for 24 hours at 35.5°C–37°C with 5% CO_2.

ii) The serial dilutions of virus are performed in 5 ml tubes using a cell culture medium without FCS as diluent. Ten-fold dilutions from 10^{-1} to 10^{-12} are prepared (0.9 ml of diluent with 0.1 ml of the previous dilution).

iii) The medium in the microtitre plates is discarded using an aspiration system. Fifty µl of each virus dilution is distributed per well. Six replicates are used per dilution. The microtitre plate is then incubated for 1 hour at 35.5–37°C with 5% CO_2. Then 200 µl of cell culture medium, containing 5% FCS, is added.

iv) Incubate in a humidified incubator for 3 days at 35.5–37°C in 5% CO_2.

4 Please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

5 http://www.woah.org/en/our-scientific-expertise/veterinary-products/reference-reagents/

v) The cells are stained using the DFA test, as detailed below. Reading is qualitative, every well that shows specific fluorescence is considered to be positive. The titre calculation is made using either the neoprobit graphic method or the Spearman–Kärber formula (WHO, 2018).

vi) The CVS titration must be performed by FAVN test to establish the infective dose in $TCID_{50}$.

2.1.4. Test procedure

i) The control plate is used for the titration of CVS (rows 1 to 4), standard sera and negative control serum are used. All other plates are used for the sera to be tested.

ii) Medium is added to the wells as follows: control plate rows 1 to 4 and cells A9 to A12: add 150 µl per well; in the other plates, rows 6 and 12: add 200 µl per well; all other wells: add 100 µl.

iii) Sera to be tested are heat inactivated for 30 minutes at 56°C. 50 µl of each undiluted serum to be tested is added to four adjacent wells.

iv) Dilutions of sera are conducted in the microplates as follows:

WOAH serum, the WHO serum, the internal control and the naive dog serum: with a 50–200 µl multichannel pipette, mix the first dilution wells by sucking in and out at least eight times, transfer 50 µl from one row to the next one, until the last one is reached. Discard 50 µl from the last row.

If there is a serum to be tested on the control plate, see below for the dilution step.

A minimum of four three-fold dilutions is required.

Sera being tested (all plates): as above, transfer successively 50 µl from one row to the following one until rows 5 and 11 (dil. $10^{-2.39}$). With a 5–50 µl multichannel pipette, transfer 10 µl from rows 5 and 11 to rows 6 and 12, respectively (from dil. $10^{-2.39}$ to dil. $10^{-4.23}$). Using a multichannel pipette adjusted to 90 µl, mix rows 6 and 12 and discard 180 µl. Then add 70 µl of medium to these rows. This final step does not lend itself to high throughput testing. To attain or exceed the recommended final dilution alternative procedures may be used. These may require modifications to the plate layout.

2.1.5. Addition of challenge virus standard

i) Stock CVS is stored in 1 ml microtubes at –80°C. One tube is thawed rapidly under cold running water, and placed in melting ice.

ii) One dilution from this tube is prepared in order to obtain 100 $TCID_{50}$ in 50 µl. Of this dilution, 50 µl is added to each serum-filled well. For virus titration, 50 µl is added to wells H1 to H4 (control plate). Next, transfer 50 µl from row to row (control plate, lines 1–4). Discard 50 µl from the last row (control plate, wells A1 to A4). No virus is added to wells A9 to A12 of control plate. The range allowed for the virus dose titre must be between 30 and 300 $TCID_{50}$/50 µl.

iii) Incubate the microplates at 35–37°C in a humid incubator with 5% CO_2 for 1 hour.

iv) Addition of cells: trypsinise a subconfluent culture of BHK-21 cells. Resuspend the cells to obtain a 4×10^5 cells/ml suspension in DMEM supplemented with 10% heat-inactivated FCS. Add 50 µl of the cell suspension to each well.

v) Incubate the microplates for 48 hours at 35–37°C in a humid incubator with 5% CO_2.

2.1.6. Fixation and staining

i) After the 48-hour incubation period, the medium is discarded, and the microplates are rinsed once in PBS, pH 7.2, and once in 80% acetone. The microplates are then fixed in 80% acetone at room temperature for 30 minutes, and are dried at room temperature for at least 30 minutes.

ii) Add 50 µl of the FITC anti-rabies conjugate, at the working dilution, to each well, gently rock the microplates and incubate at 35–37°C for 30 minutes. Discard the fluorescent conjugate

and rinse the microplates twice with PBS. Excess PBS is removed by briefly inverting the microplates on absorbent paper.

2.1.7. Reading and interpreting the results

i) The total surface of each well is observed. The reading evaluation is qualitative (plus or minus): no fluorescent cell – a minus score is recorded for the well; fluorescent cells (one cell or more) – a plus score is recorded for the well. Use a fluorescence microscope suitable for FITC fluorescence equipped with ×10 eye-piece and ×10 objective. The global magnification of the microscope ranges between ×100 and ×125 due to the extra magnification of some epi-fluorescence systems.

ii) Cell and virus controls are read first. For titration of CVS, negative control serum, and WOAH standard serum, titres are calculated according to the Spearman–Kärber method or the neoprobit graphic method (WHO, 2018).

iii) Results of titration of CVS ($TCID_{50}$), naive serum (D_{50} [median dose]) and positive standard (D_{50}) are reported on a control card for each of these three controls. The control results of the current test are compared with the accumulated control test results from previous tests using the same batch of control. The test is validated if the values obtained for the three controls in the current test are not statistically different from the mean (±2 SD) of all the values obtained in the tests conducted previously according to this technique.

iv) The result of the test corresponds to the non-neutralised virus after incubation with the reference serum or with the serum to be tested. These titres are calculated with the neoprobit graphic method or with the Spearman–Kärber formula (WHO, 2018).The comparison of the measured titre of the tested sera with that of the positive standard serum of a known neutralising titre allows determination of the neutralising titre of the tested sera in IU/ml. The conversion to IU/ml can be made by using either the log D_{50} value of the day or the mean value of the positive standard serum.

2.1.8. Formula to convert the log D_{50} value in IU/ml titre:

$$\text{Serum titre (IU/ml)} = \frac{[(10^{(\text{serum log } D_{50} \text{ value})}) \times \text{theoretical titre of positive standard serum 0.5 IU/ml}]}{(10^{(\log D_{50} \text{ of positive standard serum 0.5 IU/ml})})}$$

Example of conversion:

- log D_{50} of the serum = 2.27
- theoretical titre of positive standard serum 0.5 IU/ml = 0.5 IU/ml
- log D_{50} of positive standard serum = 1.43
  (for the log D_{50} of positive standard, the value of the day or the mean value can be considered)

$$\text{Serum titre (IU/ml)} = \frac{10^{2.27} \times 0.5}{(10^{1.43})} = 3.46 \text{ IU/ml}$$

The following parameters have to be strictly respected:

- RABV: only the CVS-11 strain should be used.
- Cells culture: only BHK-21 cells (ATCC number – CCL 10) should be used.
- The FAVN test must be performed only in 96 wells microplate.
- Control charts should be used for RABV, negative control serum and positive standard serum of dog origin.
- The back titration of the CVS virus, as well as negative control serum and positive standard serum of dog origin, must be present on control plate.

- A minimum of four three-fold dilutions of sera are required. The reading method is 'all or nothing' only.
- Four replicates of each serum should be diluted.
- For the conversion of log D_{50} in IU/ml, the laboratories should use only the log D_{50} value of the positive standard serum of dog origin.

2.2. Virus neutralisation test in cell culture: the rapid fluorescent focus inhibition test (RFFIT)

2.2.1. Preparation of seed virus suspension

i) Trypsinise one 3-day-old 150 ml flask culture of mouse neuroblastoma (MNA) cells. A similar cell line (CCL-131) may be obtained on request from the ATCC.

ii) Resuspend 3×10^7 cells in a 50 ml conical centrifuge tube in 2.7 ml of Eagle's minimal essential medium supplemented with 10% fetal calf serum (EMEM-10).

iii) Using standard rabies safety procedures, add 1×10^7 infectious units of CVS-11 RABV (previously ATCC reference VR959) and vortex/mix once. Incubate the cells and virus for 15 minutes at 37°C; vortex/mix the cells once during this time.

iv) Add 10 ml EMEM-10, vortex/mix, and centrifuge the cells at 500 ***g*** for 10 minutes.

v) Discard the supernatant. Resuspend the cells in 30 ml of growth medium and transfer to a 150 ml flask.

vi) Gently rock the flask to mix the cell suspension, and then prepare three eight-well tissue-culture chamber slides by pipetting 0.2 ml of the cell suspension into one well of each slide.

vii) Incubate the flask and slides at 37°C in a humidified incubator with 0.5% carbon dioxide (CO_2). The flask should be incubated as a closed culture (tighten the cap).

viii) At 20, 40 and 64 hours after infection, acetone fix and stain one slide using an immunofluorescence technique to determine the virus infectivity. The supernatant should be harvested 24 hours after the cells reach 100% infectivity (typically 40 hours after infection).

ix) Transfer the supernatant to a 50 ml centrifuge tube and centrifuge at 4000 ***g*** for 10 minutes.

x) Distribute the supernatant into 0.5 ml aliquots and store at –70°C.

2.2.2. Titration of seed virus suspension

i) Thaw one aliquot of the seed virus and prepare serial ten-fold dilutions (from 10^{-1} to 10^{-8}) in EMEM-10.

ii) Distribute 0.1 ml of each virus dilution into one well of an eight-well tissue-culture chamber slide. Add 0.2 ml of MNA cells suspended in EMEM-10 (concentration 5×10^4 cells per 0.2 ml) to each well.

iii) Mix the cells and virus by gently rocking the slide, then incubate at 37°C in a humidified incubator with 0.5% CO_2 for 40 hours.

iv) Acetone fix and stain the slide using an immunofluorescence technique. Evidence of virus infection should be observed at the 10^{-6} dilution of virus, indicating a virus stock suspension containing at least 1×10^6 infectious units per 0.1 ml. Prepare sufficient seed virus so that frequent serial passage of the virus is unnecessary.

2.2.3. Preparation of stock virus suspension

i) Infect 3×10^7 MNA cells with 1×10^7 infectious units of the seed virus preparation (see above).

ii) Harvest the supernatant 24 hours after the cells reach 100% infectivity (typically 40 hours after infection).

iii) Distribute the supernatant into 0.5 ml aliquots and store at –70°C.

2.2.4. Titration of stock virus suspension

i) Thaw one aliquot of the stock virus and use this to prepare serial ten-fold dilutions (from 10^{-1} to 10^{-6}) in EMEM-10.

ii) Distribute 0.1 ml of each virus dilution into one well of an eight-well tissue-culture chamber slide. Add 0.2 ml of MNA cells suspended in EMEM-10 (concentration 1×10^5 cells per 0.2 ml) to each well.

iii) Mix the cells and virus suspension by gently rocking the slide, then incubate at 37°C in a humidified incubator with 0.5% CO_2 for 20 hours.

iv) Acetone fix and stain the slide using an immunofluorescence technique.

Each well of an eight-well tissue-culture chamber slide contains 25–50 distinct microscopic fields when observed at ×160–200 magnification or 20 distinct microscopic fields when observed at x100 magnification. One unit of virus for the RFFIT is determined as the dilution at which 50% of the observed microscopic fields contain one or more foci of infected cells (the focus-forming dose, FFD_{50}). The stock virus suspension should contain at least 1×10^4 FFD_{50} per 0.1 ml (i.e. the well with cells infected with the 10^{-4} dilution of the virus should contain at least one focus of infected cells in 50% of the observed microscopic fields). For example, a stock virus suspension of this titre (1×10^4 FFD_{50} per 0.1 ml) can then be diluted to $10^{-2.3}$ to obtain a challenge virus containing 50 FFD_{50}. To calculate the working dilution subtract the log of 50 (1.7) from the log of the virus dose (e.g. in the example given 4.0 – 1.7 – 2.3).

2.2.5. Reference sera

A recognised (WHO [see footnote 3], WOAH [see footnote 5]) reference serum standard or a prepared standard verified against a recognised standard diluted to a potency of 2.0 IU/ml should be included in each test. The reference serum should be maintained as frozen aliquots in amounts sufficient for 1 week of tests. A positive serum control standard diluted to a potency of 0.5 IU/ml and a negative serum control standard with a potency of <0.1 IU/ml should also be prepared by the laboratory and included in each test.

2.2.6. Test sera

Serum samples should be heated at 56°C for 30 minutes before testing in order to inactivate complement. If sera are frozen, they should be reheated after thawing. Five-fold serial dilutions of test sera may be prepared directly in an eight-well tissue-culture chamber slide or in a 96 well plate and transferred to the chamber slide wells (final volume 0.1 ml/serial dilution). The RFFIT may be performed as a screen using 2 dilutions or as an endpoint test suing 4 dilutions. Screening dilutions of 1/5 and 1/50 are sufficient for routine evaluation of vaccination efficacy. Endpoint testing is generally performed using dilutions of 1/5, 1/25, 1/125, and 1/625.

For the screening test:

i) Prepare a 1/2.5 dilution by adding 0.1 ml of inactivated serum and 0.15 ml of EMEM-10 to one of the slides. Mix by gently rocking the slide.

ii) Transfer 0.05 ml of the 1/2.5 dilution to a second well containing 0.45 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/2.5 dilution.

iii) Mix the second well and discard all but 0.1 ml.

For the endpoint test:

i) Prepare a 1/2.5 dilution by adding 0.1 ml of inactivated serum and 0.15 ml of EMEM-10 to one of the slides. Mix by gently rocking the slide.

ii) Transfer 0.05 ml of the 1/2.5 dilution to a second well containing 0.2 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/2.5 dilution (first well).

iii) Transfer 0.05 ml of the 1/2.5 dilution to a third well containing 0.2 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/5 dilution (second well).

iv) Transfer 0.05 ml of the 1/2.5 dilution to a fourth well containing 0.2 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/25 dilution (third well).

v) Mix the fourth well containing the 1/125 dilution and discard all but 0.1 ml.

2.2.7. Addition of virus

i) Add 0.1 ml of the challenge virus preparation (working dilution) to all serum dilutions.

ii) Prepare a 1/10 and 1/100 back titration of the challenge virus working dilution and add 0.1 ml of each to a slide well.

iii) Mix and incubate at 37°C in a humidified incubator with 0.5% CO_2 for 90 minutes.

2.2.8. Addition of cells

i) During the incubation period, trypsinise a stock culture of 3- to 5-day-old MNA cells.

ii) Resuspend the cells in EMEM-10 to give a final concentration of 1×10^5 cells per 0.2 ml.

iii) Distribute 0.2 ml of the cell suspension into each well of the slide and incubate at 35°C in a humidified incubator with 0.5% CO_2 for a further 20 hours.

2.2.9. Acetone fixation and staining by immunofluorescence

i) After 20 hours, remove the slides from the incubator and pour off the medium into a virucidal solution.

ii) Rinse the slides once in PBS and then fix for 10 minutes at room temperature in cold acetone (–20°C). Note: if using plastic slides use 80% cold acetone.

iii) Leave the slides to dry for 10–30 minutes before adding FITC-conjugated anti-rabies antibody. The conjugate may be prepared in EMEM-10 or PBS; there is no need to adsorb the conjugate with tissue or cells. The working dilution of the conjugate should be determined by titration. The slides should be stained for 20–60 minutes at 37°C (optimal time determined by conjugate qualification) and then rinsed in PBS and distilled water, respectively.

iv) Observe the slides under a fluorescence microscope. Record the number of fields (out of 20 per well) where virus infection of cells is observed.

2.2.10. Calculation of virus-neutralising antibody titres

Residual virus is detected using a standard fluorescence microscope. The serum neutralisation end-point titre is defined as the dilution factor of the highest serum dilution at which 50% of the observed microscopic fields contain one or more infected cells (i.e. a 97% reduction in the virus inoculum). This value may be obtained by mathematical interpolation. Alternatively, a 100% neutralisation titre may be determined by recording the highest serum dilution at which 100% of the challenge inoculum is neutralised and there are no infected cells in any of the observed fields. For both titration methods, the titre of antibody in the test serum (in IU/ml) can be obtained by comparison with the titre of the recognised reference serum standard included in each test. It should be noted that it is also valid to perform the RFFIT using BHK-21 cells instead of neuroblastoma cells. A modified protocol for this has been published (WHO, 2018).

The following parameters have to be strictly adhered to:

i) RABV; only the CVS-11 strain should be used. Virus: CVS-11 (previously ATCC reference VR 959) strain, which is available from the ATCC or the WOAH Reference Laboratory for rabies, Nancy, France. Vials are stored at –80°C. The back titration should indicate a dose of 30–100 FFD_{50}.

ii) Cells cultures: only BHK-21 cells (ATCC number CCL10) or MNA cells (ATCC number CCL131) should be used.

iii) The test should be performed only on suitable chamber slides.

iv) Control charts should be used for RABV, naïve serum and positive standard dog serum.

v) The back titration of the CVS virus, as well as the naïve serum and positive standard dog, WOAH Reference Serum must be present on control plate.

vi) Reading method for the test: each chamber slide should contain 25–50 fields and be observed at ×160–200 magnification or 20 fields and be observed at ×100 magnification.

vii) Convert log D_{50} to IU/ml of test sera using the log D_{50} value of the recognised standard serum diluted to a potency of 2.0 IU/ml.

2.3. Enzyme-linked immunosorbent assay (ELISA)

ELISAs provide a rapid serological test that avoids the requirement to handle live RABV. Those tests detect antibodies that can specifically bind to RABV antigens, primarily the RABV glycoprotein and nucleoprotein. None of the available direct, indirect or competitive ELISAs is validated for international animal movement or trade (Wasniewski *et al.,* 2014). However, ELISAs are a useful tool for monitoring rabies vaccination campaigns in wildlife species provided they are properly validated for this purpose. A commercial ELISA has been recommended for monitoring rabies vaccination campaigns in foxes and raccoon dogs (Wasniewski *et al.,* 2016).

3. Quality assurance

Annual participation in inter-laboratory proficiency testing is highly encouraged as part of quality assurance schemes; such tests should be organised for Regional Laboratories by the National Reference Laboratories, while the latter in turn should participate in international proficiency tests organised by WOAH Reference Laboratories. Whenever possible, international accreditation of a laboratory should be considered (see Chapter 1.1.5 *Quality management and veterinary testing laboratories*).

C. REQUIREMENTS FOR VACCINES

1. General background

The prevention and control of rabies in a country is a national responsibility and, in many countries, the vaccine may be used only under the control of the Competent Authority. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements. Varying requirements relating to quality, safety and efficacy apply in particular countries or regions for manufacturers to obtain relevant regulatory approval for a veterinary vaccine. Where possible, manufacturers should seek to obtain such a licence or authorisation for their rabies vaccines as independent verification of the quality of their product. Internationally and nationally recognised regulations for animal experimentation should be followed in all stages of rabies vaccine development and production.

Virulent RABV may be used to produce inactivated rabies vaccine; consequently, the rabies vaccine production facility should operate under the appropriate biosafety procedures and practices. The facility should meet the requirements for containment outlined in chapter 1.1.4 and WHO (2005).

Rabies vaccines are defined as a standardised formulation containing defined amounts of immunogens. These immunogens are either inactivated (killed), live-attenuated or biotechnology-derived as described in chapter 1.1.8.

Authorised vaccines for the parenteral vaccination of domestic animals and oral vaccines for the immunisation of wild animals and free-roaming dogs are available. These vaccines are frequently used off-label.

Oral rabies vaccination (ORV) has been successfully used to control the disease in certain wildlife reservoir species (Cliquet *et al*., 2012; Freuling *et al.,* 2013). However, because dog-mediated human rabies is a candidate for global elimination, the dog should be considered a main target for rabies elimination; more than 99% of all human cases of rabies are caused by dogs. Countries should assess the need for both ORV of dogs and parenteral vaccination in their rabies control strategy. Parenteral vaccination of dogs should remain the foundation of mass vaccination campaigns. Apart from mass parenteral vaccination (carried out concurrently or sequentially), the use of oral vaccination, especially in free-roaming and inaccessible dogs, taking into account structure and accessibility of the dog population, should represent a complementary measure for the improvement of the overall vaccination coverage in dog rabies control programmes (WHO, 2013). For ORV of dogs, the handout and retrieve model should be used preferably unless the situation requires other means of distribution.

An optimal individual or combination vaccination strategy for both vaccination of wildlife (ORV with or without Trap-Vaccinate-Release) and dogs (Central Point Vaccination, House-to-House vaccination, with or without ORV) should

be determined by taking the size of the target species population into account. As regards ORV of wildlife and dogs, oral RABV vaccine bait candidates should be selected based on efficacy and safety profiles. Monitoring of human exposure to oral RABV vaccines and risk management should be undertaken. Under specific circumstances, vaccination of other susceptible companion animals and livestock would be beneficial and should be considered as part of any national vaccination programme.

2. Rabies vaccine for injectable use

2.1. Background

The principal rationale for the use of rabies vaccine is to protect animals and, as a consequence, humans. For injectable rabies vaccination in animals, inactivated virus (for companion animals and livestock), and recombinant vaccines (for cats) are used. As injectable live-attenuated vaccines have been documented to cause vaccine-induced rabies (Bellinger *et al*., 1983; Esh *et al*., 1982) their use should be discontinued.

Rabies virus glycoprotein biotechnology-derived vector vaccines are prepared by inserting non-infectious rabies virus nucleic acid coding for rabies virus glycoprotein into a vector such as avipox for injectable vaccine (WHO, 2018). Alternatively, non-replication competent constructs (e.g. virus like particles [VLPs], replicon vaccines, mRNA vaccines, plasmid vaccines or subunit vaccines etc.) or replication restricted rabies vaccine constructs (e.g. single cell cycle rabies vaccines), may be available for injectable use in the future (see Chapter 1.1.8). While the same efficacy requirements apply in principle to these vaccine constructs, additional safety considerations may need to be taken into account. As these vaccines do not contain live rabies virus, animals vaccinated with such vaccines should not be restricted from entry into countries (Taylor *et al*., 1991).

2.2. Outline of production and minimum requirements for conventional vaccines

2.2.1. Characteristics of the seed

i) Biological characteristics

Any RABV strain considered for vaccine production should protect against any RABV variant of phylogroup 1. Selection of master seed viruses (MSVs) should ideally be based on the ease of growth in culture, virus yield, stability and antigenic spectrum (Wu *et al*., 2011). A record of the source of the MSV should be maintained.

Biotechnology-derived vaccines are prepared in appropriate cell lines using a vector expressing the RABV glycoprotein.

ii) Quality criteria

MSVs for vaccine production must be well characterised and proven to be pure and free from all extraneous agents in accordance with Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and those listed by the appropriate regulatory authorities.

The efficacy of the resultant vaccine is assessed by studies on every target species to be vaccinated as recommended in chapter 1.1.8 and Section C.2.3.3 of this chapter.

2.2.2. Method of manufacture

i) Procedures

a) In cell culture

The virus is used to infect a suspension or monolayers of an established cell line. Such cell culture should be proven to be free from contaminating microorganisms (see chapter 1.1.8).

Cultures are infected with cell-culture-adapted MSV and incubated at the appropriate temperature for a defined period. As RABV does not normally cause cytopathic effect, this allows several harvests from the same culture. This material is processed and used to formulate vaccine. For inactivated (killed) vaccine the virus is inactivated by addition of an

inactivant of the first order, usually β-propiolactone (BPL) or ethyleneimine (EI) in the form of binary ethyleneimine (BEI). It is important that the necessary safety precautions for working with inactivants are fully observed. Other inactivants, such as formalin or phenic acid, should not be used. The inactivant is added to a virus suspension to achieve a predetermined final concentration. Inactivation must be duly validated and documented to show the inactivation kinetics and the results of the inactivation controls. The time period for inactivant treatment and temperature used for inactivation must be validated for the actual conditions and equipment used during industrial production.

Inactivated rabies vaccines are usually formulated as liquid or freeze dried. The liquid vaccine is prepared by adsorbing the antigen onto an adjuvant, for example aluminium hydroxide gel.

ii) Requirements for media and substrates

The final blend may include antifoam, phenol red dye (if permitted by the country requiring vaccine), lactalbumin hydrolysate, tryptose phosphate broth, amino acids, vitamins and buffer salts. Saponin or other polysaccharides as adjuvant could be incorporated in rabies vaccines for ruminants. Addition of preservatives is recommended for multi-dose vials. The freeze-dried vaccines should be reconstituted before injection with the appropriate solvent.

a) In cells

The cell lines used for the production of RABV vaccines should be in accordance with chapter 1.1.8.

b) In embryonated eggs

This method of culture is used for the production of live-attenuated vaccines such as the Flury LEP or the HEP variant strain. Their use should be discontinued as indicated in Section C.2.1 (Tao *et al*., 2010; Wachendörfer *et al*., 1982).

iii) In-process control

During the production process, tests are undertaken at different times before constitution of the final blend, which allows the consistency of production to be verified as in accordance with chapter 1.1.8. Tests for infectivity, sterility and inactivation are fundamental in-process controls. The formulation of the final product can be standardised using additional tests to measure viral integrity after storage, antigenic mass and glycoprotein content.

a) Inactivation test

Inactivation is verified using a test for residual live virus. For this, the inactivated harvest is inoculated into the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive. The quantity of inactivated virus harvest used is equivalent to not less than 25 doses of the vaccine. After incubation for 4 days, a subculture is made using trypsinised cells; after incubation for a further 4 days, the cultures are examined for residual live-RABV by the immunofluorescence test. The inactivated virus harvest complies if no live virus is detected (European Pharmacopoeia, 2021).

iv) Final product batch/serial tests

After combining all of the ingredients the final blend contains the definite vaccine formulation. Filling of the final blend into vials is the last step in the production process for a batch/serial. This final batch/serial undergoes the tests described below.

a) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

b) Safety

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for relevant regulatory approval.

Unless consistent safety of the product is demonstrated and approved in the registration dossier, and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8, batch safety testing is to be performed.

This final product batch/serial safety test is conducted to detect any abnormal local or systemic adverse reactions. For the purposes of batch/serial release, each of at least two healthy seronegative target animals is inoculated by the recommended route a minimum of a double dose of the vaccine. The animals are observed at least daily for 14 days. The vaccine complies with the test if no animal shows adverse reactions or dies of causes attributable to the vaccine (European Pharmacopoeia, 2021).

c) Residual live virus

The test is carried out using a pool of the contents of five containers.

For vaccines that do not contain an adjuvant, a suitable amplification test for residual live virus is carried out using the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive. The vaccine complies with the test if no live virus is detected.

For vaccines that contain an adjuvant, 0.03 ml of a pool of at least five times the smallest stated dose is injected intracerebrally into each of no fewer than ten mice, each weighing 11–15 g. To avoid interference of any microbial preservative or the adjuvant, the vaccine may be diluted more than 10 times before injection. In this case, or if the vaccine strain is pathogenic only for suckling mice, the test is carried out on 1- to 4-day-old mice. The animals are observed for 21 days. If more than two animals die during the first 48 hours, the test is repeated. The vaccine complies with the test if, from the day 3 to day 21 post-injection, the animals show no signs of rabies and immunofluorescence test carried out on the brains of the animals show no indication of the presence of RABV.

d) Batch/serial potency

For live attenuated and biotechnology-derived vaccines, virus titrations are reliable indicators of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in the target species and titres of the modified live vaccine. Virus titration should be carried out using cell culture techniques. This allows laboratories to act in accordance with the 3Rs principles (European Commission, 2010).

The potency of inactivated vaccines is tested in mice by a serological test (Krämer *et al*., 2010), or a challenge test (European Pharmacopoeia, 2021; WHO, 2018). For inactivated virus vaccines, an *in-vitro* agent identification test has been reported (Stokes *et al*., 2012).

It is not necessary to carry out the potency tests described in Section C.2.2.2.iv.d.1 Serological test, and Section C.2.2.2.iv.d.2 Challenge test, for each batch/serial of vaccine produced, provided that at least one of these tests has been carried out on a previous batch/serial of vaccine and this batch/serial has been demonstrated to meet the minimum potency requirements. Under these circumstances, an alternative validated method may be used to establish batch/serial potency, the criteria for acceptance being set with reference to the batch/serial of vaccine that has given satisfactory results in either the serological test or the challenge test as described below:

1) Serological test

In the serological test, the inactivated vaccine is compared with the standard reference vaccine by measuring the amounts of neutralising anti-RABV-specific antibodies in

mouse serum. The test vaccine passes if it induces more antibodies than the standard reference vaccine. The test should be performed as follows:

Eight to ten mice, each weighing 18–20 g are used. Each mouse is vaccinated by a subcutaneous, intramuscular or intraperitoneal route using 1/5 of the recommended dose volume. Blood samples are taken 14 days after the injection and the sera are tested individually for rabies antibodies (see Section B.2 and European Pharmacopoeia, 2021).

The vaccine meets the requirement if the rabies antibody titre of mice immunised with the test vaccine is significantly higher than that obtained with a reference vaccine that gave satisfactory results in the test described in C.2.2.2.iv.d.2 Challenge test.

2) Challenge test

In the challenge test, the test vaccine is compared with the reference vaccine by measuring the protection conferred on mice. The test vaccine passes if it induces more protection than the reference vaccine

According to the European Pharmacopoeia, the test described below uses a parallel-line model with at least 3 points for the vaccine to be examined and the reference preparation.

i) Selection and distribution of the test animals

Healthy female mice about 4 weeks old, preferably in a range of 18–20 g live weight and from the same stock should be used in the test. The mice should be distributed into at least ten groups of no fewer than ten mice.

ii) Preparation of the challenge suspension

A group of mice is inoculated intracerebrally with the CVS strain of RABV; when the mice show signs of rabies, they are killed, the brains are removed and a homogenate of the brain tissue is prepared in a suitable diluent. Gross particulate matter is separated by centrifugation and the supernatant is used as challenge suspension. The suspension is distributed in small volumes in ampoules that are sealed and stored at –80°C. One ampoule of the suspension is thawed and serial dilutions are made in a suitable diluent. Each dilution is allocated to a group of mice and each mouse is anaesthetised and injected intracerebrally with 0.03 ml of the dilution allocated to its group. The animals are observed at least daily for 14 days and the number in each group that develop signs of rabies between day 5 and day 14 is recorded. The median mouse intracerebral lethal dose ($MICLD_{50}$) of the undiluted suspension is calculated.

iii) Determination of potency of the vaccine to be examined

At least three serial dilutions of the vaccine are prepared for examination along with three similar dilutions of the reference preparation. The dilutions are prepared such that those containing the largest quantity of vaccine may be expected to protect more than 50% of the animals into which they are injected and those containing the smallest quantities of vaccine may be expected to protect less than 50% of the animals into which they are injected.

Each dilution is allocated to a different group of mice and each mouse is injected by the intraperitoneal route with 0.5 ml of the dilution allocated to its group. A suspension of the challenge virus is prepared 14 days after the injection such that, on the basis of the preliminary titration, it contains about 50 ID_{50} in each 0.03 ml. Each vaccinated mouse is injected intracerebrally with 0.03 ml of this suspension.

Three suitable serial dilutions of the challenge suspension are prepared. The challenge suspension and the three dilutions are allocated, one to each of four

groups of ten unvaccinated mice. Each mouse is anaesthetised and injected intracerebrally with 0.03 ml of the suspension of the dilution allocated to its group (Stokes *et al*., 2012). The animals in each group are observed at least daily for 14 days. The test is invalid if more than two mice of any group succumb within the first 4 days after challenge. The number in each group that develops signs of rabies between day 5 and day 14 after challenge is recorded.

The test is invalid unless:

a) For both the vaccine being examined and the reference preparation, the 50% protective dose lies between the smallest and the largest dose given to the mice;

b) The titration of the challenge suspension shows that 0.03 ml of the suspension contained at least 10 ID_{50};

c) The confidence limits (p = 0.95) are not less than 25% and not more than 400% of the estimated potency; when this validity criterion is not met, the lower limit of the estimated potency must be at least 1 IU in the smallest prescribed dose;

d) Statistical analysis shows a significant slope (p = 0.95) and no significant deviations from linearity or parallelism of the dose–response curves (p = 0.99).

The vaccine meets the WOAH requirement if the estimated potency is not less than 1 IU in the smallest prescribed dose.

iv) Application of humane end-points

Once a laboratory has established the above assay for routine use, the lethal end-point is replaced by an observation of clinical signs and the application of an end-point earlier than death to reduce animal suffering. The following scoring scheme is given as an example.

The progress of rabies infection in mice following intracerebral injection can be represented by five stages defined by typical clinical signs:

Score 1: ruffled fur, hunched back;

Score 2: slow movements, loss of alertness (circular movements may also occur);

Score 3: shaky movements, trembling, convulsions;

Score 4: signs of paresis or paralysis;

Score 5: moribund state.

Mice are observed at least twice daily from day 4 after challenge. Clinical signs are recorded at each observation. Experience has shown that using score 3 as an end-point yields assay results equivalent to those found when a lethal end-point is used. This must be verified by each laboratory by scoring a suitable number of assays using both clinical signs and the lethal end-point.

The potency test of the National Institute of Health (NIH test), as described in the US Code of Federal Regulations (9CFR), is similar to the European test, except that a second injection of vaccine is performed one week after the first injection. Reading and calculation are identical (European Pharmacopoeia, 2021; 9CFR, 2010).

2.3. Requirements for relevant regulatory approval

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.2.1 and C.2.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches/serials with a volume not less than 1/3 of the typical industrial batch/serial volume.

2.3.2. Safety requirements

Safety tests for registration of inactivated injectable rabies vaccine are identical to those described in Section C.2.2.2.iv.d.1 and need to be carried out in accordance with VICH Guideline 44, Section 2.1.2, as outlined here.

For vaccines that require a single life-time dose or primary vaccination series only, the primary vaccination regimen should be used. For vaccines that require a single dose or primary vaccination series followed by booster vaccination, the primary vaccination regimen and an additional dose should be used. For convenience, the recommended intervals between administrations may be shortened to an interval of at least 14 days. Evaluation of the one or repeat dose testing should be conducted using either a pilot or production batch containing the maximum release potency or, in the case where maximum release potency is not specified, then a justified multiple of the minimum release potency should be used.

In general, eight animals per group should be used unless otherwise justified. For each target species, the most sensitive class, age and sex proposed on the label should be used. Seronegative animals should be used. In cases where seronegative animals are not available, the use of alternatives should be justified.

If multiple routes and methods of administration are specified for the product concerned, administration by all routes is recommended. If one route of administration has been shown to cause the most severe effects, this single route may be selected as the only one for use in the study. Special attention shall be paid to the site of injection, especially for cats. Site recommendations should be followed.

Biotechnology-derived injectable vaccines do not shed virulent RABV, but other safety concerns may be evident (Roess *et al*., 2012). Specific requirements for safety of this type of vaccine are described in chapter 1.1.8 for biotechnology-derived vaccines.

Tests for reversion to virulence of modified live vaccines (MLV) should be done in accordance with chapter 1.1.8.

i) Precautions and hazards

For adjuvanted vaccines, live attenuated vaccines and biotechnology-derived vaccines, warnings should be provided by manufacturers that medical advice shall be sought in case of self-injection

2.3.3. Efficacy requirements

In herbivores, as a minimum requirement, efficacy can be demonstrated by serology (European Pharmacopoeia, 2021). In other species efficacy is demonstrated by challenge with an appropriate challenge RABV. Test animals shall be uniform and have no neutralising antibodies to rabies as determined by the serum neutralisation tests (see Section B of this chapter).

For challenge tests, challenge-dose finding studies are conducted to determine the dose and route that is sufficient to induce clinical signs of rabies in at least 80% of unvaccinated control animals. As soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using the diagnostic tests described in Section B of this chapter.

For efficacy tests in vaccinated animals, such as dogs, 25 or more animals shall be used as vaccinates. The vaccine formulation used for the efficacy trial is the minimum to be used for

routine production. Ten or more additional animals shall be added as controls. At the end of the period claimed for duration of immunity, vaccinates and controls are challenged with the predetermined dose as described above. Animals are observed at least daily for 90 days after challenge. As soon as clinical signs of rabies are observed, animals are humanely killed and rabies is confirmed using appropriate diagnostic tests. At the end of the observation period, all surviving animals are humanely killed and their brains are tested using the diagnostic tests described in Section B of this chapter.

Requirements for acceptance in challenge tests shall be death due to rabies in at least 80% of the control animals while at least 22 of 25 or 26 of 30 or a statistically equivalent number of the vaccinates remain free of rabies for a period of 90 days.

2.3.4. Stability

As described in chapter 1.1.8.

2.3.5. Duration of immunity

As part of the authorisation procedure the manufacturer should be required to demonstrate the duration of immunity of a given vaccine by either challenge or the use of a validated alternative test, such as serology at the end of the claimed period of protection.

3. Rabies vaccines for oral use

3.1. Background

All vaccines currently used for oral vaccination are either MLV or biotechnology-derived vaccines (BDVs). It should be noted that oral rabies vaccine constructs generated using reverse genetics are considered from a regulatory perspective as BDVs although they do not express a foreign gene. While there are numerous oral rabies vaccines for wildlife available (Müller & Freuling, 2020), they are perhaps the most underused of all tools in the fight against dog-mediated rabies (Cliquet *et al*., 2018; Wallace *et al*., 2020).

Of paramount consideration for oral vaccine use is safety, not only for the target animals, but for the environment and other species, including humans, who may come in contact with the vaccine (see chapter 1.1.8). Some of the MLVs have been documented to cause vaccine-derived rabies in target and non-target species (Fehlner-Gardiner *et al.,* 2008, Müller *et al.,* 2009, Hostnik *et al.* 2014, Pfaff *et al.*, 2018).

Requirements for guaranteeing the safety and efficacy of oral vaccines both for the target species and non-target species (especially humans) that might be in contact with baits or a recently vaccinated animal have been developed (European Pharmacopoeia, 2020; WHO, 2007; 2013).

As well as the requirements for oral RABV vaccines as described below, for ORV of wildlife and dogs, appropriate bait configuration and bait delivery systems are also critical and may require adaptation to local circumstances. It may be necessary to reassess efficacy with each significant variation of baits and bait delivery systems.

3.2. Outline of production and minimum requirement for vaccines

In addition to the requirements outlined in chapter 1.1.8, the following specific requirements must be met.

3.2.1. Characteristics of the seed

The seed is a pure preparation of a single immunogenic clonal strain of a highly attenuated MLV or BDV. The history of the MSV, its immunogenic properties, safety and absence of reversion to virulence shall be well characterised, including the presence of genetic markers for MLV. A full genome consensus sequence of the MSV should be submitted to the regulatory authority and deposited in a public database for verification of identity and genetic stability. For biotechnology-derived MSV, additional information on recombination should be considered, as a theoretical risk exists for the potential of genetic transfer and exchange with other viruses.

3.2.2. Method of manufacture

i) Procedure

The MSV is used to infect a suspension or monolayers of an established cell line. Ideally, these cell cultures should be non-tumorigenic and free from contaminating microorganisms.

ii) In-process control

During the production process, tests are undertaken at different times before constitution of the final blend, which allow the consistency of production to be verified. Tests for infectivity and sterility are fundamental in process controls.

iii) Final product batch/serial tests

After combining all of the ingredients the final blend contains the definitive formulation that is either used in a freeze-dried or in liquid form. Filling the final blend into sachets/capsules to be included in baits or filling directly into the bait is the last step of production of a batch/serial. This final batch/serial undergoes the tests described below.

a) Sterility

This test may be done before or after filling the bait. Tests for sterility and freedom from contamination of biological materials intended for veterinary use are described in chapter 1.1.9.

b) Identity

The identity of the immunogen is tested using rabies anti-serum monospecific for the glycoprotein G for BDV, and for MLV a test is carried out to demonstrate the presence of the genetic marker.

c) Batch/serial purity

For MLV, 1 in 10 and 1 in 1000 dilutions of the vaccine are inoculated into susceptible cell cultures. The dilutions are incubated at 37°C. After 2, 4 and 6 days, the cells are stained with a panel of monoclonal antibodies that do not react with the vaccine strain but that react with other strains of RABV (for example, street virus, Pasteur strain). Alternatively, genetic characterisation can be used. The vaccine complies with the test if it shows no evidence of contaminating RABV (European Pharmacopoeia, 2021).

d) Safety

Safety tests in target animals are not required by many regulatory authorities for the release of each batch or serial. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for relevant regulatory approval.

Unless consistent safety of the product is demonstrated and approved in the registration dossier, and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8, batch safety testing is to be performed as follows: two healthy seronegative animals of the target species are administered orally with 10 times the field concentration. In addition, a 0.5 ml dose is injected by the intraperitoneal or subcutaneous routes into eight mice. All animals are then observed for at least 14 days. If any intolerable adverse reactions attributable to the products occur in any animals during the observation period, the batch/serial is unsatisfactory.

e) Batch/serial potency

For MLV and BDV, virus titrations are reliable indicators of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in the target species and titres of the vaccine. Virus titration should be carried out using cell culture. This allows laboratories to act in accordance with the 3Rs principles (European Commission, 2010).

3.3. Requirements for relevant regulatory approval

3.3.1. Manufacturing process

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the Competent Authority. This information shall be provided from three consecutive vaccine batch/serials with a volume not less than 1/3 of the typical industrial batch/serial volume.

The in-process controls are part of the manufacturing process.

3.3.2. Safety requirements

In accordance with chapter 1.1.8, safety tests are required in each species for which the product is indicated. For purposes of this class of product, only the overdose and reversion-to-virulence safety tests are required.

Tests for reversion to virulence of MLVs and safety testing of BDV should be done in accordance with chapter 1.1.8.

i) Modified live vaccines (MLV)

a) In target species

For the overdose safety test, a 10 × maximum titre of a field dose is administered, preferably using a syringe, via the oral route to ten animals (less than 6 months of age for wild animals and less than 10 weeks for dogs), that are free of rabies antibodies. After administration, the possibility of excretion of vaccine virus in the saliva of the animals described above should be assessed by taking swabs several times within the first day and on several additional selected time points within the first week after vaccine administration. Any virus recovered should be characterised. The animals are observed for at least 90 days. Particular attention shall be paid to neurological signs and sudden death and shall be investigated using appropriate tests (see Section B of this chapter). At the termination of the study, the brain should be examined for vaccine virus presence using reference tests as described in Section B.1.3.1.

The test is satisfactory if no intolerable adverse reactions attributable to the vaccine are observed and if no virus is detected in the brain. Virus recovered in swabs should be the vaccine strain and be consistent temporally and quantitatively with limited viral replication.

b) In non-target species

A representative group of species including rodents, cats and dogs that are likely to consume the baits should be investigated. At least ten animals of each species should be tested orally with 10 × maximum titre of a field dose and observed for at least 90 days.

As testing wild animals might prove to be difficult and should be kept at a minimum, additional tests using different routes of administration in laboratory rodents (both nude and SCID mice or other immunocompromised animals) are recommended. Rodents (i.e. mice and rats) should be tested using at least 20 animals per test, inoculated orally with the amount of vaccine strain equivalent to one maximum oral dose. The same number of contact animals should be used for investigation of virus transmission. All animals should be observed daily for at least 30 days. Animals that die from causes not attributable to rabies are eliminated. After termination of the study, the brain of animals should be examined for RABV antigen using reference tests as described in Section B.1.3.1.

A risk assessment should be undertaken to evaluate directly the safety risk for humans (safety of vaccine) and the risk that humans will come in contact with the vaccine.

ii) Biotechnology-derived vaccines (BDV)

a) In target species

For the overdose safety test, a 10× maximum titre is administered, preferably using a syringe, via the oral route to ten animals that are free of rabies antibodies. After administration, the possibility of excretion of vaccine virus in the saliva of these animals should be assessed by taking swabs on the first day and on several additional selected time points within the first week after vaccine administration. Any virus recovered should be characterised. The animals should be observed for at least 90 days or according to their known incubation period for the vector used.

The test is satisfactory if no intolerable adverse reactions attributable to the vaccine are observed. Viral RNA and virus recovered from swabs should be the vaccine strain and be consistent temporally and quantitatively with limited viral replication. For vaccine intended for use in dogs, absence of the virus should be demonstrated 4 days post-immunisation.

b) In non-target species

A representative group of species including rodents, cats and dogs that are susceptible to the virus vector and likely to consume the baits should be investigated. At least ten animals of each species should be tested orally with a 10 × maximum titre and observed for at least 90 days or according to their known incubation period for the vector used.

As testing wild animals might prove to be difficult and should be kept to a minimum, additional tests in laboratory animals susceptible to the vector are recommended. Laboratory animals should be tested using at least 20 animals per test, inoculated orally with the amount of vaccine strain equivalent to one maximum oral dose. The same number of contact animals should be used for investigation of virus transmission. All animals should be observed daily for at least 30 days. Animals that die from causes not attributable to the disease caused by the vector are eliminated.

A risk assessment should be undertaken to evaluate directly the safety risk for humans (safety of vaccine) and the risk that humans will come in contact with the vaccine.

iii) Precautions hazards

The release of oral vaccines into the environment shall comply with the requirements in chapter 1.1.8. Oral rabies vaccines are innocuous when presented in bait form and present no toxic hazard to vaccinators. For leaks from ruptured sachets containing vaccines, warnings shall be provided by manufacturers that medical advice shall be sought in the event of inadvertent contact, especially when contact is with mucosal membranes, skin or skin abrasions.

Prior to initiating vaccination campaigns, public health officials should be informed and public education provided, particularly not to touch baits or be in contact with animals that have recently consumed baits.

Public health information with respect to the risk of oral vaccines in specific human population groups is provided by WHO (2005).

3.3.3. Efficacy requirements

Efficacy of the final product (vaccine bait) (see Section C.3.3.5) shall be demonstrated in each species for which the vaccine use is claimed by the manufacturer. The protection status cannot be checked by serology only; a virulent challenge with an appropriate challenge RABV is necessary. Preferably, a target species adapted RABV strain should be used. The vaccine titre should not be greater than the claimed minimum protective dose.

Test animals at least three months of age should have no rabies specific antibodies to rabies as determined by the serum neutralisation tests (see Section B of this chapter).

For challenge tests, challenge-dose finding studies should be conducted to determine the dose and route that is sufficient to induce clinical signs of rabies in at least 80% of unvaccinated control animals for each target species. As soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using the diagnostic tests described in Section B of this chapter.

For efficacy tests in vaccinated animals, at least 25 animals shall be used as vaccinates. The titre of the vaccine virus that is used in the efficacy test establishes the minimum immunising dose. At 180 days after presentation of a vaccine-bait, vaccinates and controls are challenged with the predetermined dose as described above. Animals need to be observed daily for 90 days after challenge and humanely euthanised at the first definitive clinical signs of rabies. The diagnosis of rabies must be confirmed in animals that die or have to be euthanised using appropriate diagnostic tests. At the end of the observation period, all surviving animals are humanely killed and their brain tissues tested using the virus identification tests described in Section B of this chapter.

Requirements for acceptance in challenge tests shall be death due to rabies in at least 80% of the control animals while at least 22 of 25, 26 of 30 or a statistically equivalent number of the vaccinates remain free of rabies for a period of 90 days.

Once the minimum immunising dose has been established in one species, the efficacy study for additional species can be limited to a study using vaccine-baits. The bait casing may have to be adapted to the new target species (see Section C.3.3.5).

3.3.4. Stability

A minimum of five samples of the final product are incubated at 25°C for 5 days. The vaccine is titrated three times. The mean virus titre must be at least the minimum virus titre stated on the label or as approved for end of shelf life. The bait is heated at 40°C for 1 hour, and the bait casing complies with the test if it remains in its original shape and adheres to the vaccine container (European Pharmacopoeia, 2020).

3.3.5. Bait requirements and characteristics

The bait is an integral part of the product and should ideally meet certain criteria:

i) Designed for and attractive to the target species and adapted to the mode of distribution.

ii) Adapted to the food preferences of the local dog population for ORV;

iii) The attractant should be compatible with bait and vaccine and adherent to the bait, and should remain palatable for a defined period;

iv) Keep its form and shape under a wide range of temperature and weather conditions to protect the vaccine under field conditions;

v) The shape of the bait should allow easy ingestion by all ages and sizes of target species;

vi) Optimise the release of vaccine into the oral cavity and to the target tissues;

vii) Be safe for target and non-target species;

viii) Ingredients should not be harmful, should comply with animal feed standards and should not interfere with vaccine activity;

ix) Allow the incorporation of a biomarker, topical or systemic (for example:

  a) surface markers (Rhodamine B, other dyes);

  b) tissue markers (Iophenoxic acid, etc.);

  c) calciphilic markers such as tetracycline (TC), which should be compatible with other bait components, safe for target and non-target species, detectable in target species for a defined period using technically simple, economical and locally available assay methods, absent or minimally present in subject population. In case of TC, care must be taken that the ratio of TC vs epitetracycline in the final bait formulation should be as high as possible to guarantee biomarker effectiveness.

x) Be economic to produce in standard form, possibly under local conditions;

xi) Feature a labelling system with a public warning and identification of the product.

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NB: There are WOAH Reference Laboratories for rabies (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3**).**
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for rabies

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.1.19.

RIFT VALLEY FEVER
(INFECTION WITH RIFT VALLEY FEVER VIRUS)

SUMMARY

Description and importance of the disease: *Rift Valley fever (RVF) is a peracute or acute zoonotic disease of domestic ruminants. The virus is confined to the African continent, some Indian Ocean islands including Madagascar and the Arabian Peninsula. For current information on distribution consult WAHIS[1]. It is caused by a single serotype of a mosquito-borne virus of the* Phenuiviridae *family (genus* Phlebovirus*). The disease occurs in climatic conditions favouring the breeding of mosquito vectors and is characterised by abortion, neonatal mortality and liver damage. The disease is most severe in sheep, goats, and cattle. Older non-pregnant animals, although susceptible to infection, are more resistant to clinical disease. There is considerable variation in the susceptibility to RVF of animals of different species. Camels, initially described as a low susceptibility species with inapparent infection, are now considered a fully susceptible species with sudden deaths, neonatal mortality and abortion rates that can be as high as in cattle.*

Humans are susceptible to RVFV and are mainly infected through contact with infected animal material (body fluids or tissues) or through bites from infected mosquitoes. RVFV has also caused serious infections in laboratory workers and must be handled with stringent biosafety and biocontainment measures using correct personal protective equipment.

Detection and identification of the agent: *RVFV consists of a single serotype of* Phlebovirus *that has morphological and physicochemical properties typical of this genus.*

Identification of RVFV can be achieved by virus isolation, antigen-detection enzyme-linked immunosorbent assay (ELISA) or immunohistochemistry. Viral RNA can be detected by reverse-transcription polymerase chain reaction.

The virus can be isolated from plasma/serum/blood during the febrile stage of the disease, or from organs (e.g. liver, spleen and brain tissues) of animals that have died and from the organs of aborted fetuses. Primary isolations are usually made on cell cultures of various types, such as African green monkey kidney (Vero) and baby hamster kidney (BHK) cells. .

Serological tests: *Identification of specific antibodies is mostly achieved by ELISA or the virus neutralisation test.*

Requirements for vaccines: *Live attenuated or inactivated vaccines can be used in countries where RVF is endemic or that are at risk of its introduction. These vaccines should preferably be prepared from attenuated strains of RVFV grown in cell culture.*

In RVF-free countries, vaccines and diagnostic tests should preferably be limited to those using inactivated virus. Work with live virus should be performed by trained personnel in biocontainment facilities following appropriate biosafety procedures.

There are two WOAH Reference Laboratories for RVF[2].

1 https://wahis.woah.org/#/home
2 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

A. INTRODUCTION

Rift Valley fever (RVF) is a peracute or acute, febrile, mosquito-borne, zoonotic disease caused by a virus of the order *Bunyavirales*, family *Phenuiviridae*, genus *Phlebovirus*. It is usually present in an epizootic form over large areas of a country following heavy rains and flooding, and is characterised by high rates of abortion and neonatal mortality, primarily in sheep, goats, cattle and camels. The susceptibility of different species and breeds to RVF may vary considerably. Some animals may have inapparent infections, while others have severe clinical disease with mortality and abortion. Susceptible, older non-pregnant animals often do not show signs of disease.

Signs of this arboviral disease tend to be nonspecific, rendering it difficult to recognise individual cases during epidemics (Swanepoel & Coetzer, 1994). However, the occurrence of numerous abortions and mortalities among young animals, together with clinical signs of the disease in humans, and in relation to climate conditions is characteristic of RVF. RVF has a short incubation period of about 12–36 hours in lambs. A biphasic fever of up to 41°C may develop, and the body temperature remains elevated until shortly before death. Affected animals are listless, disinclined to move or feed, and may show enlargement of superficial lymph nodes and evidence of abdominal pain. Lambs rarely survive longer than 36 hours after the onset of signs of illness. Animals older than 2 weeks may die peracutely, acutely or may recover or develop an inapparent infection. Some animals may regurgitate ingesta and may show melaena or bloody, foul-smelling diarrhoea and bloodstained mucopurulent nasal discharge. Icterus may sometimes be observed, particularly in cattle. In addition to these signs, adult cattle may show lachrymation, salivation and dysgalactia. In pregnant sheep, the mortality and abortion rates vary from 5% to almost 100% in different outbreaks and between different flocks. The death rate in cattle is usually less than 10%. Camels have been regularly involved in the RVF epidemics in East Africa, Egypt and more recently Mauritania. Clinical presentations such as sudden deaths, abortion and some early post-natal deaths have been observed in camels. Differential diagnosis includes: bluetongue, Wesselsbron disease, enterotoxemia of sheep, ephemeral fever, brucellosis, vibriosis, trichomonosis, Nairobi sheep disease, heartwater, ovine enzootic abortion, toxic plants, bacterial septicaemias, peste des petits ruminants, anthrax and Schmallenberg disease.

The hepatic lesions of RVF are very similar in all species, varying mainly with the age of the infected individual (Swanepoel & Coetzer, 1994). The most severe lesion, occurring in aborted fetuses and newborn lambs, is a moderately to greatly enlarged, soft, friable liver with a yellowish-brown to dark reddish-brown colour with irregular congested patches. Numerous greyish-white necrotic foci are invariably present in the parenchyma, but may not be clearly discernible. In adult sheep, the lesions are less severe and pinpoint reddish to greyish-white necrotic foci are distributed throughout the parenchyma. Haemorrhage and oedema of the wall of the gallbladder are common. Hepatic lesions in lambs are almost invariably accompanied by numerous small haemorrhages in the mucosa of the abomasum. The contents of the small intestine and abomasum can be dark chocolate-brown as a result of the presence of partially digested blood. In all animals, the spleen and peripheral lymph nodes can be enlarged, oedematous and may have petechiae.

Microscopically, hepatic necrosis is the most obvious lesion of RVF in both animals and humans. In fetuses and neonates of cattle and sheep, foci of necrosis consist of dense aggregates of cellular and nuclear debris, some fibrin and a few inflammatory cells. There is a severe lytic necrosis of most hepatocytes and the normal architecture of the liver is lost. In about 50% of affected livers, intranuclear inclusion bodies that are eosinophilic and oval or rod-shaped are found. Mineralisation of necrotic hepatocytes is also seen. In adult animals, hepatic necrosis is less diffuse, and in sheep, icterus is more common than in lambs (Swanepoel & Coetzer, 1994).

In humans, RVF infections are usually inapparent or associated with a moderate to severe, nonfatal, influenza-like illness (Madani *et al.*, 2003). A minority of patients may develop retinal lesions, encephalitis, or severe hepatic disease with haemorrhagic manifestations, which is generally fatal. RVF virus (RVFV) has caused serious human infections in laboratory workers. Staff should work under strict biosafety and biocontainment environments where they use correct personal protective equipment to protect themselves against possible infection. An inactivated vaccine has been developed for human use. However, this vaccine is not licensed nor commercially available. It has been used experimentally to protect veterinary and laboratory personnel at high risk of exposure to RVF. Further information about the disease and vaccination in humans is available from WHO[3]. RVFV should be handled at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Particular care needs to be exercised when working with infected animals or when performing post-mortem examinations.

3 http://www.who.int/mediacentre/factsheets/fs207/en/

RVFV consists of a single serotype of the *Phenuiviridae* family (genus *Phlebovirus*) and has morphological and physicochemical properties typical of bunyaviruses. The virus is enveloped, spherical and 80–120 nm in diameter. Glycoprotein spikes project through a bilayered lipid envelope. The virus is readily inactivated by lipid solvents and acid conditions below pH 6. RVFV has a segmented, single-stranded, negative-sense RNA genome and consists of the following segments: L (large), M (medium) and S (small), each of which is contained in a separate nucleocapsid within the virion. The S segment is ambisense, i.e. has bi-directional coding (Bird *et al.,* 2007).

No significant antigenic differences have been demonstrated between RVFV isolates and laboratory-passaged strains from many countries, but differences in pathogenicity among virus genetic lineages have been shown (Bird *et al.*, 2007b).

RVFV is endemic in many African countries and may involve several countries in the region at the same time or progressively, expand geographically over the course of a few years. In addition to Africa, large outbreaks have been observed in the Arabian Peninsula and some Indian Ocean Islands. These generally, but not exclusively, follow the periodic cycles of unusually heavy rainfall, which may occur at intervals of several years, or the flooding of wide areas favouring the proliferation of mosquitoes.

Rainfall facilitates mosquito eggs to hatch. *Aedes* mosquitoes acquire the virus from feeding on infected animals, and may potentially vertically transmit the virus, so that new generations of infected mosquitoes may hatch from their eggs (Bergren *et al.,* 2021). This provides a potential mechanism for maintaining the virus in nature, as the eggs of these mosquitoes may survive for periods of up to several years in dry conditions. Once livestock is infected, a wide variety of mosquito species may act as the vector for transmission of RVFV and can spread the disease.

Low level RVF activity may take place during inter-epizootic periods. RVF should be suspected when exceptional flooding and subsequent abundant mosquito populations are followed by the occurrence of abortions, together with fatal disease marked by necrosis and haemorrhages in the liver that particularly affect newborn lambs, kids and calves, potentially concurrent with the occurrence of an influenza-like illness in farm workers and people handling raw meat. Suspected cases should be confirmed with a diagnostic test.

During a suspected outbreak of RVF, preventive measures to protect workers from infection should be employed when animals or animal products potentially infected with RVFV are to be handled.

B. DIAGNOSTIC TECHNIQUES

The collection of specimens and their transport should comply with the recommendations in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials* of this *Terrestrial Manual*.

Proper diagnosis should always use a combination of techniques based on history, the purpose of the testing and the stage of the suspected infection and available samples. For a definitive interpretation, combined epidemiological, clinical and laboratory information should be evaluated carefully.

All the test methods described below should be validated in each of the laboratories using them (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial diseases*). The WOAH Reference Laboratories for RVF should be contacted for technical support. Table 1 provides a general guidance summary on the use of the diagnostic test methods. More detailed aspects are addressed in the test descriptions that follow.

Table 1. Test methods available for diagnosis of Rift Valley fever and their purposes

Method	Purpose					
	Population freedom from infection (unvaccinated animals)	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases[(a)]	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[(b)]						
Virus isolation in cell culture	–	–	–	+++	+	–
RT-PCR	–	++	–	+++	+	–
Antigen detection	–	++	+	++	+	–
Histopathology with immuno-histochemistry	–	–	–	++	–	–
Detection of the agent-specific immune response						
ELISA	+++	++	+++	++	+++	+++
VNT/PRNT	+++	+++	+++	++	++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction;
ELISA = enzyme-linked immunosorbent assay; VNT= virus neutralisation test; PRNT = plaque reduction neutralisation test.
[(a)]Laboratory confirmation of clinical cases should require a combination of at least two positive results from two different diagnostic test methods: either positive for virus or viral RNA and antibodies or positive for IgM and IgG with demonstration of rising titres between paired sera samples collected 2–4 weeks apart. Depending on the stage of the disease, virus or antibodies will be detected.
[(b)]A combination of agent detection methods applied on the same clinical sample is recommended.

1. Detection and identification of the agent

RVFV may be isolated from serum but preferentially from plasma or blood collected with anticoagulant during the febrile stage of the disease in live animals, or from liver, spleen and brain of animals that have died, or from aborted fetuses. Primary isolation is usually performed in cell cultures of various types.

1.1. Specimen collection

Using appropriate protective equipment to ensure biosafety of the staff, approximately 5 ml of blood with anticoagulant (preferably ethylene diamine tetra-acetic acid [EDTA]) collected during the febrile stage of the disease, or approximately 1 cm^3 of liver, spleen, brain or abortion products collected post-mortem, should be submitted for virus isolation. The samples should be kept at 0–4°C during transit. If transport to the laboratory is likely to take more than 24 hours, the samples should be frozen and sent on dry ice or frozen cold pack. In the case of a blood sample, plasma should be collected and frozen for transport.

1.2. Isolation in cell culture

A variety of cell line monolayers, including African green monkey kidney (Vero), baby hamster kidney (BHK) and AP61 mosquito cell lines (Digoutte *et al.*, 1989), may be used. They are inoculated with 1/10 dilution of the sample and incubated at 37°C for 1 hour (with mosquito cell lines, the incubation should be done at 27°C for 1 hour). It is advisable to also inoculate some cultures with a further 1/100 dilution of the inoculum. This is to avoid the production of defective particles, which follows the use of very high titre virus inoculum. The inoculum is removed and the monolayer is washed with phosphate-buffered saline (PBS) or culture medium. The wash solution is removed, replaced with fresh culture medium and cells

incubated at the appropriate temperature. The cultures are observed for 5–6 days. Mammalian cell lines are preferably used as RVFV induces a consistent cytopathic effect (CPE) characterised by slight rounding of cells followed by destruction of the whole cell monolayer within 12–48 hours. Confirmation of virus isolation should be performed preferably by using immunostaining or reverse-transcription polymerase chain reaction (RT-PCR).

1.3. Reverse-transcription polymerase chain reaction

A rapid diagnosis can also be made by detection of viral RNA (Sall *et al.*, 2001) using validated conventional or real-time RT-PCR (Drosten *et al.*, 2002; Sall *et al.*, 2001). These techniques have been very useful during RVF outbreaks. They may also be used to detect RVFV RNA in mosquito pools (LaBeaud *et al.*, 2011).

Below are proposed protocols for conventional and real-time RT-PCR. For information on specific procedures consult the WOAH Reference Laboratories.

1.3.1. Agarose gel-based RT-PCR assay

This procedure is used by some of the WOAH Reference Laboratories. The RT-PCR assay consists of the four successive steps of (a) extraction of template RNA from the test or control sample followed by (b) RT of the extracted RNA, (c) PCR amplification of the RT product and (d) detection of the PCR products with agarose gel electrophoresis.

i) Test procedure

RNA is extracted using an appropriate chemical or magnetic particle method according to the procedure recommended by the manufacturer of the commercial kit routinely used in the laboratory. When the procedure is completed, retain the extracted RNA samples on ice if the RT step is about to be performed. Otherwise store the RNA sample at –70°C. For RT-PCR, the nested RT-PCR protocol using two steps from Sall *et al*. (2001) is used. For the first RT-PCR step, NSca (5-'CCT-TAA-CCT-CTA-ATC-AAC-3') and NSng (5'-TA-TCA-TGG-ATT-ACT-TTC-C-3') primers are used. Details of reagent volumes and cycling times are given for guidance, but may need to be adapted according to the manufacturer's recommendations.

a) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (22.75 µl); RT-PCR reaction buffer, 5× conc (10 µl); $MgCl_2$, 25 mM (1 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); primer NSca, 10 µM (2.5 µl); primer NSng 10 µM (2.5 µl): reverse transcriptase/Taq polymerase enzyme mix, 5 units/µl (0.25 µl).

b) Add 40 µl of PCR reaction mix to a well of a PCR plate or to a microcentifuge tube for each sample followed by 10 µl of the RNA (prepared in step i) to give a final reaction volume of 50 µl.

c) Centrifuge the plate or tubes for 1 minute in a suitable centrifuge to mix the contents of each well.

d) Place the plate in a thermal cycler for PCR amplification and run the following programme:

45°C for 30 minutes: 1 cycle;

95°C for 2 minutes: 1 cycle;

94°C for 30 seconds, 44°C for 30 seconds, 72°C for 1 minute: 40 cycles;

72°C for 5 minutes: 1 cycle.

e) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of loading dye solution and load onto a 1.2% agarose gel containing a highly sensitive stain for visualisation of the amplicon in agarose. Also load a DNA ladder for assistance with estimation of the size of the product. After electrophoresis, a positive result is indicated by the presence of a 810 bp band (242 bp band for the strain Clone 13) corresponding to RVFV sequence in the NSs coding region of the S segment of the RVFV genome.

For the nested RT-PCR step, NS3a (5'-ATG-CTG-GGA-AGT-GAT-GAG-CG-3') and NS2g (5'-GAT-TTG-CAG-AGT-GGT-CGT-C-3') are used.

f) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (36.5 µl); RT-PCR reaction buffer, 10× conc (5 µl); $MgCl_2$, 25 mM (1.25 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); primer NS3a (5'-ATG-CTG-GGA-AGT-GAT-GAG-CG-3'), 10 µM (2.5 µl); primer NS2g (5'-GAT-TTG-CAG-AGT-GGT-CGT-C-3'), 10 µM (2.5 µl): reverse transcriptase/Taq polymerase enzyme mix, 5 units/µl (0.25 µl).

g) Add 49 µl of PCR reaction mix to a well of a PCR plate or to a microcentifuge tube for each sample followed by 1 µl of the amplicon obtained from RT-PCR reaction with NSca and NSng to give a final reaction volume of 50 µl.

h) Centrifuge the plate or tubes for 1 minute in a suitable centrifuge to mix the contents of each well.

i) Place the plate in a thermal cycler for PCR amplification and run the following programme:

95°C for 2 minutes: 1 cycle;

94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute: 25 cycles;

72°C for 5 minutes: 1 cycle.

j) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of loading dye and load onto a 1.2% agarose gel containing a highly sensitive stain for visualisation of DNA in agarose. A DNA ladder should also be loaded for assistance with estimation of the size of the amplicon. After electrophoresis a positive result is indicated by the presence of a band of 668 bp (129 bp for Clone 13) corresponding to RVFV sequence in the NSs coding region of the S segment of the genome.

1.3.2. Real-time RT-PCR assay

The real-time RT-PCR assay can use the same procedures for extraction of total RNA from the test or control sample followed by RT of the extracted RNA as for the conventional procedure. The protocol is adapted from Drosten *et al*. (2002). If commercial kits are used, the manufacturers' method should be followed.

i) Test procedure

a) Prepare the PCR mix described below for each sample. Again it is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (2.8 µl); RT-PCR reaction master mix, 2× conc. (10 µl); real-time PCR forward primer **RVS**: 5'-AAA-GGA-ACA-ATG-GAC-TCT-GGT-CA-3', 10 µM (2 µl); real-time PCR reverse primer **RVAs**: 5'-CAC-TTC-TTA-CTA-CCA-TGT-CCT-CCA-AT-3', 10 µM (2 µl); **RVP**: **FAM** 5'-AAA-GCT-TTG-ATA-TCT-CTC-AGT-GCC-CCA-A-3' **TAMRA** 20 µM (0.2 µl).

b) Add 17 µl PCR reaction mix to a well of a real-time PCR plate for each sample followed by 3 µl of the prepared RNA to give a final reaction volume of 20 µl.

c) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.

d) Place the plate in a real-time PCR machine for PCR amplification and run the following programme:

45°C for 30 minutes: 1 cycle;

95°C for 5 minutes: 1 cycle;

95°C for 5 seconds, 57°C for 35 seconds: 45 cycles.

e) Interpreting the results: assign a threshold cycle (CT) value to each PCR reaction from the amplification plots (fluorescence signal versus cycle number); different cut-off values may be appropriate for different sample types. The CT values used to assign samples as either

RVFV positive or negative should be defined by individual laboratories using appropriate internal reference material.

There are alternative methods that target the S and L segments (Bird *et al.,* 2007a; Wilson *et al.,* 2013).

1.4. Antigen detection

RVFV antigen can be detected using ELISA (Madani *et al*., 2003; Munyua *et al*., 2010) or pen-side rapid diagnostic test (lateral flow device: LFD) (Cetre-Sossah *et al.,* 2019).

The antigen detection enzyme-linked immunosorbent assay (ELISA) is an immunocapture test. Samples are tested at different dilutions with appropriate positive and negative controls. This test has been used for human and animal samples during outbreaks in Saudi Arabia and Kenya (Madani *et al*., 2003; Munyua *et al*., 2010).

1.4.1. Antigen ELISA procedure

The controls and antisera used in the performance of this assay should have been treated by the manufacturer to inactivate any viable RVF viral particles. These products are safe, within the limits of the ability to detect viable replicative viral particles. The material to be tested for the presence of RVF viral antigen is potentially contaminated with viable RVFV or other agents for which a differential determination is being sought. Samples should be inactivated using appropriate detergent and heat inactivation (56°C for 1 hour in the presence of 0.5% Tween 20 [v/v]).

i) The principle is based on a double-antibody sandwich capture assay in which the antigen is captured by an antibody on a solid phase which in turn is detected by a second antibody. A third antibody coupled with a detection system using horseradish peroxidase (HRPO)–ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) is used to determine how much of the second antibody has been retained on the solid phase of the system.

a) Capture (coating) antibody (diluted 1/2000 in PBS [no Tween], pH 7.4 and coated overnight at 4°C; control wells are coated with a similar dilution of normal fluid)

Plates are coated with a specific anti-viral antibody (available in WOAH or WHO Reference Laboratories) capable of capturing viral antigen from the test sample. In this instance it is either a polyclonal hyperimmune mouse ascitic fluid (HMAF) or monoclonal antibodies specific for RVFV. Normal serum is added to rows to serve as controls used to determine the nonspecific background or noise of the system. Incubate for 1 hour at 37°C. Wash three times with PBS/0.1% Tween 20.

b) Suspect samples and control antigen (diluted 1/4 and then diluted four-fold successively down the plate)

Suspect samples and control antigen are added in serum diluent to allow specific viral antigens to bind to the capture antibody. The serum diluent (PBS, 0.01 M, pH 7.4, with or without thiomersal) contains 5% skim milk and 0.1% Tween 20 to reduce nonspecific binding. Incubate for 1 hour at 37°C. Wash three times with PBS/0.1% Tween 20.

c) RVFV-specific second antibody

An antibody, high titrated for specific viral antigen, is added to allow detection of the bound viral antigen. In this assay, it is an anti-RVFV hyperimmune rabbit serum (available in WOAH or WHO Reference Laboratories) that has a high titre against RVFV. Incubate for 1 hour at 37°C. Wash three times with PBS/0.1% Tween 20.

d) Detection antibody – anti-rabbit antibody conjugated to HRPO (commercial product)

This is used to detect the rabbit anti-RVFV antibody that binds to the antigen. Wash six times with PBS/0.1% Tween 20.

e) Add ABTS substrate/chromogen. Incubate for 30 minutes in the dark at 22–25°C. Hydrolysis of ABTS by HRPO causes green colour development in negative wells.

f) Stop reaction by adding sodium dodecyl sulphate (SDS) 1% in distilled water. Read plate at 405 nm.

g) Criteria for determining positives

A standard control antigen has been provided and will be run in a standard dilution series. This, in effect, provides a standard curve that will determine the limits of detection of the assay. A group of normal tissues or samples, uncontaminated with antigen, are tested to determine the background of the assay and the limit at which the standard was positive. The values of these normal controls are used to generate the mean and standard deviation of the random background to be expected with negative samples. A sample is considered positive if its optical density (OD) value exceeds the mean plus 3 standard deviations of these normal controls.

1.4.2. Pen-side rapid diagnostic test (lateral flow device)

A commercial LFD for RVF antigen is available for use in the field. It has been produced and validated by Cetre-Sossah *et al.* (2019). The test strips are designed using the principle of immune-chromatography lateral flow technology employing colloidal gold-labelled monoclonal antibodies (MAbs). Sera samples are taken from suspect animals and the supplied buffer is applied to one end of a chromatographic strip. The sample mixes with a capture RVF specific MAb that recognises any RVFV antigen present in the suspect sample. Buffer flow moves along the chromatographic strip. If the sample contains RVFV antigen, the capture MAb/antigen complex is then captured by the anti-RVFV detection MAb, which is gold labelled, making a coloured line to indicate a positive result. The test has been validated against RVFV isolates from different geographical origins with 98.81% diagnostic specificity and 100% sensitivity in laboratory testing. As expected the limit of detection of this LFD, which is a test of interest in the field as a first-line diagnosis, has been found to be lower than the one defined using RT-PCR. The test takes 20 minutes and requires no additional equipment but has to be performed using appropriate protective equipment to ensure biosafety of the staff. A first-line diagnosis made using LFD needs to be confirmed using confirmatory tests (e.g. RT-PCR, virus isolation).

1.5. Histopathology and immunohistochemistry

Histopathological examination of the liver, spleen, kidney and lung of affected animals will reveal characteristic cytopathology, and immunohistochemistry staining, especially of the liver and spleen, will allow specific identification of RVFV antigen in tissues (Odendaal *et al.,* 2018). This is an important diagnostic tool because liver or other tissue placed in neutral buffered formaldehyde in the field is inactivated (subject to specimen thickness and time of fixation) and does not require a cold chain, which facilitates handling and transport from remote areas.

2. Serological tests

Samples collected from animals for antibody testing may contain live virus and appropriate inactivation steps should be performed. A combination of heat and chemical inactivation may be necessary. Immunofluorescence assays are still used, although cross-reactions may occur between RVFV and other phleboviruses. Techniques such as the agar gel immunodiffusion (AGID), radioimmunoassays, haemagglutination inhibition (HI), and complement fixation are no longer used.

Several assays are available for detection of anti-RVFV antibodies in a variety of animal species. Currently the most widely used technique is the ELISA for the detection of IgM and IgG isotypes. Virus neutralisation tests (VNT), such as plaque reduction neutralisation test (PRNT), have been used to detect neutralising antibodies against RVFV in the serum of a variety of species as well. VNTs are the most specific diagnostic serological tests, but require the use of live virus and are therefore not recommended for use outside endemic areas or in laboratories without appropriate biosecurity facilities and vaccinated personnel. However, alternative neutralisation assays not requiring handling of highly virulent RVFV and not requiring high containment, are being developed and validated.

2.1. Enzyme-linked immunosorbent assay

The ELISA is a reliable and sensitive test to detect antibodies against RVFV. Both IgG and IgM ELISAs are available for most species. IgM-capture ELISA allows diagnosis of recent infections.

A number of ELISAs using different formats are commercially available and others are under development (Cetre-Sossah *et al.,* 2009; Munyua *et al*., 2010; Paweska *et al*., 2005; Williams *et al.,* 2011). They are used routinely in many countries for single case diagnosis, outbreak management, and surveillance. Examples of blocking, indirect and antibody capture ELISAs are given below:

2.1.1. Blocking IgG ELISA

This ELISA can be used in human and ruminant sera and plasma samples (Paweska *et al.,* 2005). The reagent dilutions stated in the test procedure below may need to be optimised by each laboratory conducting the test. Inactivation of RVFV is necessary for safety purposes. For washing, blocking and diluent buffers, PBS and 0.1% Tween 20, 10% skimmed milk powder in PBS, and 2% skimmed milk powder in PBS are used, respectively. Unless otherwise stated, volumes used are 100 µl/well, and all washes are performed three times for 15 seconds using 300 µl of wash buffer per well. Note: During step ii), sera and antigen are mixed in a separate plate or diluting tubes, not the ELISA plate.

i) Coat plates with 100 µl polyclonal sheep anti-RVF capture antibody diluted 1/500 in PBS and incubate plates covered with lids at 4°C overnight. Wash plates.

ii) Add 200 µl/well of blocking buffer and incubate for 1 hour in a humid chamber at 37°C. Wash the plates. During the blocking stage, add 21 µl of each undiluted test and control sera into diluting wells containing 189 µl virus or control antigen pre-diluted 1/10. (NOTE: These volumes are for testing samples in duplicate – for testing in singles, adjust the volumes accordingly.) For conjugate control use diluent buffer without any additives.

iii) Add 100 µl of test and control sera/virus antigen mixture to rows A–D 1–12 and 100 µl of test and control sera/control antigen mixture to rows E–H 1–12 and incubate for 1 hour in a humid chamber at 37°C. Wash plates.

iv) Add 100 µl/well of rabbit anti-virus rN diluted 1/2000 in diluent buffer and incubate for 1 hour in a humid chamber at 37°C. Wash plates.

v) Add 100 µl/well of anti-rabbit IgG HRPO-conjugate diluted 1/6000 in diluent buffer and incubate for 1 hour in moist chamber at 37°C. Wash plates six times.

vi) Add 100 µl/well of ABTS peroxidase substrate. Leave plates for 30 minutes at room temperature (22–25°C) in the dark. Add 100 µl/well of 1 × concentrated SDS stop solution and read optical density at 405 nm.

vii) A specific activity of each serum (net OD) is calculated by subtracting the nonspecific background OD in the wells with control antigen from the specific OD in wells with virus antigen. Results are expressed as percentage inhibition (PI) where the mean OD readings for replicate tests are converted to PI values using the equation: [100 – (mean net OD of test sample/mean net OD of negative control) × 100].

The indirect IgG (Paweska *et al.,* 2003) and IgM capture (Williams *et al.,* 2011) ELISAs mentioned below are for use in ruminant sera and plasma. Both assays use 10% non-fat milk/Tris salt Tween (NFM/TST) as blocking and dilution buffer, and TST buffer (50 mM Tris; 150 mM NaCl; 0.1% Tween 20) as washing buffer (pH 8.0). The reactions are stopped with 2 N H_2SO_4.

The recombinant nucleoprotein (rN) of RVFV is produced and purified as described by Williams *et al*. (2011). Conjugation of the protein to HRPO is performed following the Nakane & Akira Kawaoi (1974) protocol. The rN antigen is stable for up to 1 year at 4°C.

To prepare plates for immediate use, make a checkerboard titration of the capture antibody or antigen against the conjugate in a 96-well ELISA plate to determine the minimum reagent concentration that would give an OD value of 0.5–0.6 when read at 650 nm after an incubation period of 20 minutes. This

will determine how the antibody/antigen and conjugate must be diluted for coating of the plates and detection of the antigen/antibody binding in the test.

2.1.2. IgM capture ELISA

i) Coat each well of the 96-well ELISA plates with 100 µl of the capture antibody (affinity-purified rabbit anti-sheep IgM) diluted to 1 µg/ml in PBS (that is a 1/1000 dilution if so determined by the titration), and incubate overnight at room temperature in a humid chamber.

ii) Wash the plates three times with wash buffer.

iii) Block the plates with 300 µl blocking buffer and incubate for 1 hour at 37°C.

iv) Wash the plates again three times with wash buffer.

v) Dilute both control (positive and negative) and test sera 1/100 in blocking buffer and add each serum in a designated well at volumes of 100 µl /well.

vi) Incubate the plates for 1 hour at 37°C. Avoid drying by putting plates in a humid chamber.

vii) Following the incubation step, wash the ELISA plates with wash buffer three times.

viii) Dilute the rN-HRP conjugate 1/6000 and add 100 µl of this in each well. Use blocking buffer as the conjugate control.

ix) The plates are then incubated for 60 minutes at 37°C.

x) The plates are washed, as in step ii above. Ready-to-use tetramethyl benzidine (TMB) substrate at 100 µl quantities is then transferred to each well, and the plates allowed to stand at room temperature for 15–30 minutes, until development of a blue colour change, or OD values of 0.5 when the plates are read at 605 nm. Exposure to direct light should be avoided.

xi) Stop the reaction with 100 µl stop solution (0.16 M sulfuric acid), and read the OD values using an ELISA plate reader at 450 nm.

xii) Interpretation of results: results are expressed as percentage of the positive serum control (PP) using the formula:

$$(\text{mean OD of duplicate test serum})/(\text{mean OD of positive control}) \times 100$$

where positive and negative cut-off values are determined by receiver operating characteristic (ROC) curve analysis.

It should be noted that the cut-off value for an ELISA can be adjusted for different target populations as well as for different diagnostic purposes.

2.1.3. Indirect IgG ELISA

i) Coat each well of the 96-well ELISA plate with 100 µl of rN diluted in 50 mM of carbonate buffer (pH 9.6) using the dilution ratio determined by prior titration as explained above; incubate overnight at room temperature in a humid chamber.

ii) Wash the plates three times with approximately 300 µl wash buffer per well.

iii) Block the plates with approximately 300 µl blocking buffer and incubate for 1 hour at 37°C.

iv) Wash the plates again three times with nearly 300 µl of wash buffer per well.

v) Dilute both control (positive and negative) and test sera 1/100 in blocking buffer.

vi Add 100 µl of the diluted sera in designated wells in duplicate.

vii) Incubate the plates for 1 hour at 37°C. Avoid drying by putting plates in a humid chamber.

viii) Following the incubation step, wash the ELISA plates with wash buffer three times.

ix) Dilute protein G-HRP conjugate 1/32,000 in blocking buffer and add 100 µl of the conjugate in each well.

x) Incubate for 60 minutes at 37°C.

xi) The plates are washed, as in step ii above. Add 100 µl of ready to use TMB substrate to each well and allow the plates to stand at room temperature for a few minutes, while avoiding exposure to direct light. The plates are read at 605 nm to determine if OD of 0.4–0.6 has been reached.

xii) Stop the reaction with 100 µl stop solution (0.16 M sulfuric acid), and read the plates using ELISA plate reader at 450 nm.

xiii) Interpretation of results: results are expressed as percentage of the positive serum (PP) using the formula:

(mean OD of duplicate test serum)/(mean OD of positive control) × 100

where positive and negative cut-off values are determined by receiver operating characteristic (ROC) curve analysis.

It should be noted that the cut-off value for an ELISA can be adjusted for different target populations as well as for different diagnostic purposes.

2.2. Plaque reduction neutralisation test

The plaque reduction neutralisation test (PRNT) may be used to determine the presence of neutralising antibodies in naturally infected and vaccinated animals. It has to be run under high containment biosafety facilities by trained personnel. The test is highly specific and can be used to test serum of any species. It is generally used to measure vaccine efficacy. The highly attenuated Smithburn neurotropic mouse brain strain of RVFV (Smithburn, 1949) or any other, preferably attenuated, RVFV, is used as challenge virus. The virus is stored at –80°C, or 4°C in a freeze-dried form.

The $PRNT_{80}$ (i.e. 80% reduction) conducted in a cell culture system is generally accepted as the standard assay system for the quantitative determination for neutralisation antibody activity in serum samples. The PRNT can be run in 6-well, 12-well, 24-well or 96-well plastic plates. The following technique is described using 12-well and four-fold dilutions of test sera.

2.2.1. Test procedure

i) Dilute the test sera 1/10 in cell culture media and inactivate for 30 minutes in a water bath set at 56°C (0.050 ml in 0.500 ml [0.050 ml serum+0.450 ml cell culture medium]).

ii) Make five sequential four-fold dilution tubes (for example 0.1 ml of diluted serum+0.3 ml of cell culture medium) starting with 1:40 and ending with 1:10,240 in culture media. Include known positive and negative control sera.

iii) A virus suspension, calculated to yield approximately 100 plaque forming units per 0.1 ml is prepared. Add 0.4 ml of virus suspension to all serum dilution tubes and positive and negative tubes. Cover tubes and refrigerate overnight at 4°C.

iv) Mark a 12-well plastic plate containing cell monolayers with the identification number of each test sample corresponding to worksheet: virus back-titration, and duplicate wells for dilutions 10, 40, 160, 640, 2560, and 10,240. Place plates in an incubator (37°C and 5% CO_2) until inoculation.

v) When ready, discard growth media. Starting with the highest dilution of serum-virus mixture (1:10,240) inoculate 0.1 ml (100 µl) into each of the two wells marked 1:10,240 containing confluent cell monolayers. Continue inoculation of wells down to and including 1:10 dilution. Allow inoculated plates to absorb for at least 1 hour at 37°C in a humidified atmosphere with 5% CO_2. Rock plates every 15 minutes.

vi) First overlay the nutrient-agarose media. Overlay inoculated cells with 1.5 ml of nutrient-agarose (equal volume of 2 × Eagle's basal medium (EBME) mixture to melted 2% agarose) and allow to set at room temperature. Invert plates when agarose is solidified and place in a 37°C, 5% CO_2 incubator.

vii) Twenty-four hours prior to counting plaques, a second overlay (0.75 ml) containing neutral red dye is added to all test wells. Allow the overlay to solidify for 15 minutes at room temperature (keep plates from light). Invert plates and place in 37°C and 5% CO_2 incubator.

viii) Approximately 24 hours after second overlay, count all plaques in virus controls, panels and positive and negative controls. A titre is expressed as the highest dilution of serum causing an 80% reduction in comparison to virus control count.

Positive control titre will depend on dilution format used in the test proper. If a four-fold dilution of sera is used, positive control titre must be within four-fold (+ or –) of the previously determined positive control titre. The titre that is considered positive for antibodies to RVFV is variable depending on the purpose of the test. If the purpose is to measure the immunogenicity of a vaccine, 1/80 or 1/100 is a useful threshold. If it is to check the circulation of RVFV in a population, a 1/10 or 1/20 titre in an animal can be considered as positive.

2.3. Virus neutralisation test

The virus neutralisation test (VNT) may be used to determine the presence of neutralising antibodies in naturally infected and vaccinated animals. It has to be performed in high containment biosafety facilities by trained personnel. The test is highly specific and can be used to test serum of any species. Any characterised, preferably attenuated RVFV can be used as challenge virus. The virus should ideally be stored at –80°C or 4°C in a freeze-dried form. The VNT is performed in a cell culture system and is generally accepted as one of the standard assay systems for the quantitative determination of neutralising antibodies in serum samples. The test is normally run in micro-neutralisation format in 96-well plastic tissue culture plates. The technique below describes the test conducted using twofold dilutions of test sera (Lubisi *et al.,* 2019).

2.3.1. Test protocol

i) Make a 1/5 dilution of test sera in tissue culture medium and inactivate at 56°C for 30 minutes in a water bath, and allow to cool.

ii) Make eight sequential twofold dilutions in tissue culture medium starting at 1/10 and ending at 1/1280. Include known positive and negative control sera. Dispense 50 µl of the highest (1/10) to the lowest (1/1280) dilution of each serum down the column of a 96-well plate in duplicate.

iii) Prepare a virus suspension, calculated to yield approximately 100–300 $TCID_{50}$ (median tissue culture infective dose) per ml. Add equal volumes of virus (50 µl) suspension to all wells containing test, positive and negative sera dilutions. Incubate the plate at 37°C for 1 hour in a humid chamber with 5% CO_2.

iv) When ready, discard the growth media. Starting with the highest dilution of serum/virus mixture (1/10,240) inoculate 0.1 ml (100 µl) into each of the two wells marked 1/10,240 containing confluent cell monolayers. Continue inoculation of wells down to and including 1/10 dilution. Allow inoculated plates to absorb for at least 1 hour at 37°C in a humidified atmosphere with 5% CO_2. Rock plates every 15 minutes.

v) Add 100 µl of Vero or BHK cells at a concentration of 3–4 × 10^5 cells per ml and incubate under the same conditions as above for 3–5 days. Include virus titration consisting of test virus at working dilution, and at least one dilution above and below, and cell controls on the same, or separate plate, depending on the number of samples tested.

vi) Monitor the plate daily under an inverted microscope and when the virus at working dilution in the virus titration wells shows cytopathic effect (CPE) of 80–100%, and the monolayers are intact in the cell control wells, the plates are ready to be read.

Record all CPE observed from the wells containing test sera. For confirmation of results, plates can be fixed with 10% formalin containing 0.05% crystal violet, and re-visualised using the microscope.

Serum antibody titres are taken as the reciprocal of the dilution at which the presence of either no (0%) or minute CPE (~10%) is observed. Each laboratory will determine the positive and negative cut-off titres according to the outcome of their internal test validation.

C. REQUIREMENTS FOR VACCINES

1. Background

Currently available RVF vaccines are either live attenuated or inactivated vaccines (Dungu *et al.,* 2018). National regulatory authorities can advise on availability in a particular country.

Table 2. Summary of the current RVF vaccine strains

	Smithburn live attenuated virus vaccines	Clone-13 live attenuated virus vaccine	MP-12 attenuated virus vaccine	Inactivated virus vaccines
Origin of the isolate	Mosquito isolate, Uganda, 1948	Human isolate, 1974	Egyptian human strain ZH548, 1977	Field strains (South Africa and Egypt) used
Attenuation	More than 200 passages in murine brain	Natural deletion in NSs gene	Mutagen directed attenuation (23 mutations)	Not applicable
Production substrate	BHK cell line	Vero cell line	Vero E6 cell line	BHK cell line
Target	livestock	livestock	livestock	livestock
DIVA policy	No	No	No	No

1.1. The live attenuated Smithburn RVF vaccine

The vaccine virus is derived from Smithburn's original neurotropic strain. This strain is not lethal to adult mice inoculated intraperitoneally and is safe for use in all breeds of cattle, sheep and goats (Barnard, 1979; Smithburn, 1949). However, it may cause fetal abnormalities or abortion in pregnant animals. The Smithburn RVF vaccine has been used for decades in the control of RVF in Eastern and Southern Africa and in the Middle East, and is still used to date in different endemic region.

1.2. The Clone 13 RVF vaccine

Clone 13 is a naturally attenuated strain characterised by a large deletion of the gene encoding for the main virulence factor, the NSs (Dungu *et al.,* 2018). The risk of reversion is considered unlikely. No abortion or side effects have been seen in experimental vaccine trials (Dungu *et al*., 2018; Hunter & Bouloy, 2001). It was recently introduced in South Africa for use in sheep and cattle using a single injection regimen.

1.3. The MP-12 RVF vaccine

RVFV strain MP-12 is derived from a plaque isolate of strain ZH548 of the Egyptian outbreak of 1977–1978. Its genome encodes 23 nucleotide mutations (Ikegami *et al.,* 2015), i.e. four mutations in the S segment, nine mutations in the M segment, and ten mutations in the L segment.

Master seed and vaccine lots of the MP-12 strain have been generated, and their safety and efficacy have been evaluated in ruminants (Morrill *et al*., 1997), Camelidae (Rissmann *et al*., 2017) and nonhuman primates (Morrill *et al*., 2003; 2011a; 2011b). Though MP-12 is highly immunogenic in ruminants, there is a lack of knowledge about the mechanism of MP-12 attenuation.

1.4. The inactivated RVF vaccine

The currently produced formalin-inactivated vaccines derived from a field strain of RVFV adapted to growth in cell culture (Barnard, 1979). These vaccines are currently adjuvanted in aluminium hydroxide. However inactivated RVF vaccines require a booster 3–6 months following initial vaccination, followed by yearly boosters. Inactivated RVF vaccine is also used in outbreak situations, and in pregnant animals as the attenuated Smithburn vaccine is not suitable for this group.

Many other candidate vaccines are either being developed and evaluated in target animals or are in an early stage of development.

There are a number of product characteristics that would be preferable to have in an effective and safe RVF vaccine, which should be used to define a target product profile. Elements of a target product profile for a RVF vaccine should preferably meet recommendation 2 of the report of the FAO meeting, 2011 (FAO, 2011) and as indicated below.

The main purpose of a RVF vaccine is to prevent epizootics and epidemics in species of economic interest (susceptible livestock species [ruminants] and, potentially, camelids), and limit the impact on animal and public health. In addition to the potential economic impact, it could also have some implications in international movements of animals. It is relevant to distinguish specific requirements for endemic regions and regions free of the disease.

1.5. Endemic region

The objective is the prevention and control of epizootics and epidemics in endemic areas and to contribute to the improvement of livestock production in endemic areas. In order of priorities, characteristics of the vaccines are:

i) preferably one dose, resulting in a long-lasting immunity of at least 1 year;

ii) preferably a life-long immunity after a limited number of doses.

1.6. Free or non-endemic region

Vaccines would be used either for the prevention of, or the response to an introduction of the virus. The expected characteristics of the vaccines are: safe with a quick onset of protective immunity and protection in animals of all ages and physiological status. Although DIVA (detection of infection in vaccinated animals) is an important property of any future vaccine, a requirement for DIVA should not hinder or block the development or licensing of an effective RVF vaccine.

In all cases, the vaccines should be:

i) Safe for the staff involved in the production of the vaccines and for the users, safe to all physiological stages of animals, and with minimal risk of introduction into the environment (potential vectors);

ii) Protective in multispecies and if possible in all susceptible species of economic importance, to prevent infection and transmission;

iii) Cost effective for producers and users, preferentially with a single-dose vaccination;

iv) Easy to use (e.g. needle-free delivery), suitable for stockpiling (e.g. a vaccine bank) and quick availability.

Staff handling virulent RVFV should preferably work in high containment facilities and be vaccinated, if vaccines are available, to minimise the risk of infection.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

In the following description of vaccine production, information is given on live vaccine production adjacent to information on inactivated vaccine production.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed virus

2.1.1. Biological characteristics of the master seed virus

The exact source of the isolate should be recorded and should include the type of material from which the virus was derived. The *in-vitro* passage history of the virus and details of the ingredients should be recorded in accordance with chapter 1.1.8. The master seed virus (MSV) should be

tested for identity, purity (freedom from adventitious agents) and safety. Characterisation of the MSV should be done using biological or genetic parameters, as relevant.

Assuming adequate immunogenicity and for obvious safety reasons, it is highly recommended that attenuated virus strains be used for the production of inactivated vaccines. The number of virus passages from the MSV stock to the final product should not exceed five (European Pharmacopoeia, 2012).

2.1.2. Quality criteria

The purity of the MSV and cells to be used for vaccine production must be maintained during the process. The seed virus should be free from adventitious agents, bacteria and *Mycoplasma*, using tests known to be sensitive for detection of these microorganisms (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*). The aliquot to be tested should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against a RVFV different from the seed virus, and the virus/antibody mixture is cultured on several types of cell line monolayers. Neutralised cultures should be passaged and tested for adventitious viruses that may have infected the cells or virus seed during previous passages. As an example, bovine viral diarrhoea virus (BVDV) is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems. A cell line highly permissive for BVDV types 1 and 2 is recommended as one of the cell lines chosen for evaluation of the MSV. Products of bovine origin should be obtained from countries with negligible bovine spongiform encephalopathy risk.

2.1.3. Validation as a vaccine strain

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy for the species for which it is intended.

2.2. Method of manufacture

2.2.1. Procedure

i) Live vaccines

Production of vaccines should be performed according to Chapter 1.1.8. Virus seed is produced in cell culture, see Table 2 above. The number of passages from the MSV should be restricted to five. The dose of virus used to inoculate cell culture should be kept to a minimum to reduce the potential for defective interfering viral particles. When the virus has reached its appropriate titre, as determined by CPE or other approved technique, the harvest can be clarified. Generally, the vaccine is freeze-dried, preferably in the presence of a suitable stabiliser.

ii) Inactivated vaccines

Antigens used in inactivated vaccines are generally prepared in a similar way to live vaccines. The virus present in the virus maintenance medium is inactivated using a validated inactivation method then can be eventually concentrated or purified and formulated with a suitable adjuvant.

Where a virulent RVFV is used for inactivated vaccine production, staff handling the live virus should be vaccinated, if vaccines are available, and the facilities and practices should conform to a high containment level minimising the risk of infection of the staff and release into the environment.

2.2.2. Requirements for ingredients

Cell lines used for cell culture should be demonstrated free of extraneous agents. All animal origin products used in the production and maintenance of cells (i.e. trypsin, fetal bovine sera) and growth of virus should be free of extraneous agents, with special attention paid to the presence of BVDV.

2.2.3. In-process controls

Yield can be assessed using antigenic mass or infectivity assays. Sterility of antigens should be checked throughout the process.

A validated inactivation control method is used to assure complete inactivation of the bulk material of each batch. For inactivated vaccines, samples taken at regular timed intervals during inactivation, then inoculated into a susceptible cell line (as used for production), should indicate a complete loss of titre by 2/3 of the total duration of the inactivation process.

For tests in cell cultures, not less than 150 cm^2 of cell culture monolayer is inoculated with 1.0 ml of inactivated harvest. The product complies with the test if no evidence of the presence of any live virus or other micro-organism is observed.

At the end of the production, antigen content is measured to establish that minimum bulk titres or antigenic mass have been achieved.

2.2.4. Final product batch tests

i) Sterility

The final products should be tested for absence of bacteria, *Mycoplasma* and fungal contamination (see chapter 1.1.9).

ii) Identity

The bulk live attenuated virus or the inactivated antigen as well as the final formulated product (freeze-dried or liquid) should undergo identity testing before release to demonstrate that the relevant RVF strain is present.

iii) Safety

The final product batch safety test is designed to detect any abnormal local or systemic adverse reactions.

Each of at least two healthy sero-negative target animals should be inoculated by the recommended route of administration with the recommended dose of vaccine. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days. Any adverse reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. If the potency test is performed in the target species, observation of the safety during this test can also be considered as an alternative to the batch safety test described here.

iv) Batch potency

For live vaccines, potency is based on live virus titre. For batch release of inactivated vaccines, indirect tests can be used for practicability and animal welfare considerations, as long as correlation has been validated to the percentage of protection in the target animal. Frequently indirect potency tests include antibody titration after vaccination of suitable species. Alternative methods (antigen mass) could be used if suitably validated.

v) Moisture content

The moisture content of the lyophilised attenuated vaccine should not exceed 5%.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.2.1 to C.2.2.4 of this chapter) should be submitted to the Regulatory Authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

The *in-process* controls are part of the manufacturing process.

2.3.2. Safety requirements

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily. In addition of these tests, the vaccines should be tested for safety in the field (see chapter 1.1.8 on field tests [safety and efficacy]).

i) Live vaccines

Vaccines should be tested for any pathogenic effects in each of the target species claimed on the label.

a) Safety test (overdose) in young animals

Carry out the test for each recommended route of application using in each young target animal not older than the minimum age recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Use no fewer than eight healthy young target animals without antibodies against RVFV. Administer to each animal a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the animals daily for at least 14 days. The body temperature of each vaccinated animal is measured on at least the 3 days preceding administration of the vaccine, at the time of administration, 4 hours after and then daily for at least 14 days. The vaccine complies with the test if the average body temperature increase for all animals does not exceed 1.5°C, no animal shows a temperature rise greater than 1.5°C for a period exceeding 3 consecutive days, and no animal shows notable signs of disease or dies from causes attributable to the vaccine.

b) Safety test in pregnant animals

Safety at different stages of gestation should be demonstrated if the product is to be used in pregnant animals.

Carry out the test with vaccination by a recommended route using a number of seronegative animals of the same age and origin sufficient to give a desired level of statistical certainty regarding the probability of side effects. Eight animals should be tested in each trimester of gestation (i.e. 24 animals in total) noting that the teratogenic risk of RVF is highest in the first two thirds of gestation (Botros *et al.,* 2006). Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each group a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Clinical observation of animals is carried out daily until parturition. Blood samples should be taken from newborn animals before ingestion of colostrum.

The test is invalid if the vaccinated animals do not seroconvert before parturition. The vaccine virus complies with the test if no abnormalities in the gestation or in the animals are noted. No animal shows notable signs of disease or dies from causes attributable to the vaccine.

Vaccine virus must not be present in blood samples from newborn animals.

c) Non-transmissibility

This test should be performed in the most susceptible species livestock for RVF, generally regarded as sheep.

Use a group of no fewer than 12 healthy lambs, at the minimum age recommended for vaccination and of the same origin, and that do not have antibodies against RVFV. Use vaccine virus at the lowest passage level that will be present between the MSV and a batch of the vaccine. Administer by a recommended route to no fewer than six lambs a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine.

Maintain no fewer than six lambs as contact controls. The mixing of vaccinated lambs and contact lambs is done 24 hours after vaccination.

After 45 days, euthanise all lambs. Carry out appropriate tests on the lambs to detect antibodies against RVF virus and on the control lambs to detect RVFV in the spleen and liver.

The vaccine complies with the test if antibodies are found in all vaccinated lambs and if no antibodies and no virus are found in the control lambs.

d) Reversion-to-virulence

This study is carried out using the master seed lot; the most susceptible species, age and route of inoculation should be used. If the quantity of the master seed lot sufficient for performing the test is not available, the lowest passage material used for the production that is available in sufficient quantity may be used. At the time of inoculation, the animals in all groups are of an age suitable for recovery of the strain. Serial passages are carried out in target animals using five groups of animals, unless there is justification to carry out more passages or unless the strain disappears from the test animal sooner. *In-vitro* propagation may not be used to expand the passage inoculum.

The passages are carried out using animals most appropriate to the potential risk being assessed.

Initially, the vaccine is administered by the recommended route most likely to lead to reversion-to-virulence, using an initial inoculum containing the maximum release titre. After this, no fewer than four further serial passages through animals of the target species are undertaken. The passages are undertaken by the route of administration most likely to lead to reversion-to-virulence. If the properties of the strain allow sequential passage via natural spreading, this method may be used, otherwise passage of the virus is carried out and the virus that is recovered at the final passage is tested for increase in virulence. For the first four groups, a minimum of two animals is used. The last group consists of a minimum of eight animals. At each passage, the presence of living vaccine-derived virus in the material used for passage is demonstrated. Care must be taken to avoid contamination by virus from previous passages. When the virus is not recovered from any intermediate *in-vivo* passage, the passage is repeated in ten animals using *in-vivo* passaged material from the last passage in which the virus was recovered. The virus recovered is used as the inoculum for the next passage. If the vaccine virus is not recovered, the experiment is considered to be completed with the conclusion that the vaccine virus does not show an increase in virulence.

General clinical observations are made during the study. Animals in the last group are observed for 21 days unless otherwise justified. These observations include all relevant parameters typical for the disease that could indicate increase in virulence. Compare the clinical signs and other relevant parameters with those observed in the animals used in the test for safety of the administration of 1 dose. If the last group of animals shows no evidence of an increase in virulence, further testing is not required. Otherwise, material used for the first passage and the virus recovered at the final passage level are used in a separate experiment using at least eight animals per group, to compare directly the clinical signs and other relevant parameters. This study is carried out using the route of administration that was used for previous passages. An alternative route of administration may be used if justified.

Unless otherwise justified and authorised, the product complies with the test if no animal dies or shows signs attributable to the vaccine strain and no indication of increased virulence is observed in the animals of the last group.

e) Environmental considerations

A risk assessment should be prepared where potential spread or risk of live vaccines to non-target species or spread by vector is considered.

f) Precautions (hazards)

Modified live virus vaccines may pose a hazard to the vaccinator depending on the strain and level of attenuation of the virus. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of vaccine.

ii) Inactivated vaccines

a) Safety test (of one dose and a repeated dose)

For the purposes of gaining regulatory approval, a trial batch of inactivated vaccine should be tested for local and systemic safety by each recommended route of administration in an

in-vivo test in eight animals of each target species. Single dose and repeat dose tests using vaccines formulated to contain the maximum permitted payload should be conducted. The repeat dose test should correspond to the primary vaccination schedule (e.g. two injections) plus the first revaccination (i.e. a total of three injections). The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days after each injection. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine.

b) Safety test in pregnant animals

Safety at different stages of gestation should be demonstrated if the product is to be used in pregnant animals.

Carry out the test with vaccination by a recommended route using no fewer than 16 healthy animals of the same age and origin and without antibodies against RVFV: eight in the first third of gestation and eight in the second third (periods of time where the teratogenic risk of RVF is the highest [Botros, 2006]).

Administer to each group a quantity of the vaccine equivalent to not less than the maximum antigen mass likely to be contained in 1 dose of the vaccine. Clinical observation of animals is carried out daily until parturition.

The test is invalid if the vaccinated animals do not seroconvert before parturition. The vaccine complies with the test if no abnormalities in the gestation or in the animals are noted, and no animal shows notable signs of disease or dies from causes attributable to the vaccine.

c) Precautions (hazards)

Inactivated RVFV vaccines present no danger to vaccinators, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of vaccine.

2.3.3. Efficacy requirements

Vaccine efficacy is estimated in vaccinated animals directly by evaluating resistance to live virus challenge using a controlled host animal vaccination–challenge study. In situations in which a host animal vaccination–challenge study is not possible, the elicitation of virus neutralising antibody by vaccination is considered an indication of efficacy, as neutralising antibody is considered to be protective; however, the minimum protective titre will vary with the type of neutralisation assay used and the virus used. Protective titre can be estimated by conducting a host animal vaccination–challenge study along with neutralising antibody measurements, thereby tying titre to efficacy. In general, a successful test in lamb is considered to be sufficient evidence of the quality of a vaccine to endorse its use in other species. Under circumstances where a vaccine is produced for use primarily in a species other than sheep, it may be more appropriate to test the efficacy of the vaccine in that same species. However, except for cattle, efficacy tests in other target species, such as goats or camelids have not yet been developed.

i) Immunogenicity study in young animals

The following test is applicable to sheep. For other species, appropriate modifications could be made.

A test is carried out for each route and method of administration recommended for vaccination using in each case lambs of the minimum age to be recommended. The quantity of vaccine to be administered to each lamb for a live vaccine is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine. For inactivated vaccines, a minimum antigenic dose should be used according to the recommended vaccination schedule.

For the test use no fewer than 16 lambs without antibodies against RVF.

For live vaccine, collect sera from the lambs before vaccination, 7 and 14 days after vaccination and just before challenge. For inactivated vaccine, collect sera from the lambs

before the first and second injection of the primo vaccination and at the time of the challenge.

Vaccinate no fewer than eight lambs, according to the recommended schedule. Maintain no fewer than eight lambs as controls. For live vaccines, challenge each lamb after 20–22 days by an appropriate route with a virulent RVFV. In the case of inactivated vaccines, challenge each lamb 14 days after completion of primo vaccination. Observe the lambs at least daily for 14 days after challenge and monitor for clinical signs and viral load by virus isolation and quantitative RT-PCR in blood.

The test is invalid if antibodies against RVFV in the sera of the control animals indicate that there was concurrent infection with the virus during the test.

The vaccine complies with the test if, during the observation period after challenge, there is a significant reduction in duration and titre of viraemia, and a notable reduction in clinical signs (if the challenge virus used produces such signs) in vaccinated lambs compared with controls.

ii) Immunogenicity test in pregnant animals

Unless otherwise prescribed in a specific country monograph, immunogenicity should be tested in pregnant animals. The following test is applicable to sheep. For other species, appropriate modifications could be made.

A test is carried out for each route and method of administration recommended for vaccination using, in each case, pregnant ewes of the minimum age to be recommended. The quantity of vaccine to be administered to each ewe for a live vaccine is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine. For inactivated vaccines, a minimum antigenic dose should be used according to the recommended vaccination schedule.

For the test use no fewer than 8 pregnant ewes without antibodies against RVF.

For live vaccine, collect sera from the ewes before vaccination, 7 and 14 days after vaccination and just before challenge. For inactivated vaccine, collect sera from the lambs before the first and second injection of the primo vaccination and at the time of the challenge.

Vaccinate no fewer than eight ewes, according to the recommended schedule. Maintain no fewer than eight ewes as controls. For live vaccines, challenge each ewe after 20–22 days by an appropriate route with a virulent RVFV. In the case of inactivated vaccines, challenge each ewe 14 days after completion of primo vaccination. Observe the ewes at least daily for 14 days after challenge and monitor for clinical signs and viral load by virus isolation and quantitative RT-PCR in blood.

The test is invalid if antibodies against RVFV in the sera of the control animals indicate that there was intercurrent infection with the virus during the test.

The vaccine complies with the test if, during the observation period after challenge, there is a significant reduction in duration and titre of viraemia, and a notable reduction in clinical signs (if the challenge virus used produces such signs) in vaccinated ewes compared with controls

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

There is currently no DIVA strategy available for the existing RVF vaccines.

2.3.5. Duration of immunity

As part of the authorisation procedure the manufacturer should demonstrate the duration of immunity of a given vaccine by either challenge or the use of a validated alternative test, such as

serology at the end of the claimed period of protection. The duration of immunity should be at least 1 year, with the vaccine to be administered at the start of the mosquito season.

2.3.6. Stability

The stability of all vaccines should be demonstrated as part of the shelf-life determination studies for authorisation.

The period of validity of a batch of lyophilised RVF vaccine or a batch of liquid inactivated vaccine should not be less than 1 year.

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NB: There are WOAH Reference Laboratories for Rift Valley fever (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Rift Valley fever

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.1.20.

RINDERPEST
(INFECTION WITH RINDERPEST VIRUS)

SUMMARY

In the past, classical rinderpest was an acute, viral disease of domestic cattle, yaks, wild African buffaloes (Syncerus caffer) *and Asian water buffaloes* (Bubalus bubalis *and* B. arnee). *It was characterised by high morbidity and mortality rates. Sheep, goats, pigs and wild ungulates might also be affected. Between 2002 and 2011, there were no reported field cases of rinderpest. The eradication campaign concluded in 2011 with an international declaration of global freedom from rinderpest.*

Existing collections of virulent and attenuated rinderpest viruses will remain under sequestration in approved research, diagnostic and vaccine manufacturing laboratories. To guard against the accidental release of virus from laboratory sources, FAO[1] and WOAH are collaborating in establishing the principle of international oversight and regulation of facilities holding rinderpest virus (RPV). All diagnostic testing, vaccine production and research activities that use live RPV or RPV-containing materials should be performed in an FAO-WOAH approved Rinderpest Holding Facility.

Rinderpest remains a notifiable disease and adequate surveillance systems must be maintained for the early detection of clinical cases should there be any accidental escape of the virus. WOAH (with FAO) will ensure the permanent availability of educational materials demonstrating the range of signs associated with rinderpest cases in live animals.

Description of the disease: *Clinical recognition of classical rinderpest is based on the finding of an individual dead animal or small groups of extremely sick animals showing two or more of the following signs: pyrexia, inappetance, depression, emaciation, shallow erosions of the upper and lower lip and gum, erosions or blunting of the cheek papillae, serous or mucopurulent ocular and/or nasal discharges, diarrhoea or terminal recumbency. It is more than likely that the group will contain a number of dead animals with such lesions. The introductory section of this chapter provides a more detailed description.*

Identification of the agent: *Laboratory confirmation is required, and is based on demonstrating the presence of the virus, viral RNA or antigen in samples from the spleen, tonsils, lymph nodes, white blood cells, ocular or nasal secretions of acutely infected animals.*

Serological tests: *Antibodies to RPV can be detected in serum from animals that have been infected with field virus or received rinderpest vaccine. This could be done using estimation of neutralising antibody from the results of a competition enzyme-linked immunosorbent assay (C-ELISA). Any test used must be highly specific for RPV. Such tests can only be carried out in FAO-WOAH approved Rinderpest Holding Facilities, as the tests require the use of live RPV (neutralisation tests) or antigen derived from live virus (C-ELISA).*

Requirements for vaccines: *A live attenuated rinderpest cell culture vaccine is available. Under the terms of the* Guidelines for Rinderpest Virus Sequestration*, of Resolution No. 21 (adopted by the WOAH Assembly, May 2017) governing the post-eradication era, it is not permitted to inoculate an animal with a rinderpest vaccine without prior permission from WOAH and FAO.*

1 FAO: Food and Agriculture Organization of the United Nations

In order to prepare for the possibility of a RPV re-emergence or release, FAO and WOAH, in collaboration with member countries, have developed a Global Rinderpest Action Plan for the post-eradication era that includes an international contingency plan, designation of a minimum number of Reference Centres/Reference Laboratories and an operational framework for emergency vaccine repositories to maintain preparedness. The retention and further manipulation of vaccine seed viruses is regulated jointly by FAO and WOAH.

A. INTRODUCTION

In the past, classical rinderpest was an acute, viral disease of domestic cattle, yaks, wild African buffaloes (*Syncerus caffer*) and Asian water buffaloes (*Bubalus bubalis* and *B. arnee*). It was characterised by high morbidity and mortality rates. Sheep, goats, pigs and wild ungulates might also be affected (Taylor & Barrett, 2007). Rinderpest is not a zoonotic disease, but the virus or virus-containing materials must be handled in accordance with strict biocontainment procedures as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*, and in conformity with the Guidelines for Rinderpest Virus Sequestration.

Between 2002 and 2011 there were no reported field cases of rinderpest. Further, in the period leading up to January 2011, the WOAH Scientific Commission for Animal Diseases scrutinised a comprehensive world-wide list of applications (evidence-based and historical) for national recognition of rinderpest-freedom. This process concluded in 2011 with an international declaration of global freedom from rinderpest. For the immediate future, existing collections of virulent and attenuated rinderpest viruses will remain under sequestration in research and approved vaccine manufacturing laboratories. To guard against the accidental release of virus from laboratory sources, FAO and WOAH are collaborating in establishing the principle of international oversight and regulation of facilities holding rinderpest virus (RPV) based on minimising the number of repositories. All diagnostic testing, vaccine development and research activities that use live RPV or RPV-containing materials[2] should be performed in an FAO-WOAH approved Rinderpest Holding Facility and after approval of the activity by WOAH and FAO.

Rinderpest remains a notifiable disease and adequate surveillance systems must be maintained for the early detection of clinical cases should there be any accidental escape of the virus. WOAH and FAO will ensure the permanent availability of educational materials demonstrating the range of signs associated with rinderpest cases in live animals. A recent account of the history of rinderpest, its eradication and its socio-economic impact is available (Roeder & Rich, 2009).

Rinderpest is caused by a negative-strand RNA virus of the *Morbillivirus* genus within the family *Paramyxoviridae*. The virus has a single serotype with at least three geographically restricted clades: African Lineages 1 and 2 and Asian Lineage 3, which cross-protect fully and are only differentiated by molecular characterisation. Although some strains of rinderpest evolved into a mild, nonfatal, infectious disease of cattle, all strains retain two very dangerous attributes. The first is an almost certain ability to undergo virulence modulations. The second is an ability to infect wild animal species and, in African buffaloes, eland, giraffe, lesser kudu and warthog, to cause an acute infection associated with high mortality.

An illustrated description of the disease is given in the WOAH Atlas of Transboundary Animal Diseases (Fernandez & White, 2010). Classical rinderpest has an incubation period of between 1 and 2 weeks. A peracute form is characterised by high pyrexia and sudden death in newborn or young animals. The acute disease is characterised by an acute febrile attack within which prodromal and erosive phases can be distinguished. The prodromal period lasts approximately 3 days, during which affected animals develop a pyrexia of between 40 and 41.5°C together with partial anorexia, constipation, congestion of visible mucosae, serous ocular and nasal discharges, depression and drying of the muzzle. However, it is not until the onset of the erosive phase, and the development of necrotic mouth lesions, that a tentative clinical diagnosis of rinderpest can be made. At the height of fever, flecks of necrotic epithelium appear on the lower lip and gum and in rapid succession may appear on the upper gum and dental pad, on the underside of the tongue, on the cheeks and cheek papillae and on the hard palate. Through the enlargement of existing lesions and the development of new foci, the extent of the oral necrosis can increase dramatically over the following 2–3 days. Much of the necrotic material works loose giving

2 See chapter 8.16 of the WOAH *Terrestrial Code* for definition of Rinderpest virus containing materials.

rise to shallow, non-haemorrhagic mucosal erosions. Necrotic lesions may also be found on the nares, vulva, vagina and preputial sheet.

Diarrhoea is another characteristic feature that develops 1–2 days after the onset of mouth lesions. The diarrhoea is usually copious and watery at first, but later on may contain mucus, blood and shreds of epithelium and it may be accompanied, in severe cases, by tenesmus. Anorexia develops, the muzzle dries out completely, the animal is depressed and emaciated, the breath is fetid and mucopurulent ocular and nasal discharges develop.

Deaths will occur but, depending on the strain involved, the breed of cattle infected and environmental conditions, the mortality rate may vary from 100% (acute strains in European breeds), to 20–30% (acute strains in zebu cattle), to zero (mild strains in zebu cattle). With both acute and mild strains, the mortality rate may be expected to rise as the virus gains progressive access to large numbers of susceptible animals. In the terminal stages of the illness, animals may become recumbent for 24–48 hours prior to death. Some animals die while showing severe necrotic lesions, high fever, emaciation and diarrhoea, others after a sharp fall in body temperature, often to subnormal values. In survivors, the pyrexia may remit slightly in the middle of the erosive period and then, 2–3 days later, return rapidly to normal accompanied by a quick resolution of the mouth lesions, a halt to the diarrhoea and an uncomplicated convalescence.

In cases where rinderpest is suspected, post-mortem examinations should pay particular attention to the abomasum, which may be highly engorged or show a grey discoloration; to the Peyer's patches, which may show lymphoid necrosis; and to the development of linear engorgement and blackening of the crests of the folds of the caecum, colon and rectum. Typically the carcass of the dead animal is dehydrated, emaciated and soiled. The nose and cheeks bear evidence of mucopurulent discharges, the eye is sunken and the conjunctiva congested. In the oral cavity, there is often extensive desquamation of necrotic epithelium, which always appears sharply demarcated from adjacent areas of healthy mucosa. The lesions frequently extend to the soft palate and may also involve the pharynx and the upper portion of the oesophagus; the rumen, reticulum and omasum are usually unaffected, although necrotic plaques are occasionally encountered on the pillars of the rumen. The abomasum, especially the pyloric region, is severely affected and shows congestion, petechiation and oedema of the submucosa. Epithelial necrosis gives the mucous membrane a grey colour. The small intestine is not commonly involved except for striking changes to the Peyer's patches where lymphoid necrosis and sloughing leaves the supporting architecture engorged or blackened. In the large intestine changes involve the ileocaecal valve, the caecal tonsil and the crests of the longitudinal folds of the caecal, colonic and rectal mucosae. The folds appear highly engorged in acute deaths or darkly discoloured in long-standing cases; in either event the lesions are referred to as 'zebra striping'.

The principal differential diagnoses in cattle are bovine viral diarrhoea/mucosal disease complex and malignant catarrhal fever; differentiation of these diseases requires the use of appropriate laboratory tests. Definitive diagnosis of rinderpest can currently only be undertaken in WOAH rinderpest Reference Laboratories.

In the mild form of rinderpest, which was associated with African lineage 2 strains of the virus found in endemic areas of eastern Africa, the incubation period could be between 1 and 2 weeks and the ensuing clinical disease little more than a subacute febrile attack in cattle. The fever was not invariable; it was short-lived (3–4 days) and low (38–40°C). The depression that characterised more acute forms of rinderpest was absent from mildly affected animals and, as a result, cattle often did not lose their appetite, and continued to graze, water and trek as well as unaffected animals. Diarrhoea, if present, was not marked. On close examination there might be some slight congestion of the visible mucous membranes and small, focal areas of raised, whitish epithelial necrosis might be found on the lower gum – sometimes no larger than a pin head – along with a few eroded cheek papillae. Some animals totally escaped the development of such erosions, the appearance of which was fleeting. Other animals might show a slight, serous, ocular or nasal secretion but, in contrast to the more severe forms of the disease, these did not progress to become mucopurulent.

Even though infections with mild rinderpest could pass unnoticed in cattle, the virus remained highly infectious for wildlife species, and among those generally regarded as highly susceptible (tragelaphine species such as lesser kudu and eland, African buffalo, and giraffe) it caused fever, a nasal discharge, typical erosive stomatitis, gastroenteritis, and death. Kock (2006) observed that, in addition, African buffaloes infected with lineage 2 showed enlarged peripheral lymph nodes, plaque-like keratinised skin lesions and keratoconjunctivitis. Lesser kudus were similarly affected but, whereas blindness – caused by a severe keratoconjunctivitis – was common, diarrhoea was unusual. Eland also showed necrosis and erosions of the buccal mucosa together with dehydration and emaciation. Therefore, in these circumstances, a diagnosis of rinderpest in any of these species points to the

likelihood of the simultaneous transmission of the virus, even at a subclinical level, in neighbouring cattle and possible dissemination of infection through live animal trade.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for rinderpest diagnosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	–	–	+	+++	–	–
Antigen detection (AGID)	–	–	+	+	+	–
(Real-time) RT-PCR	–	+++	+++	+++	+	–
Detection of immune response						
AGID	+	+	+	–	+	+
C-ELISA	++	–	++	–	++	++
VN	+++	–	+++	–	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
AGID = agar gel immunodiffusion; RT-PCR = reverse-transcriptase polymerase chain reaction;
C-ELISA = competitive enzyme-linked immunosorbent assay; VN = virus neutralisation.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

Special Post-Eradication Note: *there are no diagnostic tests for RPV or antibodies to RPV for which there is a positive control that does not come within the FAO-WOAH definition of a Rinderpest Virus Containing Material (RVCM). Continued storage of RVCM requires approval of the laboratory through the FAO-WOAH Rinderpest secretariat as an FAO-WOAH Holding Facility; use of RVCM for any purpose, including validation of diagnostic tests, requires explicit permission of FAO and WOAH.*

Suspect cases, that is animals with clinical signs similar to those seen in the case of infection with RPV, will still arise, and need to be tested to ensure that any future re-emergence or escape of RPV is detected in a timely manner. For the initial testing of samples from suspect cases, laboratories that are not FAO-WOAH-approved Rinderpest Holding Facilities are recommended to use (gel-based or real-time) reverse-transcriptase polymerase chain reaction (RT-PCR) using the established primer sets. The test can be run without a RPV positive control; parallel tests using (vaccine or wild type) peste des petits ruminants virus (PPRV) and published primer sets for PPRV can be used as a control for most of the stages of the assay (RNA extraction, reverse transcription and PCR reagents); alternatively the bovine actin primers can be used in parallel as an internal control reaction. For definitive diagnosis, samples should be sent to one of the FAO-WOAH approved Rinderpest Holding Facilities.

There are no circumstances where tests for anti-RPV antibodies will be required unless there is a re-emergence or escape of the virus.

1. Identification of the agent

Any suspicion of rinderpest must be viewed as a potential threat to international biosecurity and must be rapidly confirmed or differentiated. RT-PCR is the most rapid and specific test. If RPV is confirmed, back-tracing measures must be immediately instigated. In addition, samples must be sent to a WOAH Reference Laboratory for rinderpest for final confirmation of the diagnosis, and the virus origin should be identified by sequencing and comparison with known RPV genomic data. If possible, the virus should be isolated (Anderson et al., 1996), though this should only be attempted in an FAO-WOAH approved Rinderpest Holding Facility.

1.1. Virus isolation

RPV can be cultured from the leukocyte fraction of whole blood that has been collected into heparin or EDTA (ethylene diamine tetra-acetic acid) at final concentrations of 10 international units (IU)/ml and 0.5 mg/ml, respectively. Samples should be thoroughly mixed and transferred to the laboratory on ice, but never frozen. On average, the onset of viraemia slightly precedes the onset of pyrexia, and may continue for 1–2 days after pyrexia begins to wane. Consequently, animals showing a pyrexia are probably viraemic and therefore the best source of blood with which to attempt virus isolation. However, as occasional febrile animals may no longer be viraemic, samples from several febrile animals should be collected for submission. Virus can also be isolated from samples of the tonsil, spleen, prescapular or mesenteric lymph nodes of dead animals; these samples may be frozen for transportation. Transportation must be under biosecure conditions in compliance with international transport regulations described in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens*, Chapter 1.1.3 *Transport of biological materials* and with the Guidelines for Rinderpest Virus Sequestration.

To isolate the virus from blood, uncoagulated blood is centrifuged at 2500 ***g*** for 15 minutes to produce a buffy coat layer at the boundary between the plasma and erythrocytes. This is removed as cleanly as possible, mixed in 20 ml physiological saline and recentrifuged in a washing procedure designed to remove any neutralising antibody present in the plasma. The resulting cell pellet is suspended in cell culture maintenance medium and 2 ml aliquots are distributed onto established monolayers of primary calf kidney, B95a marmoset lymphoblastoid, *Theileria*-transformed bovine T lymphoblast or African green monkey kidney (Vero) cells, preferably Vero cells expressing morbillivirus receptor SLAM. These cells may be cultured in roller tubes, culture flasks or multiwell plates.

Alternatively, 20% suspensions (w/v) of post-mortem tissue may be used. These should be made by macerating the solid tissues in serum-free culture maintenance medium using standard grinding or shearing techniques and inoculating monolayers as before. The release of virus from solid tissue can be achieved in several ways. Perhaps the easiest is with a pestle and mortar, but this technique requires the use of sterile sand as an abrasive. Alternatively, tissue may be ground without an abrasive using all-glass grinders, for example, a Ten Broeck grinder. Shearing techniques are equally applicable using blenders. Virus-containing suspensions are clarified by low-speed centrifugation. The volume of the inoculum is not critical; a working volume is between 1 and 2 ml. Commonly used antibiotics are penicillin and streptomycin in combination, each at a concentration of 100 IU/ml or 100 μg/ml. A similar broad-spectrum cover can be obtained using neomycin at 50 μl/ml. Amphotericin B should be included at 2.5 μg/ml.

The inoculum should be removed after 1–2 hours and replaced with fresh medium. Thereafter, the culture maintenance medium should be decanted and replaced every 2 or 3 days and the monolayer observed microscopically for the development of cytopathic effects (CPE). These are characterised by refractility, cell rounding, cell retraction with elongated cytoplasmic bridges (stellate cells) and/or syncytium formation. The speed with which the CPE develops varies by substrate and probably by strain of virus also. Up to 12 days should be allowed in primary cells, a week in Vero and 2–5 days in B95a cells. Blind passages may be attempted before declaring an important sample negative. Isolates of virus can be partially identified by the demonstration of morbillivirus-specific precipitinogens in infected cell debris, or completely identified by either RT-PCR using RPV-specific primers (see below) or the demonstration of specific immunofluorescence using a RPV-specific monoclonal antibody.

1.2. Antigen detection by agar gel immunodiffusion

The agar gel immunodiffusion (AGID) tests may be conducted in Petri dishes or on glass microscope slides (Foreman *et al*., 1983). In either instance the surface should be covered with agar to a depth of about 4 mm using a 1% aqueous solution of any high quality agar or agarose. Wells are usually cut in a hexagonal pattern of six peripheral wells around a single central well. For slides, wells should be 3 mm in diameter and 2 mm apart. For Petri dishes, the wells can be increased to 4 mm in diameter and the distance between wells to 3 mm. The closer the wells are placed from each other, the shorter the reaction time.

Using a small volume pipette, rinderpest hyperimmune rabbit serum should be placed in the central well. In the absence of a rinderpest-containing positive control, PPRV (e.g. preparations of vaccine virus) can be used as the control, which should be placed in alternate peripheral wells (i.e. one, three and five). Negative control antigen is placed in well four. Test antigens are obtained as exudates from the cut surface of spleen or lymph nodes submitted for testing; if no exudate can be obtained a small portion of the sample should be ground with a minimum of saline. Ocular exudates may be squeezed directly from the swabs or, alternatively, by compression in a microtip (the cotton wool should be cut off the swab and placed into the wide end of a plastic 50–250 µl pipette tip; the stem of the swab may then be used to compress the cotton wool and force a small volume of exudate out of the narrow end of the tip). Test samples are added to wells two and six. Tests are best developed at 4°C or low ambient temperatures. The reaction area should be inspected from 2 hours onwards for the appearance of clean, sharp lines of precipitation between the wells forming a line of identity with the controls. Tests should be discarded after 24 hours if no result has been obtained. The result is not acceptable unless precipitation reactions are also obtained giving a line of identity with the control positive antigen preparation.

Although the test is neither highly sensitive nor highly specific, it is robust and adaptable to field conditions. A positive reaction from a large domestic ruminant should be treated as if it were rinderpest. From a small ruminant, a positive result should be treated as having been derived from a case of peste des petits ruminants (PPR) although further testing is recommended, given the lack of specificity in this test.

1.3. Nucleic acid detection and characterisation methods

RT-PCR techniques based on the amplification of parts of the N or F protein genes have been developed for the specific diagnosis of RPV (Forsyth & Barrett, 1995). This technique is extremely sensitive, specific and can detect RPV in cattle as early as two days post-infection with the advantage that results are obtained in 5 hours, including the RNA extraction. The two most commonly used protocols are given in some detail below. The PCR products are analysed on a 1.5% (w/v) agarose gel along with a suitable DNA marker to identify the specific DNA product.

A real-time RT-PCR assay for RPV diagnosis was described by Carrillo *et al.* (2010). This assay has been shown to be sensitive, to detect isolates representative of all known phylogenetic lineages of the virus, and to clearly differentiate RPV from PPRV and other clinically similar diseases (foot and mouth disease virus, bovine viral diarrhoea virus, bovine herpes virus, vesicular stomatitis virus). Comparison of samples from experimentally infected animals showed that white blood cells and conjunctival swabs are the sample of choice for this test, allowing the preclinical detection of the disease by 2–4 days post-infection. In the event of a RPV outbreak, this single-tube format real-time RT-PCR has the capability of preclinical diagnosis, thus aiding efforts to prevent further transmission of disease. It should be noted, however, that the assay was developed after the last RPV case, and was never used in practice for RPV diagnosis. Laboratories, other than the WOAH Reference Laboratories, that wish to carry out their own testing of suspect cases are advised to carry out gel-based RT-PCR using the available controls.

For both gel-based and real-time PCR methods, a positive control such as PPRV (with its specific primers) or bovine actin, and a negative control using sterile distilled water instead of RNA, must be included. Positive reactions with a RPV-specific primer set should be confirmed either by using additional RPV-specific primer sets or by sequence analysis of the DNA product.

1.3.1. Extraction of RNA from field samples

Viral RNA can be purified from lymph node or tonsil (ideal), peripheral blood lymphocytes (PBLs), swabs from eyes or mouth lesions, or from spleen (not ideal because of its high blood content). Tissue samples should be extracted with acidified guanidinum thiocyanate phenol (Forsyth & Barrett, 1995) using one of the commercial preparations available. Solid tissues (0.5–1.0 g) are minced and homogenised with 10 ml reagent, eye and mouth swabs are extracted with 1.0 ml, and purified PBLs (from 5 ml whole blood) are homogenised with 1.0 ml; RNA is then purified according to the manufacturer's procedure. For PBLs or swabs, RNA extraction spin columns are also suitable. The resulting RNA is stored at –70°C or –20°C until required.

The cDNA synthesis and PCR are carried out using a one-tube combined reaction. Suitable reagents are available from a number of manufacturers in addition to that given in the example protocol. The PCR products are analysed on a 1.5% (w/v) agarose gel along with a suitable DNA marker to identify the specific DNA products. An internal positive control such as the beta-actin primers should be included to validate the RNA extraction step and the RT-PCR reagents; if possible a parallel extraction of PPRV should be carried out and the viral RNA identified using PPRV-specific primers (Chapter 3.8.9, Section 2.4). A negative control using sterile distilled water instead of RNA must be included in each set of reactions. Positive reactions with either RPV-specific primer set should be confirmed by sequence analysis of the DNA product. In addition, positive samples should be sent to the WOAH Reference Laboratory in the United Kingdom (UK) for confirmatory testing. It is important to use more than one set of primers for the PCR step when testing for the presence of RNA viruses, as their nucleotide sequences can vary significantly and a mismatch of the primer at the 3'-end or within the primer sequence may result in failure of the primers to amplify the DNA. The FAO World Reference Laboratory[3] in the UK, which is also a WOAH Reference Laboratory for rinderpest, and the WOAH Reference Laboratory in France[4]), can advise on use of the technique for field sample analysis.

1.3.2. RT-PCR for the diagnosis of RPV based on the amplification of parts of the N and/or F genes

N and F gene amplification is based on the initial protocol described by Forsyth & Barrett (1995), reformulated as a one-step RT-PCR method. The test described requires the following materials: a commercial one-step RT-PCR kit, distilled water and primers, and a suitable PCR machine. Facilities for DNA agarose electrophoresis are also required.

i) Sequences of primers used:

Gene	Product size	Primer	Sequence (5' → 3')
RPV N	297 bp	B2	ATC-CTT-GTC-GTT-RTA-TGC-TCT-YRG
		B12	CAA-GGG-RRT-GAG-ACC-CAG-MAC-AR
RPV F	448bp	F3B	AGT-ATA-AGA-GGC-TGT-TGG-GGA-CAG-T
		F4D	TGG-GTC-TCT-GAG-GCT-GGG-TCC-AAA-T
β-Actin	275bp	BA1	GAG-AAG-CTG-TGC-TAC-GTC-GC
		BA2	CCA-GAC-AGC-ACT-GTG-TTG-GC

ii) Prepare each primer dilution by adding 5 µl of the primer stock solution (100 µM) to 45 µl of distilled water. A primer concentration of 10 µM is obtained with a final volume of 50 µl.

iii) For each test gene, prepare PCR master mix containing 0.6 µM final concentration of primers.

iv) Add 5 µl of RNA template to 45 µl of each master mix. Distilled water (5 µl) is added in place of RNA to provide a negative control which has to be included in each set of tests.

3 http://www.fao.org/docrep/004/X2096E/X2096E09.htm
4 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

v) Thermal cycler conditions are as follows:

50°C for 30 minutes	1 cycle	Reverse transcription step
95°C for 15 minutes	1 cycle	Inactivates RT and activates polymerase
94°C for 30 seconds 55°C for 30 seconds 72°C for 1 minute	40 cycles	PCR amplification of the cDNA
72°C for 5 minutes	1 cycle	Final extension
4°C (indefinite)	–	–

vi) Ten microlitres of each reaction are analysed by electrophoresis on a 1.5 % agarose gel. For all positive results, the remainder of the final product may be directly used for sequencing.

1.3.3. Real-time RT-PCR for the diagnosis of RPV

The real-time RT-PCR assay is carried out essentially as described in Carrillo *et al.,* 2010. It is typically performed as a 20 µl reaction. Several suitable reagents for one step RT-PCR are available, and the exact reaction conditions should be altered to fit with the reagents and the real-time PCR machine being used. For detailed advice on this test contact the WOAH Reference Laboratories.

2. Serological tests

2.1. The competitive enzyme-linked immunosorbent assay

A competitive ELISA is useful for the detection of rinderpest antibodies in the serum of animals of any species previously exposed to the virus. The test is based on the ability of positive test sera to compete with a rinderpest anti-H protein MAb for binding to rinderpest antigen. The presence of such antibodies in the test sample will block binding of the MAb, producing a reduction in the expected colour reaction following the addition of enzyme-labelled anti-mouse IgG conjugate and a substrate/chromogen solution. As this is a solid-phase assay, wash steps are required to ensure the removal of unbound reagents.

The rinderpest antigen is prepared from Madin–Darby bovine kidney cell cultures infected with the attenuated Kabete 'O' strain of rinderpest virus and inactivated at 56°C for 2 hours. The viral antigen is extracted from the infected cells by repeated cycles of sonication and centrifugation. The MAb was obtained by fusing the splenocytes of hyperimmunised mice with the NSO myeloma cell line, and then shown to be rinderpest H protein specific; this MAb has now been designated as C1. Kits will continue to be available commercially.

2.1.1. Test procedure

i) Reconstitute the freeze dried rinderpest antigen with 1 ml of sterile water and further dilute it to the manufacturer's recommended working dilution using 0.01 M phosphate buffered saline (PBS), pH 7.4.

ii) Immediately dispense 50 µl volumes of the diluted antigen into an appropriate number of wells of a flat-bottomed, high protein-binding ELISA microplate using two wells per test serum. Tap the sides of the microplate to ensure that the antigen is evenly distributed over the bottom of each well and, having sealed the plate, incubate it on an orbital shaker for 1 hour at 37°C. Wash the wells three times with 0.002 M PBS, pH 7.4.

iii) Add 40 µl of blocking buffer (0.01 M PBS, 0.1% [v/v] Tween 20 and 0.3% [v/v] normal bovine serum) to each test well followed by 10 µl volumes of all test sera.

iv) Follow the manufacturer's recommendations to prepare a working dilution of the MAb in blocking buffer, and add 50 µl of this to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.

v) Follow the manufacturer's recommendations to prepare a working dilution of rabbit anti-mouse immunoglobulin horseradish peroxidase conjugate in blocking buffer and add 50 µl to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.

vi) At the end of this period the plates are washed as before and immediately refilled with 50 µl volumes of substrate/chromogen mixture (1 part 3% H_2O_2 to 250 parts OPD), and incubate at room temperature for 10 minutes without shaking. Then add 50 µl of a stopping solution consisting of 1 M sulphuric acid.

vii) The test system must include known rinderpest positive and negative serum samples, a MAb control and a conjugate control.

viii) Measure the resulting absorbance values on an ELISA reader with a 492 nm interference filter and express the test results as percentage inhibition values compared with the value obtained using the MAb control. Inhibition values of 50% or more are considered to be positive and values below 50% are considered to be negative.

Lowering the positive/negative threshold to 40% or less increases the sensitivity of the test, but inevitably affects specificity by increasing the proportion of false-positive test results encountered. In practise, the 50% value is recommended by GREP at which level sensitivity is at least 70% and specificity exceeds 99%. The sensitivity needs to be taken into account when designing sampling frames for serosurveillance.

2.2. Antibody detection by agar gel immunodiffusion (AGID)

The AGID test can be used for screening bovine sera where there is suspected rinderpest disease and where PPRV is not circulating. As noted in section 1.2, the test does not distinguish between PPRV and RPV, so antibodies to either virus will give a positive reaction. Set up the AGID as described in 1.2, except that the central well contains a suspension of PPRV vaccine, while the outer wells contain known anti-RPV antisera (positions 1, 3 & 5), negative control serum (e.g. commercial bovine serum) in position 4 and test sera in positions 2 and 6. Antibodies to RPV will cross-react with the PPRV antigens, giving rise to precipitin lines.

2.3. Virus neutralisation

The virus neutralisation test (VNT) is performed in roller-tubes or culture flask cultures of primary calf kidney cells following the method of Plowright & Ferris (1961) or in 96-well microplates (Taylor & Rowe, 1984); both tests have been validated in experimentally infected cattle.

In the roller tube procedure, sera, that have not been heat inactivated, are diluted at intervals of 1 in 10 and then, starting with undiluted serum, mixed with an equal volume of $10^{3.0}$ $TCID_{50}$ per ml of an attenuated vaccine strain virus. Mixtures are held overnight at 4°C, after which 0.2 ml volumes are inoculated into each of five roller tubes, immediately followed by 1 ml of dispersed indicator cells suspended in growth medium at a rate of 2×10^5 cells per ml. Tubes are incubated at 37°C, sloped for the first 3 days, after which they are replenished with maintenance medium and placed on a roller apparatus. They are examined regularly for virus-specific cytopathology and positive tubes recorded and discarded; the final examination takes place on day 10. For calculating end-points, the virus dose is regarded as satisfactory if the final dilution falls within the range $10^{1.8}$ to $10^{2.8}$ $TCID_{50}$/tube. Under these circumstances, the presence of any detectable antibody in the 1/2 final serum dilution is considered to indicate previous infection with RPV.

In the microplate method, sera are heat-inactivated for 30 minutes at 56 ˚C before use. An initial serum dilution of 1/5 is further diluted at twofold intervals. Thereafter, 50 µl volumes of serum are incubated with 50 µl volumes of virus diluted to contain between $10^{1.8}$ and $10^{2.8}$ $TCID_{50}$ (Taylor & Rowe, 1984). Following a 45-minute to overnight incubation, 50 µl RPV-susceptible cells (between 1 and 2×10^5 primary calf or lamb kidney cells, 5×10^3 Vero or Vero-SLAM cells, or 5×10^4 B95a cells) are added as indicators. Tests are terminated after 6 or 7 days. Such tests may give indications of nonspecific neutralisation at high serum concentrations. There appear to be factors in some normal (with respect to prior rinderpest exposure) sera that bring about the failure of the virus to penetrate and replicate in indicator cells. In the tube test, these factors were probably removed during changes in maintenance medium; in the microplate method, they remain present the whole time. If the most concentrated final serum dilution is limited to 1/10, the effect disappears.

It should be noted that, since this test requires the manipulation of live vaccine virus, the VNT can currently only be undertaken in FAO-WOAH approved Rinderpest Holding Facilities with specific permission to carry out the procedure.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

The live attenuated tissue culture rinderpest vaccine (TCRV) described in previous editions of the *Terrestrial Manual* (Plowright, 1962) was developed in Kenya through the serial passage in primary bovine calf kidney cells of RBOK (rinderpest bovine old Kabete, or "Kabete O"), a virulent bovine rinderpest field strain isolated in 1910. While the modern division of rinderpest viruses into four lineages (Africa 1 and 2 and an old African one which includes Kabete O, and Asian) was unknown until 1995 (Wamwayi *et al.,* 1995), the Plowright vaccine virus undoubtedly cross-protects against all strains of all lineages. Since its development, the Plowright vaccine seed was widely distributed and hundreds of millions of doses of it were used on the Indian subcontinent, the Middle and Near East, and Africa in the control and eradication of rinderpest.

Other currently active TCRV strains, LA (Nakamura & Miyamoto, 1953) and LA-AKO (Furutani *et al.,* 1957a), were established from a previously developed lapinised vaccine strain, Nakamura III (alternatively known as L strain; Nakamura *et al.,* 1938), by repeated passages in rabbits and chick embryos. The parental Nakamura III was widely used to control the disease in East and South-East Asia. LA and LA-AKO are reported to be far less virulent than the parental strain, especially in highly susceptible cattle in Eastern Asia such as Japanese black and Korean yellow. Currently, LA-AKO is being used, at an FAO-WOAH-approved Rinderpest Holding Facility, for production of rinderpest vaccine for emergency use.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

i) Plowright (RBOK) vaccine

The vaccine strain was developed by 90 passages in primary calf kidney cells, and shown to be safe, effective and to resist reversion to virulence during 7 back passages in cattle (Plowright, 1962). The vaccine sequence has been published (Baron & Barrett, 1995) and deposited in the public databases. Seed lots used in the manufacture of Plowright TCRV must produce a cell-culture vaccine that is similarly safe and that confers an immunity in cattle lasting at least 5 years. The immunogenicity of seed virus was demonstrated up to the 122nd BK passage level, which should not be exceeded. Therefore, vaccine seed must be maintained in a seed lot system between passage levels 90 and 120. Seed lot virus must be preserved in a freeze-dried state at a temperature of -20°C or lower. The virus must be cultured in Vero cells or primary or serially cultivated kidney cells derived from a normal bovine fetus or a very young calf. Serially cultivated cells may not be more than ten passages removed from the primary cultivation.

The seed virus produces a vaccine that is safe to use in a variety of European, African and Indian cattle breeds. Its safety and efficacy have never been assessed in Chinese or Japanese cattle breeds.

ii) LA-AKO vaccine

The master seed virus (LA-AKO) was established from the lapinised "Nakamura III" vaccine strain (at the 897th rabbit passage level) by repeated passages in rabbits (29 passages) and chick embryos (456 passages). LA-AKO does not cause any clinical signs except slight hyperthermia in highly susceptible animals such as Japanese black cattle. It should be

noted however that the virus induces marked enlargement of the spleen in inoculated chick embryos (Furutani *et al.,* 1957b). The whole genome sequence of LA-AKO, and its strain of origin, Nakamura III, have been registered in the public database (Fukai *et al.,* 2011; Takamatsu *et al.,* 2015).

Seed lots should be lyophilised or frozen and stored at a temperature of –20°C or lower until use.

2.1.2. Quality criteria

i) Special considerations

Due to the fact that RPV has been eradicated worldwide, special consideration needs to be made in regards to animal inoculation to assess safety and efficacy. It is recommended to sequence a candidate vaccine virus and compare with reference strains of RPV to assess similarities that would negate the need to inoculate animals.

Subject to the above, for both Plowright and LA-AKO strains, seed lots should be shown to be:

a) Pure

Free from contamination with viruses, bacteria, fungi or mycoplasmas.

b) Safe

Inducing no abnormal clinical reaction on inoculation into rinderpest-susceptible cattle.

c) Efficacious

Inducing an immunity to rinderpest in rinderpest-susceptible cattle.

2.2. Method of manufacture

2.2.1. Procedure

Individual vaccine batches are prepared by infecting cell cultures and, after an appropriate incubation period, harvesting either the overlying media or the media and infected cells together. Virus should be harvested from cultures not more than 7 days (LA-AKO) or 10 days (Plowright) after the date that these cultures were infected. The decision to harvest should be based on the development of extensive characteristic CPEs within the cell monolayer.

To constitute a batch, infected cultures must have been inoculated with the same seed virus and incubated and harvested together.

To form a bulk suspension, the harvest should be clarified by low-speed centrifugation or by filtration before mixing with cryoprotectant.

Multiple harvests are permissible from the same set of cultures and may be pooled to form a single bulk suspension. For long-term storage and cold chain distribution, bulk suspensions are freeze dried.

Written records must accompany all stages of vaccine manufacture.

2.2.2. Requirements for substrates and media

i) Cells

Plowright vaccine may be grown in primary kidney cells from bovine embryos or calves, or cells derived by up to ten serial subcultures from either of these sources. In addition, the vaccine may be manufactured in approved continuous cell lines; Vero cells have been used for this purpose. The master seed stocks of LA-AKO are normally prepared in embryonated SPF chick eggs. Vero cells are used for the production of working/production seed stocks or vaccine of LA-AKO. In all cases, the cells should be

shown not to be infected with adventitious viruses including bovine viral diarrhoea virus (BVDV), bovine leukaemia virus (BLV), bovine rotavirus and bluetongue virus (BTV), and should be maintained in a seed lot system.

ii) Media

Calf kidney cells are grown and maintained in Earle's Balanced Salts Solution or Eagle's Minimum Essential Medium [MEM] supplemented with 0.5% lactalbumin hydrolysate and 0.1% yeast extract together with 5% new-born calf serum that must come from rinderpest-susceptible animals and originate from countries with negligible risk of bovine spongiform encephalopathy.

Vero cells are grown in Eagle's MEM supplemented with 10% heat-treated fetal calf serum and 0.295% tryptose phosphate broth (TPB), with antibiotics as required. Other formulations of medium have been used, e.g. Glasgow Modified Eagle's medium (GMEM) supplemented with 14% (v/v) TPB and 6% (v/v) non-heat-treated (rinderpest antibody-free) bovine serum, with antibiotics as required. All serum must come from rinderpest-susceptible animals and originate from countries with negligible risk of bovine spongiform encephalopathy.

iii) Cryoprotectant

For lyophilisation, the bulk suspension of virus is mixed with an equal volume of a solution containing either 5% lactalbumin hydrolysate and 10% sucrose, or 1% sodium glutamate, 0.3% polyvinylpyrolidone and 10% sucrose.

2.2.3. In-process controls

To ensure the properties of a master seed stock, a marker test should be undertaken where possible. A virus titration must be undertaken on each batch of a bulk suspension, and on the final bulk suspension itself, using tenfold virus dilutions in a microplate or roller tube system and employing four to ten replicates per dilution. Each batch of the final bulk suspension, or the final bulk suspension itself, should also be examined for adventitious viral contamination by relevant assays, including one or more of the following:

i) Samples are mixed with a neutralising titre of rabbit anti-rinderpest antiserum, added to continuous cultures of Vero cells, bovine kidney or testicular cells, and incubated at 37°C for 7 days. These cells must not develop any CPE within the incubation period.

ii) Samples are inoculated onto an African monkey-derived embryonic kidney cell line, MA-104, which is reported to be highly susceptible to Simian rotavirus (Smith *et al.,* 1979). Inoculated MA-104 cells must not develop CPE.

iii) A 10 ml of the sample from the batch of suspension clarified harvest or bulk suspension is mixed with a neutralising titre of rabbit anti-rinderpest antiserum and inoculated into a bovine leukemia virus (BLV)-susceptible sheep via an intramuscular route. The sera obtained from the sheep at 2 and 3 months after inoculation should be examined for the presence of BLV antibodies by an agarose gel immunodiffusion test.

The batch of a clarified harvest or bulk suspension may also be subjected to a marker test if available. LA-AKO vaccine induces a marked increase in the size of the spleen in inoculated chick embryos. 15 µl of 10- and 100-fold dilutions of a sample from a final bulk suspension are inoculated into a blood vessel of more than ten eggs each on day 11 to 12 after laying. Inoculated eggs are incubated at 38°C for 5 days. Spleens of inoculated chick embryos which are still alive after incubation are collected and weighed. These spleens become heavier than 15 mg in weight.

Checks for adventitious viral contamination should be undertaken on at least two uninfected control cell cultures prepared from the cell suspension used in batch production, after having been maintained using the same media and incubation conditions as the rinderpest-infected cells. They must be subjected to frequent in-process microscopic observations with negative results. After virus harvesting, the control cultures should be washed to remove bovine serum and re-incubated for 10 days in media containing bovine serum substitutes during which period

they are again subject to frequent microscopic observations for evidence of cytopathic change. At the end of this period at least one culture should be examined for the presence of noncytopathic BVD virus using an immunofluorescence or immunoperoxidase test or RT-PCR.

The control cultures may also be examined for haemadsorption activity. The uninfected cultures should be washed to remove bovine serum, and divided into two groups. Each group is overlaid with 0.1% suspension of guinea-pig or goose red blood cells (RBCs) for 1 hour, then subjected to microscopic observation. The control cultures must not adsorb RBCs from either of those species.

Prior to lyophilisation, the batch of a clarified harvest or bulk suspension may be held for not more than 5 days at 4°C, but considerably longer storage is achievable if frozen at –20°C to –80°C.

2.2.4. Final product batch tests

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

The final batch product consists of the freeze dried vials produced from a single bulk suspension; a batch may contain several filling lots. The contents of one container from each filling lot must be exposed to neutralisation by rabbit rinderpest antiserum, using a varying virus/constant serum method, and inoculated into primary bovine kidney or other susceptible cells. The identity of the product is established if no rinderpest-specific CPE develops.

ii) Safety and efficacy

Procedures may present slight variations depending on the country and system of production. For established virus seed stocks, animal-based testing of safety and efficacy may be deemed unnecessary.

Animals used in these procedures should be kept in isolation from other rinderpest-susceptible animals. At the end of the procedures they must be killed and the carcases disposed of securely. Using rinderpest-susceptible cattle, the contents of one to five randomly selected vials are pooled and used to inoculate each of two or three cattle with a volume equivalent to a single cattle field dose (where a field dose is taken to be ≥ 1000 $TCID_{50}$). In addition, one bovine may be inoculated with a volume equivalent to 100 cattle field doses. These animals are maintained in a biologically secure animal facility for the following 2–3 weeks. During this period the animals are subjected to daily temperature recording and frequent clinical inspections. At the end of this period, the cattle are examined for the presence of rinderpest neutralising serum antibodies (Section B.2.2). The vaccine is considered safe and efficacious if it does not induce any abnormal clinical reaction except for slight hyperthermia and if all vaccinated animals show a RPV-neutralising titre of 1/10 or greater.

In general terms, the safety of the Plowright vaccine has been widely demonstrated in both European and Indian breeds of cattle and Dwarf West African breeds. It has not been tested in Japanese or Chinese breeds and its safety in such animals cannot be guaranteed. The LA-AKO vaccine has been tested for the safety in a highly susceptible breed, Japanese black, as well as in Holstein breed.

iii) Batch potency

The close relationship between immunising potency and infectivity allows the latter to be used as the basis for potency estimations. Infectivity titrations are undertaken using cells of an approved continuous line or cells grown from each of three different bovine calf or embryonic kidneys. The number of estimates of the virus titre, and the number of vials pooled for each estimate, should be determined depending on the batch size and the local reproducibility of the assay. The sensitivity of the cells used in each working session must

be measured using a standard laboratory RPV preparation of an approved facility. The final titre is the geometric mean of all estimates, each undertaken using tenfold dilutions and four to ten observations per dilution. Potent vaccine should contain ≥ 100 field doses per vial.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

Plowright vaccine causes no clinical signs in rinderpest susceptible cattle or Asian water buffaloes. LA-AKO vaccine causes no clinical signs except slight pyrexia in rinderpest-susceptible cattle. Neither spreads by contact transmission to rinderpest susceptible cattle housed in close proximity to vaccinates.

ii) Reversion to virulence

Plowright vaccine virus retains its attenuated characteristics during at least five back passages in cattle and lacks the ability to spread by contact. Any sub-strain of the Plowright or LA-AKO strains used in the manufacture of rinderpest vaccine must be identifiable by written historical records that trace its origins to either of these vaccine strains.

iii) Environmental considerations

There are no environmental considerations with respect to either the manufacture or application of rinderpest vaccine.

2.3.2. Efficacy requirements

i) For animal production

Both vaccines protect vaccinated animals from clinical disease caused by virulent RPV infection.

ii) For control and eradication

For eradication purposes the object should be to use vaccine to immunise all susceptible animals in and around the vicinity of an outbreak in as short a period of time as possible (Taylor *et al.,* 2002).

2.3.3. Stability

Both the Plowright and LA-AKO strains of TCRV are highly stable when correctly freeze-dried and will keep for long periods at either +4 or –20°C provided the product retains a vacuum or is filled with nitrogen gas. The rate of degradation of lyophilised TCRV can be altered by the choice of cryoprotectant and by variations in the drying cycle. Good results have been obtained with the use of (a) a 5% lactalbumin hydrolysate/10% sucrose stabiliser, a 72–74 hour drying cycle under reduced vacuum (≤ 13 Pa), initial drying for 16 hours at –30°C, and a final shelf temperature of 35°C, or (b) a 1% sodium glutamate/0.3% polyvinylpyrolidone/10% sucrose stabiliser, a 48 hour drying cycle under reduced vacuum (≤ 10 Pa), initial drying for 24 hours at –45°C, a final shelf temperature of 22°C, and filling the vial with nitrogen gas.

Following reconstitution in either normal saline or 1M magnesium sulphate, the virus becomes much more thermolabile. The period for field distribution of reconstituted vaccine should not exceed its half-life, but as this parameter is temperature dependent and varies between 8 and 24 hours over a range from 4°C to 37°C, a universal period of 4 hours can be recommended.

3. Vaccines based on biotechnology

No biotechnology-based vaccines have so far been approved.

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NB: There are WOAH Reference Laboratories for rinderpest (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for rinderpest

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.1.21.

SURRA IN ALL SPECIES (*TRYPANOSOMA EVANSI* INFECTION)

SUMMARY

Description of the disease: Trypanosoma evansi *causes a trypanosomosis known as 'surra'. It affects a large number of wild and domesticated animal species in Africa, Asia, and Central and South America. The principal host species varies geographically, but camels, horses, buffalos and cattle are particularly affected, although other animals, including dogs, cats and wildlife, are also susceptible. It is an arthropod-borne disease; several species of haematophagous flies, including tabanids and Stomoxys, are implicated in transferring infection from host to host, acting as mechanical vectors. In Brazil, vampire bats are also implicated in a unique type of biological transmission. Peroral transmission is the main way of transmission to carnivores.*

The general clinical signs of T. evansi *infections – pyrexia directly associated with parasitaemia together with a progressive anaemia, loss of condition and lassitude – are not sufficiently pathognomonic for diagnosis. Recurrent episodes of fever and parasitaemia occur during the course of the disease. Oedema, particularly of the lower parts of the body, urticarial plaques and petechial haemorrhages of the serous membranes are sometimes observed in horses. Abortions have been reported in water buffaloes and camels. Nervous signs are common in horses and dogs. The disease causes immunodeficiencies that may be of high impact when interfering with other diseases or vaccination campaigns (foot and mouth disease and haemorrhagic septicaemia for example).*

Detection of the agent: *Laboratory methods for detecting the parasite are required. In early infection or acute cases, when the parasitaemia is high, examination of wet blood films, stained blood smears or lymph node materials might reveal the trypanosomes. In more chronic cases, or more generally when the parasitaemia is low, the examination of thick blood smears, as well as methods of parasite concentration and the inoculation of laboratory rodents are required. In apparently healthy carriers (animals without clinical signs), parasites are rarely observed and mouse inoculation gives the best results. Several primer pairs targeting the subgenus* (Trypanozoon) *or the species-specific* (T. evansi) *parasitic DNA sequences are available for diagnosis by polymerase chain reaction (PCR). PCR is more sensitive than parasitological examination, but it may give false-negative results when the parasitaemia is very low; in these cases, suspicion of potential carriers can only be confirmed by serological examination.*

Serological tests: *Infection gives rise to specific antibody responses and a variety of antibody detection tests have been introduced for laboratory and field use. Some have been partially validated, but await large-scale evaluation and standardisation. The most relevant are immunofluorescence test (IFAT), enzyme linked immunosorbent assays (ELISA) and card agglutination test (CATT/*T. evansi*). For field use, only CATT/*T. evansi *can be applied. Rapid diagnostic tests (RDT) are currently unavailable. Estimates of predictive values indicate that ELISA for detecting IgG is more likely to classify correctly uninfected animals, while the CATT is more likely to classify correctly truly infected animals. ELISA would thus be suitable for verifying the disease-free status of animals prior to movement or during quarantine. CATT can be used to target individual animals for treatment with trypanocidal drugs. For declaring a disease-free status, serial testing – CATT and ELISA followed by re-testing of suspect samples – is recommended in association with PCR. In areas where* T. cruzi, T. equiperdum *or tsetse-transmitted trypanosomes occur, cross-reactions may occur with any serological test employed.*

Requirements for vaccines: *No vaccines are available for the disease.*

A. INTRODUCTION

Infection with *Trypanosoma evansi* (subgenus *Trypanozoon*) causes a disease named *surra* in India (mainly in horses and bovines), also called, amongst others, *El Debab*, *El Gafar, Tabourit* or *MBori* in North Africa (mainly in camels), or *Mal de Caderas* or *Murrina* in Latin America (in horses). The clinical signs of surra are indicative but are not pathognomonic, thus, diagnosis must be confirmed by laboratory methods. The disease in susceptible animals, including camels (dromedary and bactrian), horses, water buffalo, cattle, sheep, goats, pigs, deer, dogs and cats is manifested by pyrexia, directly associated with parasitaemia, together with a progressive anaemia, loss of condition and lassitude. Such recurrent episodes lead to intermittent fever (as high as 44°C in horses [Gill, 1977]) and parasitaemia during the course of the disease. Oedema, particularly of the lower parts of the body, rough coat in camels, urticarial plaques and petechial haemorrhages of the serous membranes are sometimes observed in male equids, oedema of the genital organs may present a clinical feature that may be confused with dourine. In advanced cases, parasites invade the central nervous system (CNS), which can lead to neurological signs (progressive paralysis of the hind quarters and, exceptionally, paraplegia), especially in horses, but also in other host species, before complete recumbency and death. Abortions have been reported in buffalos and camels (Gutierrez *et al*., 2005) and there are clear indications that the disease causes immunodeficiency (Desquesnes *et al.,* 2013a).

There is considerable variation in the pathogenicity of different strains and the susceptibility of different host species. The disease may manifest as an acute or chronic form, and in the latter case may persist for several months, possibly years. The disease is often rapidly fatal in camels, horses and dogs. It may also be fatal in water buffalo, cattle, sheep, goat, pig and llamas, however, these host species, as well as camels, may develop mild or subclinical infections. Wild animals such as deer, capybara (*Hydrochoerus hydrochaeris*) and coati (*Nasua nasua*) can become infected and ill (including death), but they may also recover and constitute a reservoir. Animals subjected to stress – malnutrition, pregnancy, work – are more susceptible to disease.

Biologically, *T. evansi* is very similar to *T. equiperdum*, the causative agent of dourine, and morphologically resembles the slender forms of the tsetse-transmitted subspecies, *T. brucei brucei, T. b. gambiense* and *T.b. rhodesiense.* The initial molecular characterisation of *T. evansi* strains isolated from Asia, Africa and South America indicated that they were very homogeneous and suggested a single origin (Ventura *et al.,* 2002), other works suggest that *T. evansi* could have emerged from *T. brucei* in several instances (Lai *et al.,* 2008). Analysis by random amplified polymorphic DNA and endonuclease fingerprinting showed that *T. evansi* and *T. equiperdum* isolates form a closely homogeneous group. The difficulties in differentiating *T. equiperdum* from the other species of *Trypanozoon* have been stressed (Zablotskij *et al.,* 2003), and the existence of *T. equiperdum* was even questioned.

When *T. evansi* evolved from *T. brucei*, its kinetoplastic DNA was subjected to alterations or loss, in part or total; as a consequence, it can no longer implement a cycle in tsetse flies (Lai *et al,* 2008), and it is mainly mechanically transmitted by haematophagous flies. In fact, *T. evansi* was considered a malignancy of *T. brucei* (Lun *et al.,* 2015). The main mechanical vectors of *T. evansi* are tabanids (amongst which *Tabanus*, *Chrysops* and *Haematopota* are predominant) and *Stomoxys* flies (*Stomoxys* spp., *Hematobia* sp., etc); they are responsible for immediate transmission at a short distance amongst animals living, pasturing or watering together. Cats and dogs do not appear to play a significant role in ongoing transmission of the disease, but they generally act as sentinel animals. Like other members of the *Trypanozoon* subgenus, *T. evansi* is able to penetrate through mucosal membrane, which allows not only vertical transmission *in utero*, but also peroral transmission, principally observed in domestic and wild carnivores, and vampire bats in Latin America, but may also be responsible for mother-to-foal transmission in peripartum. Due to frequent peaks of parasitaemia, the risk of iatrogenic transmission must also be considered when serial treatments are applied (Desquesnes *et al.,* 2013b).

In Latin America, *T. evansi* has found a vertebrate vector: the vampire bat, such as *Desmodus rotundus*. These haematophagous mammals are infected when feeding on hosts such as infected horses or cattle, they develop the infection and may recover and stay under chronic infection; during this period, trypanosomes invade the salivary glands. They are then able to transmit *T. evansi* when biting their congeners in the bat colony, and to livestock when feeding; thus vampire bats are both hosts, reservoirs and vectors of the parasite. Several wild mammals act as potential reservoirs of *T. evansi* in Latin America, with capybara, the largest rodent in the world, being the most important sentinel to detect the presence of surra, as significant parasitaemia levels are not associated with clinical impact (Desquesnes *et al.*, 2013a; 2013b).

Like all pathogenic trypanosomes, *T. evansi* is covered by a dense protein layer consisting of a single protein called the variant surface glycoprotein (VSG). This acts as a major immunogen and elicits the formation of a series of specific antibodies. The parasites are able to evade the consequences of these immune reactions by switching the VSG, a phenomenon known as antigenic variation.

Surra should be considered for differential diagnosis when cases presenting fever and/or anaemia are observed in the field. Anaemia is a reliable indicator of trypanosome infection, but it is not in itself pathognomonic. On the other hand, animals with a mild subclinical infection can have parasitaemia without evidence of anaemia. Reliable diagnosis requires laboratory confirmation of the infection.

In enzootic areas, routine diagnoses can be made using parasitological techniques, while serological surveys can be carried out, preferably by enzyme linked immunosorbent assays (ELISA). Card agglutination test (CATT)/*T. evansi* can be used to target individual animals for treatment with trypanocidal drugs.

Where a definitive confirmation of the absence of infection is needed (e.g. for importation into a disease-free area), serial examinations are required, including the antibody detection ELISA and a sensitive agent detection method, such as polymerase chain reaction (PCR). Testing by mouse inoculation should be limited and used only if fully justified.

In areas where *T. cruzi, T. equiperdum* or tsetse-transmitted trypanosomes are present, cross-reactions may occur with any serological test employed so that the exact trypanosome status of an animal may not be established in full.

Surra is not known as a zoonotic disease, however, some rare human cases have been described, notably in India and Vietnam (Van Vinh Chau *et al*., 2016). Consequently, suspect animal samples should be handled at an appropriate biosafety and containment level determined by biorisk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

As there is no vaccine against trypanosomoses, the only option is treatment of the animals using trypanocidal drugs; WOAH has published an article setting out quality control approaches for trypanocidal drugs: https://www.ncbi.nlm.nih.gov/pubmed/25812206 or http://dx.doi.org/10.20506/rst.33.3.2320

B. DIAGNOSTIC TECHNIQUES

A variety of diagnostic tests is available and researchers are still working to improve existing tests and develop new ones. Current diagnostic tests vary in their sensitivity and specificity, the ease with which they can be applied and their costs. The choice of a particular test will be guided by economic principles, by the availability of expertise and especially by the diagnostic requirements. For example, different degrees of sensitivity and specificity are required to confirm the infection in an individual animal as compared with the detection of infection at the herd level. Similarly, the diagnostic test(s) to establish the parasitological prevalence of trypanosomosis is (are) different from those required to establish the presence or absence of the disease in an area. Reliable diagnosis may be achieved by combining appropriate diagnostic tests. Reliable interpretation of results from diagnostic tests will depend on test validity, as well as on proper sample selection/collection, the sample size, and the way the diagnostic tests are conducted (see Table 1). Detailed diagnostic techniques (including figures) of most of the tests described in Section B can be found in the "Compendium of standard diagnostic protocols for Animal Trypanosomoses of African Origin", available online: https://www.woah.org/en/document/compendium-of-diagnostic-protocols-of-the-oie-reference-laboratory-for-animal-trypanosomoses-of-african-origin/

Table 1. Test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Thin GSBS (or lymph or oedema fluid)	–	+	+	+++	++	–
DNA detection/PCR	+++	+++	+++	+++	+++	–
Wet blood film	–	–	–	++	–	–
TGSBF	–	–	–	++	++	–
HCT (Woo)	+++	+++	+++	+++	+++	–
BCT (Murray)	+	+	++	++	++	–
AECT	–	+	++	++	–	–
Detection of immune response						
CATT/ *T. evansi*	++	++	+++	+++	++	–
IFAT *T. evansi*	++	+++	+++	+++	++	–
ELISA *T. evansi*	+++	+++	+++	+++	+++	–
TL RoTat1.2 test	–	–	++	–	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
GSBS = Giemsa-stained blood smear; PCR = polymerase chain reaction; TGSBF = thick Giemsa-stained blood film;
HCT = haematocrit centrifuge technique; BCT = buffy coat technique; AECT = anion exchange chromatography technique;
CATT = card agglutination test; IFAT = indirect-fluorescent antibody test;
ELISA = enzyme-linked immunosorbent assay; TL = trypanolysis test.
[(a)]A combination of agent identification methods applied on the same clinical specimen is recommended (See Section B.3.2).

1. Detection of the agent

The classical direct parasitological methods for the diagnosis of trypanosomosis, namely microscopic examination of blood or lymph node material, are not highly sensitive, but a number of techniques, including enrichment of the sample, rodent inoculation and DNA methods may increase the sensitivity. In regions where other species of *Trypanozoon* might be present in addition to *T. evansi*, species-specific discrimination requires molecular tools and cannot be accomplished by microscopic examinations.

1.1. Direct microscopic examination

1.1.1. Blood sampling

Trypanosoma evansi is a parasite of the blood and tissues. As for other trypanosomes, it is recommended that blood for diagnosis be obtained from peripheral ear or tail vein, even if the jugular vein is most often preferred for practical reasons. However, it should be realised that less than 50% of infected animals may be identified by examination of blood.

Peripheral blood is obtained by puncturing a small vein in the ear or tail. Deeper samples are taken from a larger vein by syringe. Cleanse an area of the ear margin or tip of the tail with alcohol and, when dry, puncture a vein with a suitable instrument (lancet, needle). Ensure that instruments are sterilised or use disposable instruments to avoid iatrogenic transmission of the infection by residual blood.

1.1.2. Wet blood films

Place a small drop of blood (2–3 µl) on to a clean glass slide and place over it a cover-slip to spread the blood as a monolayer of cells. Examine by light microscopy (200×) to detect any motile trypanosomes. Improved visualisation can be obtained with dark-ground or phase-contrast microscopy (200–400×). The sensitivity of this method is low, approximately 10 trypanosomes per µl, which is frequent in early or acute infections only. This examination can be applied to case confirmation, however, due to its very low sensitivity, it is mostly used to follow-up experimentally infected animals.

1.1.3. Thick Giemsa-stained blood film (TGSBF)

Place a large drop of blood (10 µl) on the centre of a microscope slide and spread with a toothpick or the corner of another slide so that an area of approximately 1.0–1.25 cm in diameter is covered. Air-dry for 1 hour or longer, while protecting the slide from insects. Placing the slide in a horizontal position, stain the unfixed smear with Giemsa's Stain (one drop of commercial Giemsa + 1 ml of phosphate-buffered saline, pH 7.2), for 25 minutes. After washing and drying, examine the smears by light microscopy at a magnification of 500× with oil immersion. The advantage of the thick smear technique is that it concentrates the drop of blood into a small area, and thus less time is required to detect the parasites, which are more visible owing to the haemolysis of the unfixed red cells. The disadvantage is that the trypanosomes may be damaged in the process, and the method is therefore not suited for species identification in case of potentially mixed infections. Consequently, thin blood smears are generally preferred to thick blood films.

1.1.4. Giemsa-stained blood smears (GSBS)

Place a small drop of blood (3–5 µl) at one end of a clean microscope slide and draw out a thin film in the usual way. Air-dry briefly and fix in methyl alcohol for 1 minute and allow to dry. Stain the smears in Giemsa (one drop Giemsa + 1 ml PBS, pH 7.2) for 25 minutes. Pour off stain and wash the slide in tap water and dry. Nowadays, commercially available fast stains are most often used, which allow fixation and staining within a few seconds. Slides are then washed in tap water and dried. Examine at a magnification of 400–1000× with oil immersion. This technique permits detailed morphological studies and identification of the *Trypanozoon* subgenus (which, sometimes, allows the species to be defined, according to the epizootiological context), but its sensitivity is very low (it can detect parasitaemia >500,000 trypanosomes/ml of blood). Nevertheless, when positive, this examination brings a reliable and subgenus-specific confirmation of the infection.

1.1.5. Lymph node biopsies or oedema fluid

Samples are usually obtained from the prescapular or precrural (subiliac) lymph nodes, preferably when they are enlarged. Select a suitable node by palpation and cleanse the site with alcohol. Puncture the node with a suitable gauge needle, and draw lymph node material into a syringe attached to the needle. Expel lymph on to a slide, cover with a cover-slip and examine as for the fresh blood preparations. Fixed thin or thick smears can also be stored for later examination. Similar examination can be done by collection of oedema fluid.

1.2. Concentration methods

In most of its hosts, *T. evansi* can induce mild clinical or subclinical carrier state infections with low parasitaemia in which it is difficult to demonstrate the presence of the parasites. In these circumstances, concentration methods are necessary, as they increase the sensitivity of microscopic examination; being based on the observation of moving parasites, these methods should be completed no later than 2–4 hours after blood collection.

1.2.1. Haematocrit centrifugation technique (also known as Woo's technique, or HCT)

Collect blood (2 × 70 µl) into two heparinised capillary tubes (75 × 1.5 mm). Close the wet end with plasticine and centrifuge at 14,000 ***g*** for 5 minutes (generally 12,000 rpm in a haematocrit centrifuge machine). The capillary tube is examined and the value of the haematocrit is expressed as a percentage of packed blood cells to total blood volume (%packed cell volume [PCV]); this gives an indication on the anaemia of the animal. The capillary tube is then placed in a groove made with pieces of slide glued to a slide, for microscopic observation. Trypanosomes are large cells that concentrate at the junction between the buffy coat and the plasma, which is observed under the microscope (100–200×). Light conditions must be set to induce refringency of the cells to increase the visibility of the moving trypanosomes; this can be obtained by lowering the position of the light condenser or with intermediary positions of the turret light condenser. Specially designed reading chambers for HCT can be obtained at the WOAH Reference Laboratories for surra[1]; they enable submerging of the capillary tube in water to avoid light diffraction, however, they require long-focal objectives. The fresher the sample, the better is the sensitivity as strong parasitic movements make trypanosomes more visible. This technique can detect around 50–200 trypanosomes/ml of blood (Desquesnes, 2004). The buffy coat sample can also be extracted from the capillary tube to be prepared for PCR. HCT is the most employed and one of the best concentration techniques for *T. evansi* detection; its specificity is limited to the subgenus level (*Trypanozoon* versus *Duttonella*, *Nannomonas*, *Schizotrypanum*, *Megatrypanum*).

1.2.2. Buffy coat technique (also known as Murray's technique, or BCT)

This technique is very similar to the previous one. Collect blood into heparinised capillary tubes and centrifuge as above. Scratch the tube with a glass-cutting diamond pencil and break it 0.5 mm below the buffy coat layer – the upper part thus contains a small top layer of red blood cells, the buffy coat (white blood cells and platelets) and some plasma.

Partially expel the contents of this piece on to a slide; avoid expelling more than 5–8 µl of plasma, but make sure the buffy coat has been expelled (the small disk of the buffy coat should be visible to the naked eye), press on a cover-slip to spread the buffy coat and examine by dark-ground, phase-contrast or similar microscopy under the previously described refringency conditions at a magnification of 200–500×. Trypanosomes are mostly present at the periphery of the thick buffy coat material. Expelling the buffy coat from the capillary tube is a delicate step and will affect the reproducibility of the technique. HCT (Section B.1.2.1 above) is more advisable than BCT as the latter is highly dependent on the technical skill of the operator and presents a low level of reproducibility.

Both the Woo and the Murray techniques allow anaemia to be estimated by measuring the PCV and may be used in surveys of herds at risk. The value of the haematocrit (considered as low when <24% for example in cattle) can be used as an indicator to select a subset of samples to be submitted to the more expensive, but also more sensitive, PCR analysis.

1.2.3. Mini-anion exchange chromatography technique (mAECT)

When a blood sample from animals infected with salivarian trypanosomes is passed through an appropriate DEAE-cellulose (diethylamino-ethylcellulose, such as DE52 GE Healthcare) anion-exchange column, the host blood cells, being more negatively charged than trypanosomes, can be adsorbed onto the anion-exchanger (in pH and ionic strength conditions adapted to the host species), while the trypanosomes are eluted, retaining viability and infectivity (Lanham & Godfrey, 1970). This technique is mostly used for the purification of parasites from the blood (for example, for parasite antigen preparation), but miniature systems have been developed, especially for diagnosis in humans. The sensitivity of this technique can be increased by approximately tenfold by the use of buffy coat preparations rather than whole blood.

i) *Preparation of phosphate buffered saline glucose* (*PSG*), *pH 8*

Na_2HPO_4 anhydrous (13.48 g); $NaH_2PO_4.2H_2O$ (0.78 g); NaCl (4.25 g); distilled water (1 litre). Solutions of different ionic strength are made by diluting the stock PBS, pH 8, and adding

1 See the list of WOAH Reference Laboratories: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

glucose to maintain a suitable concentration. For blood of mice, domestic and wild ruminants and dogs, add four parts PBS to six parts distilled water and adjust the final glucose concentration to 1%. For blood of pigs and rabbits, add three parts PBS to seven parts distilled water and adjust the final glucose concentration to 1.5%. The PBS/glucose solution (PSG) must be sterile (however, PBS must be autoclaved before adding glucose).

ii) *Equilibration of DEAE-cellulose*

Suspend 500 g of DEAE-cellulose in 2 litres of distilled water. Mix for 20 minutes with a plastic-coated magnetic stirrer at low speed (metallic contact with DEAE-cellulose is proscribed). Adjust the pH to 8 with phosphoric acid. Allow to settle for 30 minutes. Discard the supernatant fluid containing the finest particles that might block the column. Repeat the procedure three times using PSG buffer as described above. Store the equilibrated concentrated suspension of DEAE-cellulose (slurry) at 4°C for a short period, or at –20°C for longer conservation.

iii) *Packing of equilibrated DEAE-cellulose*

Place a 2 ml syringe without the plunger on a test-tube rack, complete with a flexible tube that can be closed with a clamp to act as a tap. Put a disc of Whatman No. 41 filter paper at the bottom of the syringe and moisten by adding a few drops of PSG. Pour 2–2.5 ml of the slurry of equilibrated cellulose into the syringe and allow packing for 5 minutes before elution of the buffer. The height of the sediment should be approximately 3 cm. Wash and equilibrate the column with 2 ml of PSG without disturbing the surface.

iv) *Adsorption of blood eluate of the trypanosomes*

Gently place 100–300 µl of heparinised blood (or preferably buffy coat) on the surface of the cellulose column; allow it to penetrate the cellulose, but do not let the cellulose dry before pouring on the eluting buffer. Progressively add 1.5 ml of PSG and start collecting the eluate into a finely tapered Pasteur pipette with a sealed end. The cellulose column should remain wet throughout the procedure. Put the filled pipette, protected by a conical plastic pipette tip, in a tube and centrifuge at 525 ***g*** (or up to 1000 ***g***) for 10 minutes. Examine the bottom of the pipette under the microscope (100× or 200×) using a special mounting device. Alternatively, the eluate could be collected into 50 ml plastic tubes, with conical bottoms, centrifuged at 1000 ***g*** and the sediment examined by dark-ground microscopy.

A similar method used in cattle, pig and goat is also referred to as the miniature anion exchange chromatography method. However, this technique, being time consuming and expensive, is rarely used for animal diagnosis. Conversely, large amounts of blood or buffy coat from experimentally infected rodents can be applied to large columns, for massive parasite isolations, to produce parasites/antigens for serological tests.

1.3. Animal inoculation

Due to increasing bioethical concerns and the tendency to eliminate the use of animals for biological testing, animal inoculation should be limited as much as possible and only used if fully justified. Laboratory animals may be used to reveal subclinical (non-patent) infections in domesticated animals. *Trypanosoma evansi* has a broad spectrum of infectivity for small rodents, and so rats and mice may be used. Rodent inoculation is not 100% sensitive (Monzon *et al.,* 1990) but further improvement in its efficacy can be obtained by the use of buffy coat material. Such a procedure is able to detect as few as 1.25 *T. evansi*/ml blood. This technique is suitable when highly sensitive detection is required.

Inoculate heparinised blood intraperitoneally into rats (1–2 ml) or mice (0.25–0.5 ml). Inoculate a minimum of two animals. Bleed animals from the tail every 48 hours post-infection to detect and/or monitor the parasitaemia. The incubation period before the initial appearance of the parasites and their virulence will depend on the trypanosome strain, on the concentration of the inoculum, and the strain of laboratory animal used; however in most cases it is very short (5 ± 2 days), but can extend to 2 weeks in some cases (Monzon *et al.,* 1990). Sensitivity of this *in vivo* culture system may be increased by the use of immunosuppressed laboratory animals. Drugs such as cyclophosphamide or hydrocortisone acetate may be used for this purpose. Such a procedure is only justified when the detection of a potentially

infected host is of high importance, for example, to confirm a suspected case in high value animals, or to isolate a parasite for further characterisation.

1.4. Detection of trypanosomal DNA

Detection of minute amounts of trypanosomal DNA is a possible means of identifying animals with active infections as the parasitic DNA does not remain for more than 24–48 hours in the blood of the host after the trypanosomes are killed (Desquesnes, 2004).

1.4.1. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) based on DNA sequences of various taxonomic levels is used. The gold standard, to date, for detection of the *Trypanozoon* subgenus are the TBR primers (Masiga *et al.,* 1992). Other primers (see Table 2) have been reported and are being evaluated; some of them are specific for *Trypanozoon* and others for *T. evansi* ± *T. equiperdum* (Desquesnes & Davila, 2002; Holland *et al*., 2001; Panyim *et al.,* 1993). To date, the most sensitive test is that of satellite DNA using TBR primers (Masiga *et al.,* 1992); the sensitivity of the other primers is being compared under various conditions, including in laboratory rodents, but can only be validated with a sufficient batch of field samples from natural hosts. The use of TBR primers is recommended, at least in the first instance, and, if necessary, confirmed by other sets of *Trypanozoon*-specific primers such as ESAG6/7 (Holland *et al*., 2001) or TEPAN (Panyim *et al*., 1993).

In areas and host species potentially infected with other *Trypanozoon* such as *T. brucei brucei*, species confirmation can be obtained with more specific primers such as RoTat 1.2 (Claes *et al*., 2004; Verloo *et al.,* 2001) or EVAB, for non-RoTat 1.2 strains (Ngaira *et al.,* 2005). RoTat1.2 primers are good candidates for a *T. evansi*-specific diagnosis, however (i) they exhibit a lower sensitivity than TBR primers (Elhaig & Sallam, 2018), (ii) they fail to detect *T. evansi* type B (Njiru *et al*, 2006), and, (iii) they amplify a similar 205 bp product as expected for *T. evansi* with some *T. brucei* and *T. equiperdum* strains (Abou El-Naga *et al*., 2012). If *T. equiperdum* is suspected, other epizootioological information must be considered (amongst which clinical signs and mode of transmission are determinant) as there is not, so far, a simple and reliable molecular test that can distinguish *T. evansi, T. equiperdum* and *T. brucei* sspp. (Gizaw *et al*., 2017).

When trypanosome subspecies that affect human are suspected, more specific primers can be used to detect *T.b. gambiense* (Radwanska *et al*., 2002b) or *T. b. rhodesiense* (Radwanska *et al*., 2002a); however, targeting single copy genes, they exhibit a much lower sensitivity than TBR, potentially leading to inconclusive results when the PCR is negative.

If *Trypanosoma* spp. of other subgenera may be suspected, primers amplifying the internal transcribed spacer 1 (ITS1) of the ribosomal DNA may be used since they can detect several subgenera and species in a single reaction (Njiru *et al*., 2005), however with limited sensitivity. Other techniques such as the loop-mediated isothermal amplification (LAMP) (Thekisoe *et al.,* 2005) and real-time PCR (Sharma *et al*., 2012), have been developed; they need to be further evaluated and validated, in comparison with the standard PCR using TBR primers; be that as it may, these techniques are more expensive than the classical PCR.

DNA preparation is an important step that determines the success and the sensitivity of the PCR. It can be done on plain blood (generally collected with anticoagulant), or, preferably, on the buffy coat to increase the sensitivity of the test (Desquesnes & Davila, 2002). Several classical techniques are available (Penchenier *et al*., 1996), including commercial kits and the classical phenol–chloroform preparation. Blood conserved 1/1 in 70% alcohol, or on dry filter paper can also be used (Hopkins *et al*., 1998).

Being dependent on the amount of DNA available, the sensitivity of the PCR is proportional to the parasitaemia. PCR is thus more sensitive in highly susceptible hosts (camels, horses, dogs, etc.) than in hosts of mild or low susceptibility (cattle, buffalo, pigs, etc.). Using a suitable DNA preparation and the most sensitive primers available (TBR), PCR can detect as few as 1–5 trypanosomes/ml of blood (Panyim *et al.,* 1993; Penchenier *et al.,* 1996), or only 10 per ml in buffaloes with a quantitative real-time PCR.

Table 2. Primers for* Trypanozoon,T. evansi *and* Trypanosoma *spp. characterisation

Specificity	Primer sequences (5' → 3')	References
Trypanozoon: *T. b. brucei, T. b. gambiense, T. b. rhodesiense, T. evansi & T. equiperdum*)	TBR1: CGA-ATG-AAT-ATT-AAA-CAA-TGC-GCA-G	Masiga *et al.,* 1992
	TBR2: AGA-ACC-ATT-TAT-TAG-CTT-TGT-TGC	
T. evansi (type A) and some *T. brucei & T. equiperdum*	RoTat1.2F: GCG-GGG-TGT-TTA-AAG-CAA-TA	Claes *et al*., 2004
	RoTat1.2R: ATT-AGT-GCT-GCG-TGT-GTT-CG	
T. evansi (type B)	EVAB1: CAC-AGT-CCG-AGA-GAT-AGA-G	Njiru *et al.*, 2006
	EVAB2: CTG-TAC-TCT-ACA-TCT-ACC-TC	
T. evansi and some *T. brucei & T. equiperdum*	TEPAN1: AGT-CAC-ATG-CAT-TGG-TGG-CA	Panyim *et al*., 1993
	TEPAN2: GAG-AAG-GCG-TTA-CCC-AAC-A	
T. evansi and some *T. brucei & T. equiperdum*	ESAG6/7F: ACA-TTC-CAG-CAG-GAG-TTG-GAG	Holland *et al*., 2001
	ESAG6/7R: CAC-GTG-AAT-CCT-CAA-TTT-TGT	
T. brucei gambiense	Tgs-GP F: GCT-GCT-GTG-TTC-GGA-GAG-C	Radwanska *et al.*, 2002b
	TgsGP R: GCC-ATC-GTG-CTT-GCC-GCT-C	
T. brucei rhodesiense	Tbr F: ATA-GTG-ACA-AGA-TGC-GTA-CTC-AAC-GC	Radwanska *et al.*, 2002a
	Tbr R: AAT-GTG-TTC-GAG-TAC-TTC-GGT-CAC-GCT	
Pan-tryp.: *T.vivax, Trypanozoon, T.congolense* savannah forest, Kilifi, *T. lewisi,* etc.	TRYP1S: CGT-CCC-TGC-CAT-TTG-TAC-ACA-C	Desquesnes *et al*., 2002
	TRYP1R: GGA-AGC-CAA-GTC-ATC-CAT-CG	
Pan-tryp.: *T. vivax, Trypanozoon, T. congolense* savannah forest, Kilifi, etc.	ITS1 CF: CCG-GAA-GTT-CAC-CGA-TAT-TG	Njiru *et al.*, 2005
	ITS1 BR: TTG-CTG-CGT-TCT-TCA-ACG-AA	

PCR offers high sensitivity and specificity required for detection of trypanosome infection (Masiga *et al.,* 1992), but it may give false-negative results. Experimental studies in sheep have shown that PCR can remain negative for long intervals during aparasitaemic periods (Desquesnes, 2004), while in buffalo the diagnostic sensitivity of a PCR was only 78%, which is similar to mouse inoculation (Holland *et al*., 2001). Nevertheless, PCR is the most sensitive technique for detection of active infection.

1.5. Antigen detection

Circulating antigen detection in blood or serum is also a way to detect active infection. Several attempts to develop such tests have not yet reached a satisfactory level to be recommended for routine diagnosis (Desquesnes, 2004).

2. Serological tests

Many historic serological methods have been used but are no longer recommended. The IFAT (Desquesnes 2004) is still useful for small-scale surveys. The trypanolysis test (Van Meirvenne *et al*., 1995) is used for individual confirmation of positivity because of its high specificity. The other tests are no longer used because they have been replaced by the more easily standardised techniques of ELISA (Desquesnes, 2004; Reid & Copeman, 2003) and CATT (Bajyana Songa & Hamers, 1988; Njiru *et al*., 2004) are the methods of choice in most circumstances.

Evaluations of ELISA and CATT have been carried out in camels, horses, cattle, buffaloes and pigs (Desquesnes *et al.,* 2009; Holland *et al*., 2005; Reid & Copeman, 2003, amongst others). Tests should preferably be carried out on plasma or serum, but the collection of samples can be simplified by using filter paper blood spots for later use in the ELISA, while for the CATT whole blood can be substituted for serum (Hopkins *et al*., 1998). It is vitally important that serological tests be validated and standardised if they are to be suitable for correctly identifying infected animals; cross evaluation in different laboratories is thus required. Standard criteria for interpreting the tests might have to be developed for each animal species and standardised at least at a regional level (Desquesnes, 2004). As

T. evansi is considered to be polyphyletic, having evolved from *T. brucei* (Lai *et al.,* 2008), it is necessary to take into account the various strains that may be present in a given area (RoTat *versus* non-RoTat for example).

2.1. Indirect immunofluorescent antibody test (IFAT)

Although the technique is not adapted to large-scale surveys, it is still useful to screen a small number of samples in laboratories that are carrying out the test for other purposes and/or that are not carrying out the ELISA. Cost of reagents is medium, around 0.5€/test, but the technique is time consuming.

2.1.1. Test procedure

The antigen consists of dried blood smears containing from five to ten *T. evansi* per field at magnification 500×, collected from a highly parasitaemic mouse or rat (3–4 days post-infection). Smears are dried at room temperature for 1 hour and fixed with acetone (± ethanol) for 5 minutes. When kept dry, the fixed smears may be stored at –20°C for several months. Better results are obtained using purified trypanosomes separated from the rat's buffy coat on a DEAE-cellulose column (Lanham & Godfrey, 1970) using a mixture of 80% cold acetone and 0.25% formalin in a normal saline solution.

On testing, the slides are first subdivided into several circles of 5 mm diameter with nail varnish using mounting media (Teflon-coated multispot slides may also be used), then washed in PBS, pH 7.2, at room temperature for 10 minutes.

After washing, a positive and a negative control serum and the field sera to be tested (diluted 1/50 in PBS), are added and allowed to react at 37°C for 30 minutes in a humid chamber. The slides are washed three successive times in PBS for 5 minutes each. A rabbit or goat anti-bovine IgG (for tests on bovine sera) conjugated to fluorescein isothiocyanate or other fluorescein-conjugated antiserum specific to the animal species tested is then added at a suitable dilution and left at 37°C for 30 minutes in a humid chamber. The slides are rewashed in PBS, mounted with 50% glycerol in PBS with immunofluroescence mounting media, and examined by fluorescence microscopy. The glycerol solution should be stored at 4°C and renewed every 2 weeks.

The fluorescein conjugate should be stored at –20°C in small aliquots to avoid repeated freezing and thawing. The tube should be shielded from light in some way, for example by wrapping in aluminium foil. The conjugate is diluted in PBS, pH 7.2, or in PBS containing Evans blue 1/1000 (w/v) as a counterstain to facilitate discrimination between positive (green) and negative (red) fluorescence. In general, monospecific anti-IgG (gamma-chain) conjugates give the most specific results.

The IFAT-*T. evansi* seroconversion can take 60–90 days. Compared with the CATT, IFAT is more sensitive, probably because it can detect more aparasitaemic animals, but its specificity is lower. In borderline cases, the interpretation is subjective and reproducibility has sometimes been questioned. For these reasons, ELISA is a more advisable technique.

2.2. Enzyme-linked immunosorbent assay (ELISA)

The principle of this technique is that specific antibodies to trypanosomes can be detected by enzyme-linked anti-immunoglobulins using solid-phase polystyrene plates coated with soluble antigen. The enzyme may be peroxidase, alkaline phosphatase or any other suitable enzyme. The enzyme conjugate binds to the antigen/antibody complex and then reacts with a suitable substrate to yield a characteristic colour change either of the substrate itself or of an added indicator (the chromogen).

The antigen for coating the plates is derived from the blood of a heavily parasitaemic rat. The trypanosomes are concentrated in the buffy coat by centrifugation and separated on a DEAE-cellulose column and washed three times by centrifugation in cold PSG, pH 8 (PBS with 1% glucose). The final pellet is suspended in cold PSG to a concentration of 5%, together with a protease inhibitor cocktail[2] subjected to five freeze–thawing cycles, and ultrasonicated three times for 2 minutes on ice to ensure complete disintegration of the organisms. This preparation is centrifuged at 4°C and 10,000 ***g*** for 10 minutes. The

2 For example: complete solution for protease inhibitor; Roche Molecular Biochemicals®

supernatant is collected and the protein concentration estimated by UV readings at 260 and 280 nm or by colorimetry. The soluble antigen obtained can be stored in small aliquots at –80°C for several months or at –20°C for shorter period. It can also be freeze-dried and stored at –20°C.

2.2.1. Test procedure

i) Dilute the soluble antigen at 5 µg/ml in freshly prepared 0.01 M carbonate/bicarbonate buffer, pH 9.6. Add 100 µl to each well of a 96-well microtitre plate and incubate overnight at 4°C or for 1 hour at 37°C on a shaker-incubator (300 rpm). For this step immunoplates ensuring that the specific activities of the epitopes are preserved during binding to the plate surface[3], are preferred to other plates that may allow epitopes to be obscured or impaired due to more specific binding characteristics.

ii) Remove antigen and add 150 µl of blocking buffer (BB: 0.01 M PBS containing 0.1% Tween 20 and 5% skim milk powder for 1 hour at 37°C. The quality of the skim milk is very critical[4]; optimal skim milk concentration may vary from 0.5 to 7% depending on the skim milk origin. Bovine serum albumin may also be used as blocking agent.

iii) Add test serum dilutions in BB (100 µl), in duplicate or triplicate. Include control negative and positive sera. Final dilution is made at 1/100. Incubate plates at 37°C for 30 minutes. Discard contents and wash five times with washing buffer (PBS-0.1% Tween 20).

iv) Add a specific peroxidase conjugated species-specific anti-globulin (100 µl) appropriately diluted in BB (usually between 1/5000 and 1/20,000). If species-specific conjugates are not available, protein A or protein G conjugates can be used. Incubate the plates at 37°C for 30 minutes, discard contents and wash three times with washing buffer.

v) For peroxidase conjugates a number of substrate/chromogen solutions can be used, consisting of hydrogen peroxide with a chromogen, such as tetramethylbenzidine (TMB), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or ortho-diphenylene-diamine (OPD). A suitable substrate/chromogen solution for peroxidase conjugates is 30% hydrogen peroxide (0.167 ml and 35 mg) in citrate buffer (100 ml), pH 6.0. The citrate buffer is made up as follows: Solution A (36.85 ml): (0.1 M citric acid [21.01 g/litre]); Solution B (65.15 ml): (0.2 M, Na_2HPO_4 [35.59 g/litre]); and distilled water (100 ml). Dissolve 10 mg TMB in 1 ml dimethyl sulphoxide and add to 99 ml of the citrate buffer. A number of these combinations are available commercially in ready-to-use formulations that remain stable at 4°C for up to 1 year. Add the substrate chromogen (100 µl) to the plates and incubate in the dark, at room temperature for 20–30 minutes.

vi) Read the plates or stop the reaction by adding 50 µl 1 M sulphuric acid. Read the absorbance of each well at 450 nm for TMB chromogen. Other chromogens may require the use of a different wavelength. All tests should include three known high, medium and low positive and negative control sera, and a buffer control. Results are expressed in relative percentage of positivity (RPP) based on the optical densities of the control samples (Desquesnes, 2004; Desquesnes et al., 2009).

A large variety of other test procedures exists, for example, using purified native antigen or, more recently, using recombinant antigens (Tran *et al.,* 2009). For closely related animal species, cross-reacting reagents may often be used (e.g. anti-bovine immunoglobulin for buffalos) and the use of monospecific anti-IgG conjugates is generally recommended. However, when specific conjugates are not available, nonspecific proteins able to fix on the Fc fragment of the immunoglobulins can be used, such as protein A or protein G. Protein A conjugate has been validated for use in camels (Desquesnes *et al*., 2009).

There is a number of methods that can be used to determine a cut-off point to discriminate between positive and negative results. The simplest method is to base the cut-off on visual inspection of the test results from known positive and negative populations (Desquesnes, 2004). These results are likely to show some overlap. The operator can choose the most appropriate point to adjust the rates of false-positive and false-negative results depending on the required application of the assay. An alternative is to base the cut-off on the mean + 2 standard deviations (SD) or + 3 SD values from a large sample of

3 For example: Polysorp Nunc® immunoplates

4 For example: ref: 190-12865, Wako Pure Chemical Industries Ltd, Osaka, Japan.

negative animals. Finally, if no suitable negative/positive samples are available, a cut-off can be based on the analysis of the data from animals in an endemic situation. If a bimodal distribution separates infected from uninfected animals, then an appropriate value can be selected. The ELISA is likely to correctly identify uninfected animals (while the CATT would correctly qualify infected ones). A new ELISA/RoTat 1.2 based on the VSG from a *T. evansi* RoTat 1.2 clone – a predominant antigen in *T. evansi* (Verloo *et al.,* 2001) – was successfully used in the field in Vietnam; protocols and reagents are available from the WOAH Reference Laboratory in Belgium for use in equines, camelidae and water buffaloes. Another test based on invariant surface glycoprotein has recently been developed at the ITM (Tran *et al.,* 2009) and should proceed to inter-laboratory evaluation.

The VSGs may be too specific to be used as antigen in a universal ELISA *T. evansi* (see below RoTat *versus* non-RoTat parasites), while the ELISA using soluble antigens is not strain specific and this qualifies it as a universal test. Soluble antigens from whole lysate of *T. evansi* are able to detect immunoglobulins directed against *T. evansi* strains present in various host species and geographical areas; they can also detect infections in heterologous systems owing to strong cross reactions with *T. vivax*, *T. congolense* and even *T. cruzi. Trypanosoma evansi* soluble antigen must then be considered as a universal reagent for detection of *T. evansi*, but consideration must be given to species specificity in multispecies areas. The cost of reagents is low, around 0.1€/test, and the technique is fast, allowing 500 samples to be tested a day by experienced technicians.

2.3. Card agglutination tests

It is well known that certain predominant variable antigen types (VATs) are expressed in common in different strains of salivarian trypanosomes from different areas. This finding was used as a basis for a test for the diagnosis of *T. evansi*, the card agglutination test – CATT/*T. evansi* (Bajyana Songa & Hamers, 1988). The test makes use of fixed and stained trypanosomes of a defined VAT known as RoTat 1.2. Both variable and invariable surface antigens can be aggregated by specific antibodies (so-called agglutinins), responsible for the agglutination reaction. The CATT/*T. evansi* is commercially available in kit form from the WOAH Reference Laboratory in Belgium. It consists of lyophilised stained parasites ('antigen'), PBS, pH 7.4, plastic-coated cards, spatulas, lyophilised positive and negative control sera and a rotator. The lyophilised antigen can be stored at 2–8°C for up to 1 year. Reconstituted antigen can be stored at 2–8°C for 1 week, but preferably should be used within 8 hours when kept at 37°C.

For screening, dilute test sera 1/4 or 1/8 in PBS. Add 45 µl of the prepared antigen suspension (previously well shaken to homogenise the parasite suspension) onto circles inscribed on the plastic cards. Add 25 µl of each diluted test serum. Mix and spread the reagents with a spatula and rotate the card for 5 minutes using the rotator provided in the kit (or at 70 rpm on a classical rotor agitator). Compare the pattern of agglutination with the illustrations of different reactions provided in the kit. Blue granular deposits reveal a positive reaction visible to the naked eye. The cost of reagents is medium, around 200 tests can be carried out a day by one technician.

The CATT/*T. evansi* is suitable for the detection of acute and chronic infections with a high positive predictive value. CATT/*T. evansi* is more likely to classify correctly truly infected animals; it can be used to target individual animals for treatment with trypanocidal drugs. The sensitivity of CATT *T. evansi* is variable from a host species to another; it is generally high in horses, medium in camels and buffaloes, and lower in pigs and cattle (Desquesnes *et al.,* 2011; Hagos *et al.*, 2009; Holland *et al.*, 2005), and it may be affected by the presence of non-RoTat 1.2 strains in some areas (Hagos *et al*., 2009; Njiru *et al.,* 2004). Animals infected by *T. evansi T. equiperdum, T. bucei* and even *T. vivax* can give a positive response to the CATT/*T. evansi* (Birhanu *et al.*, 2015; Desquesnes, 2004; Gizaw *et al.,* 2017). CATT/*T. evansi* is consequently not considered as species specific. Besides this, nonspecific agglutination can be observed in populations of hosts that are not infected by trypanosomes, with variable rates depending on the host species. Nevertheless, CATT/*T. evansi* being (i) the only kit available for surra diagnosis, (ii) possibly used in all mammal species, and (iii) presenting a high positive predictive value, it is recommended in the serodiagnosis of surra.

2.4. Immune trypanolysis test RoTat1.2 (TL RoTat1.2)

Immune trypanolysis test detects specific 'trypanolytic' antibodies directed against a given parasitic strain able to induce trypanolysis in the presence of complement. It is performed with T. evansi variable antigen type RoTat 1.2 and may therefore be positive only with hosts that produce trypanolytic

immunoglobulins directed against RoTat 1.2 VAT (Van Meirvenne *et al.,* 1995). Sera are tested at a 1/4 dilution. Live trypanosomes are incubated for 60 minutes with test serum in the presence of guinea-pig serum as the source of complement. When variant specific antibodies are present in the serum, lysis of the RoTat 1.2 trypanosomes occurs. The sample is considered positive for the presence of anti-RoTat 1.2 antibodies when 50% or more of the trypanosomes are lysed. This test requires the production of complement and the growth of trypanosomes in rodents and is thus costly and ethically concerning. At present, it is mostly used to confirm samples suspected to be positive using other tests. It can be carried out at the ITM, Antwerp, on request. The cost of the test is high.

2.5. Formol-gel test

The formol-gel test is the test of choice in camels but has not been validated in other species. It is carried out by adding two drops of concentrated formalin solution (40% formaldehyde [w/v]) to 1 ml of serum. The test is positive if the serum coagulates immediately and turns white. In negative reactions, the serum remains unchanged or coagulation may take up to 30 minutes to appear.

3. Test applications

Like the majority of biological tests, the methods described above are limited both in terms of sensitivity and specificity. Moreover, test performances and parameters are highly variable, depending on the host species and breed, the species diversity of trypanosomes in the geographical area in which the host occurs, and the epidemiological situation (epizootic/enzootic/sporadic). To date, there is no common test (parasitological, serological or even molecular) that is capable of distinguishing *T. evansi* from the other *Trypanozoon* species or sub-species (Gizaw *et al.,* 2017). The final diagnosis of surra will depend on epizootiological information, clinical signs and laboratory results. For these reasons, a number of test combinations adapted to the different circumstances relevant to a particular host species and geographical area are currently recommended. These are guidelines that should be helpful in achieving the correct diagnoses. Combining at least two of the four methods below is recommended to optimise reliable and specific diagnosis.

3.1. Characteristics and performances of recommended tests for the diagnosis of surra

i) *Microscopic examination:* Microscopic observation (×400–1000 in oil immersion) of a Giemsa-stained thin blood smear (GSBS) from the host, or from a mouse inoculation test, allows identification of the subgenus *Trypanozoon* based on morphology and morphometry of parasites. When fresh samples are available, it is recommended to combine with HCT (or BCT) to increase the sensitivity of microscopic examinations. Observation of a parasite provides a certain diagnosis, however, species identification requires complementary tests if other pathogenic *Trypanosoma* spp. may potentially be present in the host investigated.

ii) PCR-TBR: DNA must be prepared from blood with a commercial kit, a resin, or phenol chloroform method, using a buffy coat obtained by 8000 ***g*** centrifugation of 0.5 ml of blood. PCR is carried out as described above, with TBR primers (Masiga *et al.,* 1992). Result is positive for *Trypanozoon* when a 177 bp product is visible on the agarose gel. Complementary primers can be used (i) to confirm the subgenus (TEPAN or ESAG), (ii) to characterise type A/B: (RoTat1.2 and EVAB), (iii) to characterise human strains (Tgs-GP and Tbr), or (iv) as evidence of other *Trypanosoma* subgenera or species (ITS1 primers); however, the sensitivity of all these primers being lower than that of TBR primers, when negative, they may lead to inconclusive results.

iii) ELISA *T. evansi:* Serum or plasma samples are tested in ELISA-*T. evansi* (soluble antigens from whole *T. evansi* lysate) as described above. A sample is positive when its RPP is above the cut off value established for the host species (appropriate conjugates are defined for each species; see below). Its negative predictive value is very high, unless the host was very recently infected.

iv) CATT/*T. evansi:* Serum or plasma are diluted 1:4 and tested as described by the manufacturer. Positive samples are samples presenting results = or > to one + (doubtful samples are considered as negative samples). Positive predictive value is high, however, nonspecific agglutination may occur. It is generally recommended to combine ELISA *T. evansi* and CATT/*T.evansi* to increase sensitivity and diagnosis reliability; however, in case of discrepancy, it is recommended to repeat sampling and testing.

Conjugates to be used in ELISA *T. evansi* for each host species are: Cattle and buffalo: anti-bovine IgG whole molecule; Pig and elephant: Protein G conjugate; Camels: Protein A conjugate; Dog: anti-dog conjugate; Goat and sheep: anti-goat and sheep conjugates; Rat: anti-rat IgG whole molecule. Conjugates remain to be defined for other host species.

3.2. Association of recommended tests for the diagnosis of surra in animals

3.2.1. Recommended method for sensitive agent detection

A combination of the three techniques GSBS, HCT and a sensitive PCR (based on satellite DNA detection) is recommended for agent detection; when positive, they should be completed with more specific PCR tests. In case of potential mixed trypanosome infections, other primers should be deployed, as indicated in Chapter 3.4.14 *Nagana: infections with salivarian trypanosomoses* (*excluding* Trypanosoma evansi *and* T. equiperdum).

3.2.2. Recommended methods for antibody detection

A combination of ELISA *T. evansi* using soluble antigens from whole trypanosome lysate, and CATT *T. evansi* (RoTat1.2 based test) is recommended in order to potentially detect antibodies directed against all types of *T. evansi*. In case of potential mixed trypanosome infections, because of bilateral cross-reactions amongst trypanosomes, inferences on species-specificity must preferably be based on PCR results.

C. REQUIREMENTS FOR VACCINES

No vaccines are available for this disease.

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* *

NB: There are WOAH Reference Laboratories for surra (*Trypanosoma evansi* infection)
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for surra

NB: FIRST ADOPTED IN 1991 AS SURRA (*TRYPANOSOMA EVANSI*). MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.1.22.

TRICHINELLOSIS (INFECTION WITH *TRICHINELLA* SPP.)

SUMMARY

Eating raw or undercooked meat of domestic animals or game containing infective Trichinella *spp. larvae can cause trichinellosis in humans. Animals can become infected by feeding on* Trichinella*-infected tissues. Ingested infective larvae mature and reproduce in the small intestine of host species including humans, pigs, rats, bears, walruses, horses and many other not-strictly herbivorous mammals, birds and reptiles. The adult worms survive less than 1 month. The larvae they produce migrate to and persist in the muscles of their hosts, serving as a source of infection for susceptible new hosts.*

Detection and identification of the agent: *Tests for detecting* Trichinella *spp. fall into two categories: 1) direct detection of first-stage larvae encysted or free in striated muscle tissue, and 2) indirect detection of infection with, or exposure to,* Trichinella *spp. by tests for specific antibodies.*

Tissue digestion and tissue compression methods have been used for the direct detection of Trichinella *larvae in tissues.* Trichinella *larvae usually localise in higher concentrations in preferred muscles, which may vary by host species. It is important that preferred muscles be sampled to maximise test sensitivity. For example, in pigs, the diaphragm (crus),* masseter *and tongue muscles are the most preferred sites in order of preference, whereas in horses, the tongue, masseter, and diaphragm typically harbour the most larvae, followed by neck muscles.*

The artificial digestion methods involve enzymatic digestion of individual or pooled muscle tissue samples incorporating mechanical homogenisation or grinding, stirring, and incubation. This is followed by filtration and sedimentation procedures to recover and concentrate any larva that are released from the muscle tissue during digestion. Samples processed by these methods are examined under a stereomicroscope for the presence of larvae. Digestion tests can detect <1 larva per gram (lpg) of tissue, but at these low levels of infection, the amount of digested muscle and uneven distribution of larvae within tissues (as well as reduced digestibility of some tissues and frozen or otherwise non-optimal wildlife samples) are limiting factors. This can be compensated for by testing larger samples per carcass, such as a minimum of 3–5 g for pigs and 5–10 g for horses, game and indicator wildlife species such as foxes. Digestion methods are recommended for the inspection of individual carcasses of food animals such as pigs, horses and game.

The compression method (trichinoscopy) is less sensitive than artificial digestion and is not recommended as a reliable test for inspection of carcasses for either food safety or surveillance purposes.

Serological tests: *Serological assays are used for indirect detection. The sensitivity and specificity of serological methods are mainly dependent upon the type and quality of antigens used. Most serological test performance (validation) data are from pigs. False-negative serological results may occur 1 week or longer after muscle larvae become infective in pigs with light or moderate infections. False-positive results have also been reported for serological tests. For surveillance or verification of* Trichinella*-free herds, serological methods are acceptable. For individual carcass inspection, only direct methods can be recommended. Pigs harbouring as few as one larva/100 g of tissue have been detected by enzyme-linked immunosorbent assays (ELISA). Excretory/secretory antigens collected by short-term (18 hours) maintenance of* T. spiralis *muscle larvae* in vitro *currently provide the most specific source. It is critical that appropriate positive and negative control sera be used to ensure that ELISAs being performed have acceptable sensitivity and specificity. Positive results obtained by*

ELISA should be confirmed by Western blot. The digestion of 100 g or more of tissue is also recommended as a confirmatory test for serologically positive animals.

Requirements for vaccines: *There are no available vaccines for* Trichinella *infection in food animals.*

A. INTRODUCTION

Clinical signs of *Trichinella* infection are not generally recognised in animals, and its main importance is as a zoonosis. Eating raw or undercooked meat of domestic animals or game containing infective *Trichinella* spp. larvae can cause trichinellosis in humans (Gajadhar *et al.*, 2006). The short-lived adult worms reside in the small intestine of host species including humans, pigs, rats, bears, walruses, horses, many other not-strictly herbivorous mammals, and some birds and reptiles. The parasite has a direct life cycle completed within a single host. Within hours following consumption of tissue with infective larvae by a suitable host, first-stage muscle larvae (L1) are released by digestion and burrow into the villi of the small intestine. They develop into adults (males up to 1.8 mm long, females up to 3.7 mm long) and survive for less than 1 month. The ovo-viviparous females release new-born larvae (NBL), which migrate via venules and lymphatics into the general circulation. The NBL are distributed throughout the body where they invade striated muscle cells and develop into infective first-stage larvae, with a predilection for specific muscle groups, which vary by host species.

For example, in pigs, the diaphragm pillar, masseter and tongue usually contain the highest concentrations of larvae and are thus sampled in that order of preference, while in horses the order of preference is tongue, masseter, diaphragm, and neck muscles. Predilection sites vary by host species, but in most species, diaphragm, masseter and tongue are optimal sites for sampling. Current knowledge on predilection sites is available for several host species (Gajadhar *et al*. 2019; ISO, 2015). In cases of severe infection, striated muscles contain high numbers of larvae. The larvae of most *Trichinella* species become encapsulated in collagen in host musculature where they can remain infective for years.

Within the genus *Trichinella*, thirteen taxa have been identified, ten of which have been designated as species (Pozio & Zarlenga, 2021; Zarlenga *et al.*, 2020). Taxa in this genus are separated into two groups (clades); one characterised by larvae that encapsulate only in mammalian muscles, and one characterised by larvae that do not encapsulate in the muscles and infect both mammalian and avian hosts or mammalian and reptilian hosts. Encapsulating taxa include the following: *Trichinella spiralis* (T1), which has a widespread distribution and is commonly associated with domestic pigs. It is highly infective for domestic and sylvatic swine, mice and rats, but it has also been detected in mammalian carnivores and horses; *Trichinella nativa* (T2) occurs commonly in mammalian carnivores of arctic and sub-arctic regions of North America, Europe and Asia (Oksanen *et al*., 2022). It is highly resistant to freezing and has been experimentally shown to have poor infectivity for pigs, however it has been found in wild boar; *Trichinella britovi* (T3) is found predominantly in wild mammals and pigs, and occasionally in horses and occurs in temperate regions of Europe, Western Asia, and in Northern and Western Africa. *Trichinella murrelli* (T5) is found in mammalian carnivores of North America. It has low infectivity for domestic pigs, and has been reported in horses (Scandrett *et al*., 2018). *Trichinella* T6 is cold-climate adapted, is closely associated with *T. nativa* in northern North America, and is also highly resistant to freezing (Zarlenga *et al*., 2020). *Trichinella nelsoni* (T7) has been isolated from mammalian carnivores and sporadically from wild pigs in Eastern and South Africa. *Trichinella* T8 has been detected in mammalian carnivores of Namibia and South Africa and *Trichinella* T9 in mammalian carnivores of Japan (Zarlenga *et al*., 2020). T8 and T9 share some intermediate characteristics with *T. britovi* and *T. murrelli*, respectively. *Trichinella patagoniensis* (T12) has been isolated from mountain lions of Argentina, and experimentally shown to have poor infectivity for pigs and rodents (Krivokapich *et al*., 2012). In 2020, a new taxon, named *Trichinella chanchalensis* (T13), was described from wolverines (*Gulo gulo*) of north-western Canada (Sharma *et al*., 2020). Non-encapsulated taxa include the following: *Trichinella pseudospiralis* (T4) is widespread in distribution and has been recovered from raptorial birds, wild carnivores and omnivores, including domestic and wild pigs, and rats and marsupials in Asia, North America, Europe and Australia (Pozio, 2016); *Trichinella papuae* (T10) has been reported from wild and domestic pigs and farmed crocodiles in Papua, New Guinea, Thailand and Australia; *Trichinella zimbabwensis* (T11) has been described in farmed and wild crocodiles of Zimbabwe, South Africa, Ethiopia and Mozambique, in monitor lizards of Zimbabwe and in mammalian carnivores of South Africa. Experimentally, it has high infectivity for a wide spectrum of mammalian hosts including pigs and rats (Pozio & Zarlenga, 2021). Most species and genotypes of *Trichinella* have been detected in humans, and it is generally accepted that all taxa of *Trichinella* are highly infective for people, representing a significant public health risk. The risk of establishing *Trichinella* infection in pig herds is presented mainly by *T. spiralis,* and to a lesser degree by *T. britovi, T. nelsoni, T. pseudospiralis, T. papuae*, and *T. zimbabwensis,* whereas there is no evidence that other species and genotypes can play such a role.

Human trichinellosis can be a debilitating disease and may result in death. The short-lived adult worms in the intestine can cause transient gastroenteritis, but the most severe signs and symptoms result from the migration and establishment of the larvae in striated muscle. The disease is transmitted primarily by eating meat of infected pigs or game that has not been sufficiently cooked (or otherwise treated to inactivate the parasite). Although the prevalence of *Trichinella* infection in horses is low, consumption of raw or undercooked horsemeat is a well documented source of human trichinellosis (Boireau *et al.*, 2000). Prevention of human infection is accomplished by meat inspection, by adequate processing (thorough cooking is the most reliable means of inactivating *Trichinella* spp.; freezing or curing of meat can also be effective depending on the genotype being targeted and if a validated procedure is used), and by preventing the exposure of food animals to tissues harbouring *Trichinella* larvae, including uncooked food waste, rodents and wildlife (Gajadhar *et al.*, 2006; Noeckler *et al*., 2019). Meat from non-strictly herbivorous game species should always be considered a potential source of infection, and should be tested or properly cooked. *Trichinella* found in game meats (mainly *T. nativa*, T6, and to a lesser degree *T. britovi*) may be resistant to freezing and therefore untested, frozen game poses a public health risk. *Trichinella* parasites circulate mainly among wild animals; among domestic animals, they usually infect only free-ranging and backyard pigs and rarely horses; *Trichinella*-infected horse and game meat has been implicated in outbreaks linked to international trade. The illegal importation of pig and wild boar meat in personal baggage has been the source of many trichinellosis outbreaks (Pozio, 2021).

Testing methods for the detection of *Trichinella* infection in pigs and other species include either: (a) direct demonstration of the parasite in tissue samples; or (b) indirect demonstration of the presence of, or exposure to, the parasite by detecting specific antibodies to *Trichinella* spp. in blood, serum or tissue fluid samples (Gajadhar *et al.*, 2009).

Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). The risk of laboratory-acquired infection for analysts is minimal if good laboratory practices are followed. Transmission occurs by the ingestion of muscle larvae in tissues or freed by artificial digestion. Naked larvae die quickly when exposed to the environment or commonly used disinfectants. Contaminated glassware and other surfaces should be cleaned with water at ≥85°C or other suitable processes to lyse and remove all larvae. Laboratory waste, including sample remnants, should be treated by boiling, autoclaving, incineration or other suitable processes to kill larvae and prevent their re-introduction into the environment. This is particularly critical when testing proficiency samples containing live larvae in a non-endemic region.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for detecting* Trichinella *infections in pigs and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of positive cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[a]						
Artificial digestion	+	–	–	+++	+++	–
PCR[b]	–	–	–	++	++	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of positive cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
ELISA	+++	+	+	–	++	–
Western blot[(c)]	++	+	+	–	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.
[(b)]PCR is used as a confirmatory test and for species-determination.
[(c)]Western blot is used as a confirmatory test for ELISA positives.

1. Detection and identification of the agent

The only recommended procedures for the detection of *Trichinella* larvae in muscle tissues are enzymatic digestion assays. A number of digestion assays are officially recognised in various countries for trade purposes. The International Commission on Trichinellosis (ICT, http://www.trichinellosis.org; Gajadhar *et al*., 2019; Noeckler *et al*., 2019) recommends several of these assays, which are documented standards in the European Union (EU), Canada and elsewhere (European Commission, 2015; Forbes & Gajadhar, 1999). Other methods are not recommended because of their lack of efficiency or reliability. Modern diagnostic assays should meet internationally accepted standards of quality assurance, which include scientifically derived validation data and a design that allows routine monitoring and documentation of critical control points. An International Organization for Standardization (ISO) standard (18743:2015) for the detection of *Trichinella* larvae in animals has also been published (ISO, 2015). The digestion assay recommended here is based on desirable innovations inherent in some digestion assays that are accepted for international trade purposes.

1.1. Recommended direct procedure for testing muscle tissue

1.1.1. Sensitivity

The sensitivity of direct testing methods depends on the amount of tissue examined and the site from which the sample was obtained. Direct methods can identify infected pigs, horses or other animals infected with *Trichinella* sp. as early as 17 days after exposure, coincident with the time that muscle larvae become infective for a new host. Direct methods are most sensitive on fresh samples. The number of larvae that can be recovered from samples declines unpredictably after prolonged storage, putrefaction and freezing (particularly for freeze-susceptible taxa). Samples tested for food safety-related purposes should be stored at 4°C and tested as soon as possible. For wildlife, larger samples (≥10 g) should be tested to compensate for a possible decrease in sensitivity due to unknown variation of predilection sites in these host species, as well as more variable storage conditions. Current methods for testing fresh samples for food safety or individual animal inspection by artificial digestion and employing a 1 g sample have a sensitivity of approximately three larvae per gram (lpg) of tissue, and testing of a 5 g sample increases sensitivity to 1 lpg of tissue (Gajadhar *et al*., 2019; Noeckler *et al*., 2019). Where large amounts of tissue (up to 100 g) are available for digestion, the sensitivity is further increased.

1.1.2. Sampling

Tests are conducted on carcass samples collected post-mortem. Muscle samples are taken from predilection sites, usually the diaphragm pillars, masseter, or tongue of pigs, and tongue, masseter, or diaphragm muscles of horses. For wildlife species in which predilection sites are unknown, tongue (preferred), masseter or diaphragm should be taken. The anterior tibial muscle is a predilection site for foxes. The sample sizes for food safety testing are based on the reliable detection of animals harbouring ≥1 lpg in tissue, but for surveillance purposes, a higher sensitivity

is required to provide more accurate infection prevalence data and to overcome sampling limitations such as those encountered with wildlife. Surveillance samples taken from predilection sites (if known) should be ≥10 g. Samples of 100 g would enable the detection of as low as 0.01 lpg in the source tissue and if the sample was obtained from a predilection site, a low larval burden or negative result would indicate a negligible load in the rest of the carcass, with an associated low risk for transmission. For food safety testing, each digestion assay can accommodate up to 100 g of muscle tissue. Individual samples of 100 g may be taken from a single animal, or multiple samples of lesser amounts may be collected from a number of animals to make a 100 g pool. The size of the samples from each carcass that contribute to the pool will determine the sensitivity of the method per sample. The ICT recommends 5 g samples per pig for testing in endemic areas (Gajadhar *et al.*, 2019). For testing horsemeat, a minimum of 5 g per carcass is required. For horses originating from endemic areas, or if horsemeat is consumed raw, a 10 g sample is recommended (Gajadhar *et al.*, 2019). Testing these amounts of muscle should prevent trichinellosis in humans but will not prevent asymptomatic infections from the consumption of meat infected with very low numbers of larvae.

1.1.3. Confirmatory testing of pooled digestion samples and serologically positive animals

When a pool of samples from different animals is digested and yields a positive result, additional digestion tests should be used to retest pools of samples from fewer animals and eventually individual animals to determine the identity of the infected animal(s). Animals that are positive on serological testing should have tissues tested by digestion to confirm infection status and to facilitate recovery of larvae and species identification.

1.1.4. Digestion and detection

i) Determine the volume of digestion solution required for the digestion (2000 ml for 100 g of meat, and 1000 ml for 50 g or less).

ii) Digestion solution for 100 g of meat: Prepare the solution, in a 3 litre glass beaker, by adding 16 ml of 25% hydrochloric acid to 2 litres of tap water preheated to 45°C ± 2°C. Stock solutions of hydrochloric acid are available in formulations other than 25% and are to be adjusted accordingly (e.g. use 11 ml of a 37% stock solution). Place a stirring rod in the beaker, place the beaker on a magnetic stirrer, and commence the stirring. Add 10 g of powdered or granular pepsin (1: 10.000 NF) or 30 ml of liquid pepsin (660 U/ml). Once the solution is prepared, retain a sufficient amount in a separate vessel to be used for rinsing as described in step vii below. A serine protease-based *Trichinella* digestion assay kit, that does not use hazardous reagents, is commercially available as an alternative to the pepsin/HCl assay and has been approved by the EU for the testing of pigs only (further information is available from the WOAH Reference Laboratory in Italy[1]).

iii) Remove as much fat, fascia and non-muscle tissue as possible from each sample of meat, including the tongue epithelium when that sample is used.

iv) Weigh the appropriate amount of trimmed meat from each sample. Cut each sample into 1–2 g pieces and pool with other samples into a 100 g amount.

v) Place the pooled meat sample into a blender and add a small amount (50 to 100 ml per 100 g meat) of digest fluid to facilitate homogenisation.

vi) Cover and blend the meat only until it is homogeneous (no chunks of meat should be present and the sample should be the consistency of pureed baby food or coarse paté), do not over-blend as that risks damaging any larvae.

vii) Transfer the homogenised sample into the glass beaker containing the stir bar and digestion solution. To avoid loss of larvae due to adhering muscle tissue, the blending equipment should be thoroughly rinsed with the digest fluid that was set aside in step ii) above and which is then poured back into the beaker.

viii) Place the beaker on a preheated magnetic stirrer hotplate or in an incubation chamber set at 45 ± 2 °C. Cover the beaker with aluminium foil. Activate the stirrer at a sufficiently high speed to create a deep vortex without splashing. Note: If the digest temperature at the

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories

beginning of digestion is below 45 ± 2°C, the sample should be allowed to warm to this temperature before the timing of the digestion is started, if feasible. Regularly monitor the temperature of the digestion fluid using a thermometer, particularly when using a hotplate.

ix) Allow the digestion to proceed for 30 minutes. For samples from host species other than domestic pigs, or that are less digestible than diaphragm, the minimum period that enables complete digestion may be longer. As well, if the temperature of the digest has fallen below 45 ± 2°C, additional digestion time may be required to complete the digestion. This can be determined by observing the digestion mixture. If pieces of undigested muscle tissue are present, the digestion time can be increased but should not exceed 60 minutes in total. Care should be taken to ensure that the digestion temperature range is not exceeded (45 ± 2°C).

x) Within 5 minutes of removal from the magnetic stirrer on hotplate or from incubation chamber, pour the digestion fluid through a 180–200 μm sieve into a 2.5 litre or larger separatory funnel with a height to width ratio of about 2:1 and preferably with polytetrafluoroethylene (PTFE) safety plugs (stopcocks). Rinse the beaker with sufficient room temperature tap water from a squirt bottle and pour this through the sieve into the separatory funnel.

xi) Rinse the sieve into the separatory funnel by squirting a small volume of room temperature tap water through the top of the sieve. The digestion process is considered satisfactory if residual debris remaining on the sieve consists primarily of indigestible non-muscle tissue (typically consisting of fascia and connective tissue) of no greater than 5 % of the original sample mass. Allow the fluid in the separatory funnel to settle undisturbed for 30 minutes. (Although not necessary, gentle tapping of the funnel wall [e.g. every 10 minutes] can facilitate the larvae settling to the bottom of the funnel.)

xii) Drain 40 ml of digestion fluid from the separatory funnel into a 50 ml conical tube or measuring cylinder (Pilsner flask) and allow to stand for 10 minutes.

xiii) At the end of 10 minutes use a pipette to remove 30 ml of the upper part of the fluid (supernatant), leaving the bottom 10 ml in the tube undisturbed (do not pour off the upper 30 ml, as this will disturb the sediment). If the analyst considers the remaining digest fluid not clear enough to be examined, a washing step can be performed as follows: add an additional 30 ml of tap water to the 10 ml of digest fluid, allow to stand for another 10 minutes, followed by removal of 30 ml of supernatant.

xiv) Gently swirl the remaining 10 ml of digest fluid and quickly transfer it into a gridded Petri dish or larval-counting basin. Rinse the tube or cylinder into the Petri dish using an additional 10 ml of tap water. The layer of fluid in the Petri dish should not be more than a few millimetres deep.

xv) Wait a minimum of 1 minute to allow larvae to settle to the bottom of the Petri dish or counting basin, then use a stereomicroscope at 10× to 20× magnification to systematically examine each grid for the presence of *Trichinella* larvae. The detection of any suspect larvae must be confirmed by the identification of morphological details at a higher magnification such as 40×. If the sediment is still too cloudy or otherwise difficult to examine at this stage, it will require further clarification as described in step xviii).

xvi) Examination should be performed immediately after digestion; if not possible, the Petri dish containing the digest fluid can be stored refrigerated for examination later that same day.

xvii) If the digest fluid is not examined within 30 minutes of preparation, and is allowed to cool down (or has been refrigerated) it may become too cloudy to examine accurately, and may require clarification as described below.

xviii) Sample clarification: transfer the contents of the Petri dish into a 50 ml conical tube using a pipette. Rinse the Petri dish thoroughly with tap water, adding the rinse water to the conical tube, then cover the Petri dish and set aside. Add additional tap water to the conical tube to bring the volume to 45 ml. Let the contents in the tube settle undisturbed for 10 minutes.

At the end of 10 minutes use a pipette to withdraw the supernatant, leaving the bottom 10 ml undisturbed (do not pour off the supernatant, as this will disturb the sediment). Retain the supernatant for disposal or decontamination after the sample has been examined.

Repeat steps xiv and xv using the same Petri dish that contained the original sample and was set aside in step xviii)

xix) In the event of a positive or suspicious result, an additional sample should be tested from each carcass contributing to the original pooled digest. These should be digested individually or as successively smaller pools until the individual infected animals are identified.

1.1.5. Identification of the larvae

First-stage larvae, digested free from muscle cells, are approximately 1 mm in length and 0.03 mm in width. The most distinguishing feature of *Trichinella* larvae is the stichosome, which consists of a series of discoid cells lining the oesophagus and occupying the anterior half of the worm's body. *Trichinella* larvae may appear coiled (when cold), motile (when warm) or C-shaped (when dead). In case of doubt, larvae should be viewed at higher magnification and further samples should be digested. If the numbers of larvae are high, appropriate dilutions must first be made to obtain accurate counts.

Larvae recovered from muscle digestion may be stored in a small vial (1–2 ml) filled with 70–90% ethyl alcohol (final concentration) for subsequent genotyping by polymerase chain reaction (PCR) (see Section B.1.3) by a qualified laboratory.

1.1.6. Quality assurance

Laboratories using artificial digestion methods should maintain a suitable quality assurance system to ensure test sensitivity. Components of a quality assurance system for digestion testing are described elsewhere and should include regular use of proficiency testing (Forbes *et al.*, 2005; Gajadhar *et al.*, 2009; 2019).

1.2. Other direct detection methods

1.2.1. The double separatory funnel method

This assay is an alternative to the commonly used digestion procedure described above. The method was designed to operate under strict conditions of quality control, minimise technical error, and has been extensively validated for use on pork and horsemeat (Forbes & Gajadhar, 1999; Forbes *et al.*, 2008). It includes a spin-bar digestion technique and sequential separatory funnels for sedimentation of the larvae. The procedure has fewer steps, requires less time and seldom needs further clarification steps. An incubation chamber equipped with transparent glass doors and set at 45°C is used to perform the digestion. The digestion is conducted in 3 litres of digest fluid on a magnetic stirrer. Following digestion, the suspension is poured into a 4 litre separatory funnel through a 177–180 μm sieve, which is rinsed thoroughly into the separatory funnel with tap water. The suspension is allowed to settle for 30 minutes, and the bottom 125 ml then dispensed directly into a 500 ml separatory funnel. The volume in this smaller separatory funnel is increased to 500 ml by adding 375 ml of tap water, and the resultant suspension allowed to settle for 10 minutes. Finally, 22–27 ml of sediment is dispensed into a Petri dish and observed for larvae as previously described.

1.2.2. Mechanically assisted pooled sample digestion method/sedimentation technique

This method uses a heated Stomacher blender for the digestion phase, and a separatory funnel for sedimentation of the larvae (*Equivalent method A, Regulation* [*EC*] *No. 2015/1375*) (European Commission, 2015).

1.2.3. Mechanically assisted pooled sample digestion method/'on filter isolation' technique

This method uses a heated Stomacher blender for the digestion phase, and a Gelman funnel mounted on an Erlenmeyer flask connected to a filter pump for the recovery of the larvae. (*Equivalent method B, Regulation* [*EC*] *No. 2015/1375;* European Commission, 2015).

1.2.4. Automatic digestion method for pooled samples of up to 35 g

This method involves an automated digestion chamber and a membrane filter for the recovery and examination of larvae (*Equivalent method C, Regulation* [*EC*] *No. 2015/1375;* European Commission, 2015). Critical steps in digestion and larval recovery are difficult to control in the automatic method and it is not recommended by the ICT or WOAH.

1.2.5. Magnetic stirrer method for pooled sample digestion/'on filter isolation' and larva detection by a latex agglutination test

This method is only considered equivalent for the testing of meat of domestic swine. The method combines the typical digestion procedure with detection of larvae by latex agglutination (*Equivalent method D, Regulation* [*EC*] *No. 2015/1375*, European Commission, 2015).

1.2.6. Artificial digestion commercial test kit for *in-vitro* detection of *Trichinella* spp. larvae in meat samples

This serine protease-based method is considered equivalent for testing meat of domestic swine only (*Equivalent method E, Regulation* [*EC*] *No. 2015/1375*, European Commission, 2015). The kit shall be used according to the manufacturer's instructions .

1.3. Other tests

1.3.1. Polymerase chain reaction

Limited studies have shown that PCR can be used to detect the nucleic acid of larvae in the musculature of infected animals. However, this method lacks sensitivity and is not practical for routine testing of food animals. Identification of the species or genotype of *Trichinella* recovered from muscle tissue is useful in understanding the epidemiology of the parasite in animals, in assessing the relative risk of human exposure and to trace back infection to the farm of origin. Specific primers have been developed that enable the identification of single larva collected from muscle tissues at the species or genotype level by PCR (Pozio & La Rosa, 2010; Pozio & Zarlenga, 2021). Detailed guidelines for this identification of *Trichinella* muscle stage larvae have been developed by the ICT (http://trichinellosis.org/; Pozio & Zarlenga, 2019). Requests for speciation or genotyping of *Trichinella* larvae can be made to the WOAH Reference Laboratories[2]).

2. Serological tests

A variety of immunological assays have been described for the diagnosis of *Trichinella* infections in domestic and wild animals (Gamble *et al.,* 2004). An immunochromatographic strip (ICS) assay using excretory/secretory (ES) antigens derived from larval and pre-adult *T. spiralis* to detect infection in pigs has also been recently described (Wang *et al.,* 2021). Only ELISA and Western blot have been validated in accordance with WOAH standards (Bruschi *et al*., 2019). Requests for reference pig sera can be made to the WOAH Reference Laboratory in Italy (Gomez-Morales *et al.,* 2015). The ICT has provided a uniform set of recommendations for the development and use of serological tests for the detection of circulating antibodies (Bruschi *et al*., 2019). The ELISA (with confirmatory testing by Western blot as feasible) is the only immunological assay recommended by the ICT. It is only approved as an epidemiological surveillance tool to detect anti-*Trichinella* antibodies in pigs; it is not reliable for the detection of *Trichinella* infection in individual animals for food safety or other purposes.

Although other serological tests may have some practical applications, the ELISA is generally acknowledged as the test of choice based on economy, reliability, adaptability to good quality assurance practices, increasing body of validation data and good sensitivity and specificity when conducted under appropriate conditions. It is a useful tool for testing populations and is routinely used for surveillance programmes and disease outbreak investigations. Testing by a validated Western blot is recommended to confirm any ELISA-positive results (Bruschi *et al*., 2019). The digestion of 100 g or more of tissue is recommended as a confirmatory test for serologically positive animals.

2 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

2.1. Enzyme-linked immunosorbent assay (ELISA)

2.1.1. Sensitivity and specificity

Infection levels as low as one larva/100 g of tissue can be detected by ELISA in pigs (Gamble *et al.*, 2004). Thus, serological testing by ELISA is useful for detecting anti-*Trichinella* antibodies at the farm level or for more broadly based surveillance programmes. Disadvantages of serology include the low rate of false-negative results observed in infected animals as well as false-positive results, which can be quite high among backyard and free-ranging pigs and wild boar. The false negatives can be attributed to the lag time of the immune response following the ingestion of infective larvae, and false-positive results to cross-reactions with other parasites and microorganisms. Detectable antibody levels are not usually present in pigs until 3–5 weeks or longer following exposure (Gamble, 1996; Gamble & Patrascu, 1996; Pozio *et al.*, 2020). For this reason, serological methods are not recommended for individual carcass testing, particularly for food safety purposes. Serological responses in pigs persist for a long time after infection with no decline in titre (Pozio *et al*., 2020); however, antibodies have been reported to decline in horses within a few months following infection. Serological tests may therefore be of little practical use in horses as antibody titres eventually drop below detectable levels despite the presence of infective larvae in muscle (Hill *et al.*, 2007; Pozio *et al.*, 2002). Little is known of antibody responses to *Trichinella* infection in game animals and other wildlife, but high quality serum samples should be obtained to reduce the likelihood of false positive reactions. Serology validation data are available for domestic pigs, but only limited data have been reported for other animals, including studies on ELISA and Western blot for wild boar and dogs (Bruschi *et al*., 2019).

2.1.2. Samples

The use of ELISA to detect the presence of parasite-specific antibodies provides a rapid method that can be performed on serum, whole blood, plasma or tissue fluid collected before or after slaughter (Gamble & Patrascu, 1996). The dilutions used are different for serum than for tissue fluid as the antibody concentrations are typically higher in serum than in tissue fluid (Nockler *et al.*, 2005).

2.1.3. Antigens

The sensitivity and specificity of ELISA are largely dependent on the antigens used in the test. TSL-1 (*T. spiralis* L1) antigens are the main components of ES antigens and are specifically secreted from the stichocyte cells of live larvae. TSL-1 bear a common immunodominant carbohydrate epitope, which is recognised by all *Trichinella*-infected animals. The *T. spiralis* ES antigens used in the ELISA appear to be conserved in all species and genotypes of *Trichinella*, even though some differences have been detected, and should therefore enable detection of specific antibodies in pigs or other animals harbouring any of the known taxa. ES antigen preparations have been developed that provide a high degree of specificity for *Trichinella* infection in pigs (Bruschi *et al*., 2019; Gamble *et al.*, 1988).

2.1.4. Antigen production

Detection of anti-*Trichinella* antibodies by ELISA can be accomplished by using ES products of *Trichinella* larvae in culture (Gamble *et al.*, 1988). For purposes of standardisation, it is recommended that *T. spiralis* be used for antigen production for food animal testing. However, as indicated above, it has been demonstrated that antigens prepared from any of the *Trichinella* species can be used for detection of antibodies in infected animals regardless of the infecting species (Bruschi *et al*., 2019). Parasites to be used for antigen preparation may be maintained by serial passage in mice, rats or guinea-pigs.

To prepare antigens for use in the ELISA (Gamble *et al.*, 1988), *T. spiralis* first-stage larvae are recovered from skinned, eviscerated, ground mouse or rat carcasses by digestion in 1% pepsin with 1% HCl for 30 minutes at 37°C (as otherwise described in Section B.1.1.4). These larvae are washed (three times for 20 minutes each) in Dulbecco's modified Eagle's medium (DMEM) with penicillin (500 units/ml) and streptomycin (500 units/ml), and then placed (at a concentration of 5000 L1 per ml) into DMEM supplemented with HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) (10 mM), glutamine (2 mM), pyruvate (1 mM), and penicillin (250 units/ml)/streptomycin (250 μg/ml) (complete DMEM) at 37°C in 10% CO_2 in air. Culture medium is recovered after not more than 18 hours, worms are removed by filtration, and the fluid

is concentrated under pressure with a 5000 Da molecular weight retention membrane or by a centrifugal filter device using a similar molecular weight cut-off. ES antigens should be supplemented with protease inhibitors to preserve quality and may be stored frozen for short periods at –20°C or for longer at –70°C; they consist of approximately 25 protein components as determined by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis), many of which bear the diagnostic TSL-1 carbohydrate antigen epitope.

Antigen purity is critical to the specificity of the ELISA. Steps should be taken to monitor growth of bacteria visually, either by phase microscopy or by plating a sample of media. Cultures showing any bacterial growth should be discarded. Larvae should not be maintained in culture longer than 18 hours; worm deterioration after this time contributes to leaking of somatic antigens that reduce test specificity. Antigens, produced as described, should have a 280:260 nm absorbance ratio of >1.0. The antigens obtained from *in-vitro* culture of *Trichinella* larvae should be tested against a panel of known negative and positive sera before use.

2.1.5. Test procedure

An example of an ELISA for detecting *Trichinella* infection in pigs is given below. It is essential that all reagents used in the assay be standardised for optimal concentration to obtain reliable results. Typical values are indicated in the example.

i) Coat 96-well microtitre plates with 100 µl/well of *T. spiralis* ES antigens diluted to 5 µg/ml in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6). Coating is performed for 60 minutes at 37°C or overnight at 4°C.

ii) Wash antigen-coated wells three times with wash buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5.0% non-fat milk powder and 1.0% Triton X-100. Following each washing, plates are blotted dry.

iii) Dilute pig sera 1/50 or 1/100 in wash buffer. Alternative sources of antibodies that may be used in place of sera include whole blood or tissue fluids at a dilution of 1/5 or 1/10 (Nockler *et al.,* 2005). Add 100 µl of diluted sera to antigen-coated wells. A known positive and negative serum sample should be used on each plate at the same dilution as the test sera. Incubate at room temperature for 30 minutes.

iv) Wash wells three times as in step ii.

v) Add 100 µl/well of an affinity-purified rabbit anti-swine IgG–peroxidase conjugate at an appropriate dilution in wash buffer. Following the addition of the secondary antibody, incubate the plates for 30 minutes at room temperature.

vi) Wash wells three times as in step ii. Rinse once with distilled water.

vii) Add 100 µl of a suitable peroxidase substrate (e.g. 5'-aminosalicylic acid 0.8 mg/ml with 0.005% hydrogen peroxide, pH 5.6–6.0).

viii) After 5–15 minutes, read plates for colour density at 450 nm on an automated microplate reader. Values obtained in the ELISA above the established cut-off value are considered to be positive (Jacobson, 1998).

Commercial adaptations of the ELISA are available. The manufacturer must validate the kit prior to licensure and the user should also evaluate the performance of the kit, prior to use, by using selected negative and positive reference samples.

The test should be conducted within an environment in which internationally accepted standards of quality management, such as ISO 17025, have been implemented.

In addition to the use of standard reference sera, all commercial and in-house ELISAs should be evaluated against a bank of negative control sera that represents the population under test, and a group of positive animals that represents different stages of infection, as per ICT guidelines.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available for prevention of *Trichinella* infections.

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NB: There are WOAH Reference Laboratories for trichinellosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for trichinellosis

NB: FIRST ADOPTED IN 1989 AS SWINE TRICHINELLOSIS. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.1.23.

TULAREMIA

SUMMARY

Description of the disease: *Tularemia is a zoonosis caused by* Francisella tularensis*. The causative bacterium is a Gram-negative coccoid rod, 0.2–0.5 μm × 0.7–1.0 μm, non-motile and non-spore-forming organism that is an obligate aerobe with optimal growth at 37°C. It is oxidase-negative, weakly catalase-positive, and cysteine is required for growth. Tularemia is primarily a disease of the orders Lagomorpha and Rodentia, but a wide range of other mammals and several species of birds have also been reported to be infected. Haematophagous arthropods have a substantial role both in the maintenance of* F. tularensis *in nature and in disease transmission.*

The disease is characterised by fever, depression and often septicaemia. In humans, there may be ulcers or abscesses at the site of exposure (*this is rarely seen in animals*) *and swelling of the regional lymph nodes. Oropharyngeal and pneumonic infections can be caused by ingestion of contaminated food and water or inhalation of aerosols, respectively. On post-mortem examination, lesions may include caseous necrosis of lymph nodes and multiple greyish-white foci of necrosis in the spleen, liver, lungs, pericardium, kidneys and other organs. The spleen is usually enlarged in septicaemic cases.*

The disease spreads through vectors such as mosquitoes, horseflies, deer flies, and ticks. Humans have a high risk of acquiring the disease through direct contact with sick animals, infected tissues, consumption of infected animals, drinking or direct contact with contaminated water, and inhalation of bacteria-loaded aerosols. Infection control precautions (*including personal protective equipment*) *based on an assessment of the risks should be in place when handling suspected or confirmed infective materials. All laboratory manipulations with live cultures or potentially infected or contaminated material must be performed at an appropriate biosafety and containment level as determined by biological risk analysis* (*see Chapter 1.1.4* Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities).

Detection of the agent: *Polymerase chain reaction is a safe and convenient method of detection and identification of* F. tularensis *in clinical specimens. The bacterium can be demonstrated in impression smears or in fixed specimens of organs using a specific fluorescent antibody test or immunohistochemistry. With Gram staining, the bacteria appear as very small punctiform Gram-negative rods, often difficult to distinguish as bacteria.*

The organism is highly fastidious. For growth it is necessary to use Francis medium, McCoy and Chapin medium, or Modified Thayer-Martin agar. In certain cases, e.g. isolation from tissues or carcasses, the use of selective medium containing antibiotics or mouse inoculation is needed to aid successful isolation. The colonies are small, round and transparent, and do not appear before 48 hours incubation at 37°C. If transportation is necessary, samples should be inoculated into sterile nutrient broth and stored at 4–10°C for a few hours or on dry ice if transit is likely to be prolonged.

Serological tests: *Serological tests are useful for diagnosing human infection, but are of limited value in the more susceptible animal species that usually die before developing antibodies. Serological surveys can be conducted in relatively resistant species that survive the infection and develop antibodies, such as sheep, cattle, pigs, dogs, cats, wild ungulates, foxes and wild boars. Relatively resistant species of rodents and lagomorphs* (*e.g. European brown hare in Central Europe*) *can also be included in serological surveys.*

Requirements for vaccines: *The attenuated* F. tularensis *subsp.* holarctica *live vaccine strain* (*LVS, NCTC 10857*) *was used for decades as a tularemia vaccine, especially in laboratory workers handling large volumes of* F. tularensis *cultures. This vaccine is no longer used because of its overall limited*

efficacy and concern about reversion to virulence. Novel vaccines against tularemia are under development for human or animal use.

A. INTRODUCTION

Tularemia is a zoonosis caused by *Francisella tularensis.* It occurs naturally in lagomorphs (rabbits and hares) and rodents, especially microtine rodents such as voles, vole rats and muskrats, and also in beavers. In addition, a wide variety of other mammals have been reported to be infected, and the organism has been isolated from birds, fishes, amphibians, arthropods, and protozoa (Anda *et al.,* 2001*;* Gyuranecz, 2012; Morner & Addison, 2001; Yeni *et al.,* 2020). Tularemia occurs endemically in the northern hemisphere. The disease can occur as epizootic outbreaks in many countries in North America and Europe, while it occurs only as sporadic cases in some other countries in Europe and Asia. It is rarely reported from the tropics or the southern hemisphere.

The two clinically most relevant types of *F. tularensis* are recognised on the basis of culture characteristics, epidemiology, and virulence. *Francisella tularensis* subsp. *tularensis* (Type A) is mainly associated with lagomorphs in North America and is primarily transmitted by ticks or biting flies or by direct contact with infected animals. It is highly virulent for humans and domestic rabbits, and most isolates ferment glycerol. *Francisella tularensis* subsp. *holarctica* (Type B) occurs mainly in aquatic rodents (beavers, muskrats) and voles in North America, and in lagomorphs (hares) and rodents in Eurasia. It has also been found in possums in Australia. It is primarily transmitted by direct contact or by arthropods (primarily ticks and mosquitoes) but may also be transmitted through inhalation or through infected water or food. It is less virulent for humans and domestic rabbits, and does not ferment glycerol (Ellis *et al.*, 2002; Keim *et al.*, 2007; Morner & Addison, 2001).

In sensitive animals, clinical signs of severe depression are followed by a fatal septicaemia (Morner & Addison, 2001). The course of the disease lasts approximately 2–10 days, and animals are usually dead when presented for diagnosis. Most domestic species do not usually manifest signs of tularemia infection, but they do develop specific antibodies to the organism following infection. Outbreaks with high mortality caused by the Type A organism have occurred in sheep (Morner & Addison, 2001). Among domestic pets, *F. tularensis* infection can result in clinical illness in cats but less commonly in dogs (Feldman, 2003). Both have been implicated in transmission of the disease to humans. Transmission from cats to humans occurs most commonly via bites or scratches and from dogs via close facial contact, ticks, and retrieved carcasses, as well as bites (Kwit *et al.,* 2019).

At necropsy, animals that have died from acute tularemia are usually in good body condition, but signs of septicaemia characterised by whitish foci of necrosis randomly distributed in the liver, bone marrow and spleen, are evident (Morner & Addison, 2001). In addition, the spleen is usually enlarged. Necrotic foci vary in size, with some barely visible to the naked eye. The lungs are usually congested and oedematous, and areas of consolidation and fibrinous pneumonia or pleuritis may be present. Fibrin may be present in the abdominal cavity. Foci of caseous necrosis are often present in one or more lymph node(s); those most often affected are lymph nodes in the abdominal and pleural cavities and lymph nodes draining the extremities. In less sensitive species, the macroscopic picture can resemble that of tuberculosis with subacute or chronic granulomas in the lungs, pericardium, kidneys, spleen and liver. Macrophages are the dominant constituent cell type in the granulomas, but other cells including lymphocytes, heterophil granulocytes, multinucleated giant cells and fibrocytes are also found occasionally. Focal or multifocal necrosis is often observed in the centre of these lesions (Gyuranecz, 2012; Gyuranecz *et al.*, 2010).

There is a high risk of human infection from *F. tularensis*, as the infective dose is extremely low and infected animals excrete bacteria in urine. Species that are moderately susceptible to tularemia, and maintain the infection for a prolonged time, may serve as reservoirs of infection to others (Hestvik *et al.,* 2015). Infection can occur by simple contact with sick animals, infected tissues, consumption of infected animals, drinking or direct contact of contaminated water and via inhalation of infective aerosols. Hunters and forest rangers should take precautions before opening dead animals as they are at risk of infection. Suitable personal protective equipment (e.g. gloves, particulate-filtering masks or respirators, and eye-shields) must be worn during any manipulation of pathological specimens or cultures to avoid human infection. All laboratory manipulations with live cultures or potentially infected or contaminated material must be performed at an appropriate biosafety and containment level as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Experimentally inoculated animals and their excreta are especially hazardous to humans.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of tularemia and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Bacterial isolation	–	–	–	++	–	–
Antigen detection	–	–	–	+++	–	–
Conventional PCR[c]	+	–	–	+++	+	–
Real-time PCR[b]	+	–	–	+++	+++	–
Detection of immune response[d]						
SAT	+++	+++	+++	++	+++	_
TAT	++	+++	++	+++	+++	_
MAT	++	+++	++	+++	+++	_
ELISA	++	+++	++	++	+++	_

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; SAT = slide agglutination test; TAT = tube agglutination test;
MAT = microagglutination test; ELISA = enzyme-linked immunosorbent assay.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]Versage *et al.,* 2003; [c]Barns *et al.,* 2005.
[d]Serology is of limited value in susceptible animals, which usually die before development of specific antibodies.

1. Detection of the agent

Francisella tularensis can be demonstrated in smear preparations or in histological sections using specific immunological or immunohistochemical methods of identification. If reagents are not readily available, fixed specimens can be analysed at laboratories equipped with proper reagents and methods. Bacterial isolation followed by identification via immunological or molecular methods is also used, however, *F. tularensis* may be difficult to isolate from dead animals and carcasses due to overgrowth of other bacteria. Selective culture media or animal inoculation can be used to enhance recovery of the organism. Polymerase chain reaction (PCR) is a safe and convenient way to detect and identify *F. tularensis* in clinical samples.

1.1. Smear preparations for antigen detection

Impression smears of organs, such as the liver, spleen, bone marrow, kidney, lung or blood, are made on microscope slides. The bacteria are abundant in such smears, but may be overlooked because of their very small size (0.2–0.7 μm). The bacteria can be demonstrated by direct or indirect fluorescent antibody staining. This is a safe, rapid and specific diagnostic tool (Karlsson *et al*., 1970; Morner, 1981).

Gram staining of smears reveals a scattering of small, punctiform Gram-negative bacteria near the limit of visibility. The use of oil microscopy increases the visibility of the bacteria. The bacteria may be difficult to distinguish from precipitates of stain.

1.2. Histological sections for antigen detection

Bacteria can be demonstrated in sections using immunohistochemical methods, such as the fluorescent antibody test (FAT) (Morner, 1981) or immunohistochemistry (Gyuranecz *et al.,* 2010). The tests are performed on organ samples fixed in neutral buffered formalin and paraffin embedded. Slides are first treated with rabbit or mouse anti-tularemia serum, washed and thereafter treated with a fluorescein isothiocyanate-conjugated or a horseradish peroxidase-labelled anti-rabbit or anti-mouse serum. The samples are examined under a fluorescence or light microscope. Large numbers of bacteria can be seen in necrotic lesions and in the blood.

1.3. Bacterial isolation

The bacteria can be isolated from heart blood, liver, spleen, bone marrow or tularemic granulomas (from lungs, pericardium, kidney, liver, spleen, etc.) from moribund animals, however it is highly fastidious; it will not grow on ordinary media, although an occasional strain can sometimes grow on blood agar on initial isolation. Culture preparations are incubated at 37°C, in ambient air or in 5% CO_2.

1.3.1. The culture media listed below are all appropriate for isolating *F. tularensis*

i) Francis medium

Peptone agar containing 0.1% cystine (or cysteine) and 1% glucose, to which is added, before solidification, 8–10% defribrinated rabbit, horse or human blood.

ii) McCoy and Chapin medium

This consists of 60 g egg yolk and 40 ml normal saline solution, carefully mixed and coagulated by heating to 75°C.

iii) Modified Thayer–Martin agar

Glucose cysteine agar (GCA)-medium base supplemented with haemoglobin and a commercially available enrichment additive containing nicotinamide adenine dinucleotide with other factors.

Colonies that form on McCoy and Chapin medium are small, prominent, round and transparent. A more abundant growth is obtained on Francis medium and modified Thayer–Martin agar, with confluent colonies that have a milky appearance and a mucoid consistency. On either medium, colonies do not appear until after 48 hours' incubation at 37°C.

1.3.2. Selective media

Cystine heart agar broth with blood (CHAB) supplemented with 7.5 mg colistin, 2.5 mg amphotericin, 0.5 mg lincomycin, 4 mg trimethoprim and 10 mg ampicillin per litre (WHO, 2007) is commonly used for complex clinical specimens. Growth in CHAB medium enables the presumptive identification of *F. tularensis* by characteristic growth at 24–48 hours of round and smooth green opalescent shiny colonies, 2–4 mm in diameter.

1.3.3. Identification of isolates

The bacteria are nonmotile, nonsporulating, bipolar staining, and of uniform appearance in 24-hour cultures, but pleomorphic in older cultures. Biochemical tests can provide a presumptive identification of isolates, but confirmation using immunological or molecular methods is typical. Type A subspecies may be biochemically distinguished from Type B subspecies by the fact that most Type A ferment glycerol.

Francisella tularensis species can be identified using matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) if corresponding reference spectra are available (Lopez-

Ramos *et al.*, 2020). The preparation is carried out using the ethanol/formic acid method. After 20 minutes in 70% ethanol, the bacteria are killed. The preparation is centrifuged and the supernatant removed. Equal volumes of formic acid and acetonitrile are then added and centrifuged again and the supernatant is dropped on the metal sample plates for spectrometry. Picking and direct application of the colonies without ethanol inactivation is not recommended.

1.4. Molecular techniques

PCR-based assays are useful for the detection of *F. tularensis* DNA directly from human, animal and environment samples. They can also determine the *F. tularensis* subspecies or genotypes, either from isolated strains or directly from clinical samples.

Methods for detection of *F. tularensis* DNA include conventional PCR (Barns *et al.*, 2005; Sjostedt *et al.*, 1997) and real-time PCR systems (Versage *et al.*, 2003). It is to be noted that PCR testing of ticks must use specific gene targets or PCR fragment sequencing to differentiate *F. tularensis* from *Francisella*-like endosymbionts (Kreizinger *et al.*, 2013; Kugeler *et al.*, 2005; Michelet *et al.*, 2013).

A conventional PCR system targeting the 16S rRNA gene followed by PCR product sequencing was designed to detect *F. tularensis* and *F. philomiragia,* as well as the *Francisella*-like tick endosymbionts by Barns *et al.* (2005) with the following primer pair:

Fr153F0.1: 5'-GCC-CAT-TTG-AGG-GGG-ATA-CC-3'
Fr1281R0.1: 5'-GGA-CTA-AGA-GTA-CCT-TTT-TGA-GT-3'.

Cycling conditions consist of initial denaturation for 10 minutes at 95°C followed by 30 and up to 40 amplification cycles of denaturation for 30 seconds at 94°C, primer annealing at 60°C for 1 minute and extension at 72°C for 1 minute.

A real-time PCR system targeting the *tul4* gene was designed by Versage *et al.* (2003) to detect specifically only *F. tularensis* with the following primers and probe:

Tul4F: 5'-ATT-ACA-ATG-GCA-GGC-TCC-AGA-3',
Tul4R: 5'-TGC-CCA-AGT-TTT-ATC-GTT-CTT-CT-3'
Tul4P: FAM-5'-TTC-TAA-GTG-CCA-TGA-TAC-AAG-CTT-CCC-AAT-TAC-TAA-G-3'-BHQ.

The probe is synthesised with a 6-carboxy-fluorescein reporter molecule attached to the 5'end and a black hole quencher attached to the 3' end. The PCR consists of initial denaturation for 10 minutes at 95°C followed by 45 amplification cycles of denaturation for 15 seconds at 95°C, primer annealing at 60°C for 30 seconds.

Appropriate methods for differentiation of *F. tularensis* subspecies and genotypes include certain PCR assays (Birdsell *et al.*, 2014; Johansson *et al.*, 2000; Kugeler *et al.*, 2006; Tomaso *et al.*, 2007), canonical single nucleotide polymorphism analysis (canSNP; Vogler *et al.*, 2009a), typing of canonical insertions and deletions (canINDELs; Svensson *et al.*, 2009), DNA microarray analysis (Broekhuijsen *et al.,* 2003) and multi-locus variable-number tandem repeat analysis (MLVA; Johansson *et al.*, 2004; Vogler *et al.*, 2009b).

1.5. Animal inoculation

Animal inoculation is not recommended because of welfare and biosafety concerns. It should only be undertaken when culture enhancement in a laboratory animal is considered unavoidable, and where proper animal biosafety facilities and cages are available (see chapter 1.1.4).

Tularemic granuloma or a piece of septicaemic organ (e.g. spleen, liver) is excised and about 1 g of tissue sample is homogenised and suspended in 2 ml of normal saline. A laboratory animal (preferably mouse) is injected subcutaneously with 0.5 ml of suspension. Diseased animals will die after 2–10 days of injection. Heart blood and bone-marrow samples are inoculated on culture media on the day of the laboratory animal's death (Gyuranecz *et al.*, 2009).

2. Serological tests

Serology is carried out for diagnosis of tularemia in humans but is of limited value in susceptible animal species, which usually die before specific antibodies can develop. Serology may be conducted either on sera or on lung extracts (Morner *et al*., 1988) in epidemiological surveys of animals that are resistant or relatively resistant to infection, such as sheep, cattle, pigs, moose, dogs, foxes, wild boars, birds or the European brown hare in Central Europe (Gyuranecz *et al*., 2011; Morner *et al*., 1988; Otto *et al*., 2014). As there is no antigenic difference between Type A and Type B strains, the less virulent *F. tularensis* ssp. *holarctica* and its attenuated live vaccine strain (LVS, NCTC 10857) could be used as antigen in all serological tests. The most commonly used serological test for diagnosis of tularemia is the microagglutination test. Other tests (immunofluorescence and enzyme-linked immunosorbent assay [ELISA]) have comparable sensitivity and specificity, but may detect antibody earlier than agglutination tests (Maurin, 2020).

2.1. Agglutination tests

The antigen used in agglutination tests is typically a culture of *F. tularensis* on Francis medium. The culture is harvested after 5–6 days. Younger cultures yield a poorer antigen. The colonies are typically inactivated by suspension in saline with 0.5% formaldehyde. After centrifugation, the pellet is resuspended in an equal volume of saline containing 0.5% formaldehyde and 0.005% safranine (Sato *et al.,* 1990). The suspension is calibrated with positive and negative sera and adjusted by adding normal saline to provide an antigen that when tested on a slide gives readily visible stained agglutination reactions against a clear fluid background.

Possible cross-reactions with S-type *Brucella* species and *Legionella* spp. have to be taken into consideration. Agglutination tests primarily detect IgM, although IgG contributes to the agglutination.

2.1.1. Slide agglutination

Slide agglutination is a useful field method (Gyuranecz *et al.*, 2011). In the slide agglutination test 1 drop of whole blood (approx. 0.04 ml) is mixed with 1 drop of antigen and the reaction is considered positive if flakes appear within 1–3 minutes at 20–25°C.

2.1.2. Tube agglutination

The test is performed in tubes containing a fixed amount of antigen (0.9 ml) and different dilutions of serum commencing with 1/10, 1/20, 1/40, etc. The results are read after 20 minutes of shaking, or after 1 hour in a water bath at 37°C followed by overnight storage at room temperature. The agglutinated sediment is visible to the naked eye or, preferably, by using a hand lens. The positive tubes are those that have a clear supernatant fluid.

2.1.3. Microagglutination

The test is performed in microtitre-plates. Serial two-fold dilutions of sera (25 µl) are mixed with an equal volume of formalin-inactivated whole cell suspension (Chaignat *et al*., 2014). The plates are read after incubation at 37°C for 18 hours. The agglutinated sediment is visible to the naked eye or, preferably, by using a hand lens. The positive wells are those that have a clear supernatant fluid.

2.2. Enzyme-linked immunosorbent assay

Another serological test, the ELISA, also allows an early diagnosis of tularemia (Carlsson *et al*., 1979; Chaignat *et al*., 2014). Different antigens, whole bacteria as well as subcellular components (e.g. purified lipopolysaccharide), have been used as recall antigens against immunoglobulins IgA, IgM and IgG. Two weeks after the onset of tularemia, specific antibodies can be detected in the serum (Chaignat *et al*., 2014; Fulop *et al*., 1991). Because IgM is sustained for a long period, it cannot be used as an indicator of recent infection (Bevanger *et al.,* 1994). For routine diagnosis, whole, heat-killed (65°C for 30 minutes) bacteria can be used as antigen. Bacteria can be coated to plastic plates, using the usual procedures (Carlsson *et al*., 1979) followed by serial dilutions of the serum to be tested. Positive reactions can be visualised by anti-antibodies labelled with enzyme. The test should also be read in a photometer with positive and negative sera as controls.

C. REQUIREMENTS FOR VACCINES

The attenuated *F. tularensis* subsp. *holarctica* live vaccine strain (LVS, NCTC 10857) was developed in the 1950s and used for decades to protect laboratory workers handling large volumes of *F. tularensis* cultures, however, it is no longer used because of its overall limited efficacy and concern about reversion to virulence. A number of novel vaccines against tularemia are under development using different approaches, but none have yet received regulatory approval for human or animal use (Carvalho *et al.,* 2014; Conlan, 2011).

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NB: At the time of publication (2022) there was no WOAH Reference Laboratory for tularemia (please consult the WOAH Web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.1.24.

VESICULAR STOMATITIS

SUMMARY

Vesicular stomatitis (*VS*) *is a vesicular disease of horses, cattle and pigs caused by vesiculoviruses of the family* Rhabdoviridae*. This disease is clinically indistinguishable in relevant susceptible species from foot and mouth disease* (*FMD*)*, vesicular exanthema of swine* (*VES*)*, or swine vesicular disease* (*SVD*)*. Sheep, goats and many other wild species can be infected. Humans are also susceptible. The disease is endemic in the Americas, but has occasionally spread to other continents.*

Virus is transmitted directly by the transcutaneous or transmucosal route and has been isolated from sandflies and mosquitoes. Experimental transmission has been shown from black flies to both pigs and cattle. There is seasonal variation in the occurrence of VS: it disappears at the end of the rainy season in tropical areas, and at the first frosts in temperate zones. The pathogenesis of the disease is unclear, and it has been observed that the specific circulating antibodies do not always prevent infection with different VS serogroups.

Although VS may be suspected when horses are involved as well as pigs and cattle, prompt differential diagnosis is essential because the clinical signs of VS are indistinguishable from FMD when cattle and pigs are affected, and from SVD, VES and senecavirus A when only pigs are affected.

Diagnosis of VS is by virus isolation or by the demonstration of VS viral antigen or nucleic acid in samples of tissue or fluid. Detection of virus-specific antibody against structural proteins, in paired serum, can also be used as an indicator of infection.

Detection of the agent: *Virus can be readily isolated by the inoculation of several tissue culture systems, or embryonated chicken eggs. Viral RNA can be detected from epithelial tissue and vesicular fluid by conventional and real-time reverse transcription polymerase chain reaction* (*RT-PCR*)*. Viral antigen can be identified by an indirect sandwich enzyme-linked immunosorbent assay* (*IS-ELISA*) *– this is the least expensive and most rapid test. The complement fixation test* (*CFT*) *is also a good alternative. The virus neutralisation test* (*VNT*) *may be used, but it is elaborate and time-consuming.*

Serological tests: *Convalescent animals develop specific antibodies within 4–8 days of infection that are demonstrated by a liquid-phase blocking ELISA* (*LP-ELISA*)*, a competitive ELISA* (*C-ELISA*) *and VNT. Other described tests are CFT, agar gel immunodiffusion and counter immuno-electrophoresis. The demonstration of specific antibodies to structural proteins in nonvaccinated animals is indicative of prior infection with VS virus.*

Requirements for vaccines: *Inactivated virus vaccines with aluminium hydroxide or oil as adjuvants have been tested in the United States of America and in Colombia, respectively. Both vaccines generated high levels of specific antibodies in the sera of vaccinated cattle. However, it is not yet clear if serum antibodies would prevent the disease. An attenuated virus vaccine has been used in the field with unknown efficacy.*

A. INTRODUCTION

Vesicular stomatitis (VS) was described in the United States of America (USA) by Oltsky *et al*. (1926) and Cotton (1927) as a vesicular disease of horses, and subsequently of cattle and pigs. Vesicles are caused by virus on the tongue, lips, buccal mucosa, teats, and in the coronary band epithelium of cattle, horses, pigs, and many other species of domestic and wild animals. Natural disease in sheep and goats is rare, although both species can be experimentally infected. Mixed infections of foot and mouth disease (FMD) and VS viruses have occurred in the same herds of cattle and can be induced experimentally. Many species of laboratory animals are also susceptible. The disease is endemic in the Americas, but has occasionally spread to other continents.

Influenza-like signs, normally without vesicles, have been observed in humans who are in contact with animals with VS or who handle infective virus. Laboratory manipulations involving live virus, including handling infective materials from animals, should be carried out at an appropriate biosafety and containment level determined by risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

The pathogenic agents causing vesicular stomatitis belong to the *Rhabdoviridae* family, genus *Vesiculovirus* with four species as follows: *Indiana vesiculovirus* – VSIV (formerly IND-1), *Cocal vesiculovirus* – COCV (formerly IND-2), *Alagoas vesiculovirus* – VSAV (formerly IND-3) and *New Jersey vesiculovirus* – VSNJV. They have been extensively studied at the molecular level (Fowler *et al.,* 2016; ICTV, 2020; Pauszek *et al.,* 2011). Several other closely related rhabdoviruses have been isolated from sick animals over the past decades. The VSIV, Cocal virusand Alagoas virus are serologically related (Federer *et al.,* 1967). Strains of the VSNJV and VSIV are endemic in livestock in areas of southern Mexico, Central America, Venezuela, Colombia, Ecuador and Peru, with VSVNJ causing the majority of the clinical cases. Sporadic activity of VSNJV and VSIV has been reported in northern Mexico and the western United States. *Cocal vesiculovirus* was isolated from domestic animals only in Argentina and Brazil and only from horses (Salto-Argentina/63, Maipú-Argentina/86, Rancharia-Brazil/66, Ribeirão-Brazil/79) (Alonso *et al.,* 1991; Alonso Fernandez & Sondahl, 1985). This finding confirms the first descriptions, in 1926 and 1927 (Cotton, 1927; Oltsky *et al.,* 1926) of the VSNJV and VSIV, COCV, VSAV in horses, and subsequently in cattle and pigs; this same predilection has been observed in other VS outbreaks. The Alagoas virus, (Alagoas-Brazil/64), has been identified, endemically in North-eastern Brazil, in cattle, horses, swine, sheep and goats (Alonso *et al.,* 1991; Alonso Fernandez & Sondahl, 1985; Cargnelutti *et al.,* 2014; PANAFTOSA-OPS/OMS, 2019; Rocha *et al.,* 2020).

The mechanism of transmission of the virus is unclear. The viruses have been isolated from sandflies, mosquitoes, and other insects (Comer *et al*., 1992; Francy *et al*., 1988; Mason, 1978). Experimental transmission of VSNJV has been demonstrated to occur from black flies (*Simulium vittatum*) to domestic swine and cattle (Mead *et al*., 2004; 2009). During the 1982 epizootic in the western USA, there were a number of cases where there was direct transmission from animal to animal (Sellers & Maarouf, 1990). VSV has historically been considered to be endemic in feral pigs on Ossabaw Island, Georgia, USA (Boring & Smith, 1962), but subsequently may have disappeared from the island (Killmaster *et al*., 2011).

The incidence of disease can vary widely among affected herds. Usually 10–15% of the animals show clinical signs. Clinical cases are mainly seen in adult animals. Cattle and horses under 1 year of age are rarely affected. Mortality is close to zero in both species. However, high mortality rates in pigs affected by VSNJV have been observed. Sick animals recover in about 2 weeks. The most common complications of economic importance are mastitis and loss of production in dairy herds (Lauerman *et al*., 1962). Recent VS outbreaks in the USA have been associated primarily with horses and VSNJV.

B. DIAGNOSTIC TECHNIQUES

VS cannot be reliably differentiated clinically from the other vesicular diseases in the relevant susceptible species, such as FMD, vesicular exanthema of swine (VES), and swine vesicular disease (SVD). An early laboratory diagnosis of any suspected VS case is therefore a matter of urgency.

The sample collection and technology used for the diagnosis of VS must be in concordance with the methodology used for the diagnosis of FMD (chapter 3.1.8), VES and SVD (chapter 3.9.8), in order to facilitate the differential diagnosis of these vesicular diseases.

Vesicle fluid, epithelium covering unruptured vesicles, epithelial flaps of freshly ruptured vesicles, or swabs of the ruptured vesicles are the best diagnostic samples. These samples can be collected from mouth lesions, as well as from the feet and any other sites of vesicle development. It is recommended to sedate the animal before collection of samples to avoid injury to animals and people Tissue and fluid samples, including swabs from all species should be placed in containers of Tris-buffered tryptose broth (TBTB) minimal essential medium (MEM) or 0.08 M phosphate buffer (with phenol red and antibiotics [1000 units/ml penicillin, 100 units/ml nystatin, 100 units/ml neomycin, and 50 units/ml polymyxin B], and adjusted to pH 7.2–7.6. Tissue samples can also be placed in glycerol/phosphate buffer with phenol red pH 7.2–7.6. (Note: glycerol is toxic to cell cultures and decreases the sensitivity of virus isolation.) Samples should be sent to the laboratory on ice packs if they can arrive at the laboratory within 48 hours after collection. If samples require more than 48 hours transit time, they should be sent frozen on dry ice with precautions to protect the sample from direct contact with CO_2. There are special packaging requirements for shipping samples with dry ice (see Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* for further information on shipping of diagnostic samples).

Considering the need for differential diagnosis with FMD, if it is not possible to collect epithelium from unruptured or freshly ruptured vesicles or vesicular fluid, oesophageal–pharyngeal (OP) fluid samples, can be collected by probang cups from ruminants only, as an alternative source of virus. Mix probang fluid with an equal volume of transport fluid (see Chapter 3.1.8 *Foot and mouth disease*). The container should be capable of withstanding freezing above dry ice (solid carbon dioxide) or liquid nitrogen (Kitching & Donaldson, 1987).

When it is not possible to collect samples for identification of the agent, serum samples can be used for detecting and quantifying specific antibodies. Paired sera from the same animals, collected 7–14 days apart, may be needed depending on the serological assay being used and prior history of vesicular stomatitis in the animals.

Specific reagents for VS diagnosis are not commercially available and each laboratory must produce its own or obtain them from a Reference Laboratory. The WOAH Reference Laboratories for vesicular stomatitis[1] produce and distribute diagnostic reagents on request.

Table 1. Test methods available for the diagnosis of vesicular stomatitis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination[(a)]
Detection of the agent[(b)]						
Virus isolation[(C)]	–	+	–	+++	–	–
IS-ELISA[(C)]	–	+	–	+++	–	–
CFT[(C)]	–	+	–	++	–	–
RT-PCR[(C)]	–	+	–	++	–	–
Detection of immune response[(d)]						
LP-ELISA[(e)]	++	++	++	++	++	++
C-ELISA[(e)]	+++	++	++	–	+++	++
VNT[(e)]	+++	+++	+++	++	++	+++
CFT[(e)]	–	+	+	++	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
IS-ELISA = indirect sandwich enzyme-linked immunosorbent assay; CFT = complement fixation test; RT-PCR = reverse transcription polymerase chain reaction; LP-ELISA = liquid-phase blocking ELISA; C-ELISA = competitive ELISA; VNT = virus neutralisation test.
[(a)]Indicates the presence of antibodies only; does not indicate protection from infection.
[(b)]A combination of agent identification methods applied on the same clinical sample is recommended.
[(C)]Should only be used on animals demonstrating clinical signs compatible with VSV. A positive result is meaningful. A negative result could mean the animal is no longer shedding virus, the virus level is too low to detect, or, for virus isolation samples that the samples were not maintained at appropriate temperatures and received in an appropriate time period following collection for virus isolation (virus inactivated).
[(d)]One of the listed serological tests is sufficient
[(e)]The presence of VSV antibodies only indicates prior exposure to VSV. It does not determine whether the antibodies are due to current or past infection. Paired sera from the same animals, collected at least 7–14 days apart, may be needed to evaluate seroconversion depending on the serological assay being used and prior history of VS. Interpretation of results needs to be based on serological results, clinical presentation, and epidemiology. CF antibody duration in an animal is generally less than 1 year. Antibodies detected by the VNT and competitive ELISAs can be detected for years following infection. The difference in sensitivity of the serological assays has an effect on detection during the acute phase of infection; combination testing, such as C-ELISA and CF or paired sampling showing four-fold titre change (CFT, VNT, LP-ELISA), is therefore necessary when an animal presents with acute clinical signs of VS.

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

1. Detection of the agent

1.1. Direct visualisation

Due to the different morphological characteristics of the rhabdovirus (VS serogroup viruses), picornavirus (FMD virus, SVD virus and senecavirus A), calicivirus (VES) and the large number of virus particles present in vesicular fluids and epithelial tissues, electron microscopy can be a useful diagnostic tool for differentiating the virus family involved.

1.2. Virus isolation in cell culture

For identification of VS viruses and the differential diagnosis of vesicular diseases, clarified suspensions of field samples suspected to contain virus should be submitted for testing. For virus isolation, the samples are inoculated into appropriate cell cultures. The inoculation of African green monkey kidney (Vero), baby hamster kidney (BHK-21) and IB-RS-2 cell cultures with the same sample permits differentiation of the vesicular diseases: VS viruses cause a cytopathic effect (CPE) in all three cell lines; FMD virus causes a CPE in BHK-21 and in IB-RS-2, while SVD virus causes a CPE in IB-RS-2 only. Many other cell lines, as well as most primary cell cultures of animal origin, are susceptible to VS serogroup viruses.

1.2.1. Test procedure

i) Where tissue has been collected in phosphate-buffered saline (PBS)/glycerol solution, it should be blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures, and weighed. A suspension is then prepared using a tissue grinder or by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added until a final volume of nine times that of the original sample has been added, giving a 10% suspension. For swabs samples, a 10% solution is made using the cell culture medium. The sample is clarified on a bench centrifuge at 2000 ***g*** for at least 10 minutes.

ii) The clarified suspension of tissues, swab or vesicular fluid from field samples suspected to contain VSV are inoculated onto cell culture vessels (BHK-21, IB-RS-2 or Vero cells).

iii) Incubate inoculated cell cultures at 37°C for 1 hour (adsorption).

iv) Discard inoculum and wash cell cultures three times with cell culture medium and replace with cell culture medium containing 2.5% fetal bovine serum (FBS).

v) Incubate plates, plastic tubes or flasks cell cultures at 35–37°C and observe for cytopathic effect (CPE). The cell cultures should be examined for CPE for 48–72 hours. If, after 72 hours, no CPE has been detected, a blind passage must be made. The cell culture is freeze–thawed and clarified by centrifugation, and the supernatant is used for inoculation of fresh monolayers or cell suspension. The sample is considered to be negative if there is no evidence of a CPE after on average of three blind passages in cell cultures; longer passages of up to 7 days may be conducted using fewer passages.

vi Reverse-transcription polymerase chain reaction (RT-PCR) may be used to identify virus recovery, using appropriate sets of primers (Rainwater-Lovett *et al.,* 2007; Sepulveda *et al.,* 2007). Alternatively, immunological methods for the identification of the viral antigens can be used such as enzyme-linked immunosorbent assay (ELISA) (Alonso et al., 1991; Ferris & Donaldson, 1988), the complement fixation test (CFT) (Alonso *et al.,* 1991; Jenny *et al.,* 1958) or fluorescent antibody staining. The virus neutralisation test (VNT), with known positive antisera against VSNJV and VSIV, may be used in tissue cultures or embryonated eggs, but it is more time-consuming.

1.3. *In-ovo* testing

The virus replicates and can be isolated in 8- to 10-day-old chicken embryos by inoculation into the allantoic sac.

1.4. Enzyme-linked immunosorbent assay

The indirect sandwich ELISA (IS-ELISA) (Alonso *et al*., 1991; Ferris & Donaldson, 1988) is a common diagnostic method of choice for identification of VS and other vesicular diseases. Specifically, the ELISA procedure with a set of polyvalent rabbit/guinea-pig antisera, prepared against virions for representative strains of VSIV, COCV, and VSAV (Alonso *et al*., 1991). For detection of VSNJV, a monovalent set of rabbit/guinea-pig antisera is suitable (Alonso *et al*., 1991; Ferris & Donaldson, 1988).

1.4.1. Test procedure

i) *Solid phase:* ELISA plates are coated either for 1 hour at 37°C or overnight at 4°C with rabbit antisera and normal rabbit serum (as described in Alonso *et al*., 1991), and optimally diluted in carbonate/bicarbonate buffer, pH 9.6. Subsequently, the plates are washed once with PBS and blocked for 1 hour at room temperature with 1% ovalbumin Grade V (grade of purification) in PBS. After washing, the plates can be used immediately or stored at –20°C for future use.

ii) *Test samples:* Antigen suspensions of test samples (10–20% epithelial tissue suspension, in PBS or MEM or undiluted clarified cell culture supernatant fluid) are deposited in the corresponding wells and the plates are incubated for 1 hour at 37°C on an orbital shaker.

iii) *Detector:* Monovalent guinea-pig antisera to VSNJV and polyvalent guinea-pig antisera to VSIV, COCV and VSAV, that are homologous to coated rabbit serum and that have been diluted appropriately in PBS containing 0.05% Tween 20, 1% ovalbumin Grade II, 2% normal rabbit serum, and 2% normal bovine serum (PBSTB) are added to the corresponding wells and left to react for 30–60 minutes at 37°C on an orbital shaker.

iv) *Conjugate:* Peroxidase/rabbit or goat IgG anti-guinea-pig Ig conjugate, diluted in PBSTB, is added and left to react for 30–60 minutes at 37°C on an orbital shaker.

v) *Substrate:* H_2O_2-activated substrate is added and left to react at room temperature for 15 minutes, followed by the addition of sulphuric acid to stop the reaction. Absorbance values are measured using an ELISA reader.

Throughout the test, 50 µl reagent volumes are used. The plates are washed three–five times between each stage with physiological saline solution or PBS containing 0.05% Tween 20. Controls for the reagents used are included.

vi) *Interpretation of the results:* Absorbance values of positive and negative antigen control wells should be within specified values for acceptance. Sample wells giving an absorbance ≥0.3 are considered to be positive for the corresponding virus subtype. Absorbance values <0.3–0.2 are considered suspicious and values <0.2 are considered negative for the corresponding virus subtype. Suspicious and negative samples should be inoculated in cell culture and passages re-tested in ELISA.

1.5. Complement fixation test

The IS-ELISA is preferable to the CFT because it is more sensitive and it is not affected by pro- or anti-complementary factors. When ELISA reagents are not available, however, the CFT may be performed. The CFT in U-bottomed microtitre plates, using the reagents titrated by CF50% test, is described.

1.5.1. Test procedure

i) *Antisera:* Monovalent guinea-pig antisera to VSNJV and polyvalent guinea-pig antisera to VSIV, COCV and VSAV, diluted in barbital buffer or an alternative CF buffer at a dilution containing 2.5 CFU_{50} (50% complement fixation units) against homologous virus, are deposited in plate wells. Those antisera are the detectors used in ELISA.

ii) *Test samples:* The antigen suspension of test samples, prepared as described for IS-ELISA, is added to the wells with serum.

iii) *Complement:* 4 CHU_{50} (50% complement haemolytic units) are added to the serum and antigen. (An alternative is to use 7.5, 10 and 20 CHU_{50} with the goal of reaching 4 CHU_{50} in the test.) The mixture of antisera, test samples and complement is incubated at 37°C for 60 minutes.

iv) *Haemolytic system:* A suspension of sheep red blood cells (SRBC) in CF buffer, sensitised with 10 HU_{50} (50% haemolytic units) of rabbit anti-SRBC serum, is added to the wells. The haemolytic system has an absorbance of 0.66 read at 545 nm, in the proportion of two volumes of haemolytic system + three volumes of distilled water. The mixture is incubated for 30 minutes at 37°C. Subsequently, the plates are centrifuged and the reaction is observed visually.

Volumes of 25 µl for antisera, test samples and complement, and 50 µl of haemolytic system, are required. Appropriate controls for the antisera, antigens, complement and haemolytic system are included.

It is possible to perform the CF50% test in tubes (Alonso *et al*., 1991) using reagent volumes of 200 µl (eight times greater than those indicated for the CFT in microtitre plates). With the CF50% test, the reaction can be expressed as absorbance read spectrophotometrically at 545 nm.

v) *Interpretation of the results:* When controls react as expected, samples with haemolysis <20% for one antiserum in comparison with the other antiserum and controls are considered to be positive for the corresponding type.

Field samples that are negative by the ELISA or CFT should be inoculated into cell culture. If there is no evidence of viral infection after three passages, the specimen is considered to be negative for virus.

1.6. Molecular methods

The RT-PCR can be used to amplify small genomic areas of the VS virus (Hofner *et al*., 1994; Hole *et al.,* 2010; Rodriguez *et al*., 1993; Wilson *et al*., 2009). This technique will detect the presence of virus RNA in tissue and vesicular fluid samples and cell culture, but cannot determine if the virus is infectious.

The diagnosis of VS in samples of epithelium, vesicular fluid and cell culture by molecular methods is the most suitable for confirmation of the disease. No screening protocol that allows the detection of all species of vesiculovirus of economic interest has been described. However, there are some multiplex tests described below that allow the detection of more than one viral species. Therefore, for a differential diagnosis, different protocols are needed to detect all VS viruses.

Extraction of nucleic acid from the sample must be done according to the manufacturer's instructions.

Methods for detecting and typing the four VS viruses have been described using both real-time and conventional RT-PCR.

1.6.1. Real-time RT-PCR detection and typing

VSNJV, VSIV, COCV and VSAV can be detected and typed using the real-time RT-PCR methods described by Hole *et al.* (2010) with adaptations, de Oliveira *et al.* (2018) and Sales *et al.* (2020), and the primers and probes described in Table 2.

Hole *et al.* (2010) developed a real-time RT-PCR assay that allows differentiation between VSIV and VSNJV with good performance. Primer concentrations must be 0.2 mM except the reverse VSNJV, which is 0.8 mM, and probe concentrations are 0.2 mM for VSNJV and 0.1 mM for VSIV. The real-time RT-PCR protocol consists of reverse transcription cycles at 50°C for 30 minutes and denaturation at 95°C for 1 minute, followed by 45 cycles of 95°C for 15 seconds, 54°C for 30 seconds and 72°C for 60 seconds. The reaction is performed according to the kit manufacturer's instructions, adding $MgSO_4$ (4 mM).

Due to the extent of genetic variation of this virus in some regions, de Oliveira *et al.* (2018) developed a method for detecting VSAV by multiplex real-time RT-PCR with excellent sensitivity and specificity. The test is performed according to the kit manufacturer's instructions and the concentration of the primers in a final volume of 25 µl was 0.4 µM. The real-time RT-PCR protocol consists of reverse transcription cycles at 50°C for 10 minutes and denaturation at 95°C for 5 minutes followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute.

Sales *et al.* (2020) described a real-time RT-PCR protocol for the diagnosis of COCV. The test is carried out according to the kit manufacturer's instructions, with the concentration of each primer 0.1 µM and probe 0.4 µM in a final volume of 25 µl. The real-time RT-PCR protocol consists of reverse transcription cycles of 45°C for 10 minutes and denaturation of 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute.

Table 2. Oligonucleotides target for real-time RT-PCR of VSV

Real-time RT-PCR for vesicular stomatitis		
Multiplex New Jersey vesiculovirus* and *Indiana vesiculovirus		
Name	**Sequence (5'→3')**	**Reference**
VSNJV 7230-7254	Forward: TGA-TTC-AAT-ATA-ATT-ATT-TTG-GGA-C	Hole *et al.,* 2010
VSNJV 7476-7495 R	Reverse: AGG-CTC-AGA-GGC-ATG-TTC-AT	
VSNJV 7274-7296 P 1	Probe: FAM-TTT-ATG-CAT-GAC-CCW-GCA-ATA-AG-NFQ-MGB	
VSNJV 7334-7353 P 2	Probe: FAM-TTG-CAC-ACC-AGA-ACA-TTC-AA-BHQ1	
VSIV 7230-7254 IN F	Forward: TGA-TAC-AGT-ACA-ATT-ATT-TTG-GGA-C	
VSIV 7433-7456 IN R	Reverse: GAG-ACT-TTC-TGT-TAC-GGG-ATC-TGG	
VSIV 7274-7290 P	Probe: VIC-ATG-ATG-CAT-GAT-CCA-GC-NFQ-MGB	
Alagoas vesiculovirus		
Name	**Sequence (5'→3')**	**Reference**
VSAV-3.GP.95.F	Forward: GGG-TWA-ACA-TCC-GTG-CTA	de Oliveira *et al.,* 2018
VSAV-3.GP.95.R	Reverse: GTC-ACA-AGT-GGT-GAT-CCA	
VSAV-3.GP.95.S	Probe: FAM-cac+Atc+Cat+Cca+Tcagc-IowaBlack	
VSAV.LP.78.F	Forward: GTC-CAT-CAA-CCC-ATT-GTT-CC	
VSAV.LP.78.R	Reverse: ATC-AAT-CCA-TCT-GCG-ACT-CC	
VSAV.LP.78.S	Probe: FAM-CGC-GAT-TCT-TAA-GTG-AGT-TCA-AAT-CAG-GA-IowaBlack	
VSAV.GP.87.F	Forward: GAG-TGT-GGA-TCA-ACC-CAG	
VSAV.GP.87.R	Reverse: CTG-TGG-CTT-GAA-CRA-TCA	
VSAV.GP.87.S	Probe: FAM-CTGC+GGTTATG+CC+TCCA-IowaBlack	
Cocal vesiculovirus		
Name	**Sequence (5'→3')**	**Reference**
COCV.GP.81.F	Forward: CGT-TGC-TGT-GAT-TGT-YCA	Sales *et al.,* 2020
COCV.GP.81.R	Reverse: GGG-AAC-TGG-GAG-TCA-ATC	
COCV.GP.81.S	Probe: FAM-ctc+Atc+Cac+Caa+Cacat -BHQ1	

1.6.2. Conventional RT-PCR detection and typing

VSNJV, VSIV, COCV and VSAV can also be detected and typed using the RT-PCR methods described by Rodriguez *et al.* (1993) and Pauszek *et al.* (2008; 2011), and the primers described in Table 3. After obtaining the reverse transcription cDNA, the conventional PCR is undertaken. The protocol for all RT-PCR described consists of cycles of denaturation at 94°C for 3 minutes followed by 40 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, and the extension step should be 72°C for 5 minutes.

de Oliveira *et al.* (2018) developed an RT-PCR to detect Cocal vesiculovirus. The test is performed according to the kit manufacturer's instructions, with the concentration of each primer being 1 µM in a final volume of 20 µl. The RT-PCR protocol consists of reverse transcription cycles at 50°C for 30 minutes and denaturation at 95°C for 15 minutes followed by 40 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute.

Table 3. Primers and references for conventional RT-PCR of VSV

Conventional RT-PCR for vesicular stomatitis virus		
New Jersey vesiculovirus and Indiana vesiculovirus		
Name	**Sequence (5' → 3')**	**Reference**
VSNJV P102	Forward: GAG-AGG-ATA-AAT-ATC-TCC	Rodriguez *et al.,* 1993
VSNJV P744	Reverse: GGG-CAT-ACT-GAA-GAA-TA	
VSNJV P179	Forward: GCA-GAT-GAT-TCT-GAC-AC	
VSNJV P793	Reverse: GAC-TCT-(C/T)GC-CTG-(A/G)TT-GTA	
Cocal vesiculovirus and Alagoas vesiculovirus		
Name	**Sequence (5' → 3')**	**Reference**
COCV P66	Forward: AAT-TGG-ATG-ACG-CMG-TCC-A	Pauszek *et al.,* 2008
COCV P711	Reverse: CCT-CCD-ACH-GAR-ATG-AAY-TCT-CC	
VSAV PJX	Forward: TAT-GAA-AAA-AAI-TAA-CAG-IIA-TC	
VSAV P711	Reverse: CCT-CCD-ACH-GAR-ATG-AAY-TCT-CC	
Nested PCR for *Cocal vesiculovirus and Alagoas vesiculovirus*		
Name	**Sequence (5' → 3')**	**Reference**
COCV P169	Forward: TTA-CCA-AAA-TCA-GGA-GGA-TGA	Pauszek *et al.,* 2011
COCV P686	Reverse: GCC-TCC-CAC-CGA-GAT-G	
VSAV P163	Forward: AGA-GCA-GCT-CCY-TCT-TAT-TAT	
VSAV P691	Reverse: TCA-TCA-TTC-CAT-TTC-CTC	
One step RT-PCR for *Alagoas vesiculovirus*		
Name	**Sequence (5' → 3')**	**Reference**
VASV - P.722 F	Forward: GGG-GCC-ATT-CAA-GAG-ATA-GA	de Oliveira *et al.,* 2018
VASV -P.722 R	Reverse: TGA-TAT-CTC-ACT-CTG-GCC-TGA-TTA-T	

2. Serological tests

For the identification and quantification of specific antibodies in serum, the ELISA and the VNT are preferable. The CFT may be used for quantification of early antibodies. Antibody can usually be detected between 5 and 8 days post-infection; the length of time antibody persists has not been accurately determined for the three tests but is thought to be relatively short for the CFT and for extended periods for the VNT and ELISA (Katz et al., 1997).

The competitive ELISA (C-ELISA) may be preferable to the CFT because it is more sensitive and it is not affected by pro- or anti-complementary factors; however during an outbreak where there may be previously exposed animals, appropriate assay selection, and paired serum samples collected at least 7–14 days apart are important to ensure ability to distinguish recent from past exposure. When C-ELISA reagents are not available however, the CFT may be performed. The CFT in U-bottomed microtitre plates, using the reagents titrated by CF50% test, is described.

2.1. Liquid phase blocking enzyme-linked immunosorbent assay

The liquid-phase blocking ELISA (LP-ELISA) is a method for the detection and quantification of antibodies to VS serogroup viruses. The use of viral glycoproteins as antigen is recommended because they are not infectious, allow the detection of neutralising antibodies, and give fewer false-positive results than the VNT (Allende *et al*., 1992).

2.1.1. Test procedure

i) *Solid phase:* As described above in Section B.1.5 for the IS-ELISA.

ii) *Liquid phase:* Duplicate, two- to five-fold dilution series of each test serum, starting at 1/4, are prepared in U-bottomed microtitre plates. An equal volume of VSNJV or VSIV glycoprotein, in a predetermined dilution, is added to each well and the plates are incubated for 1 hour at 37°C. 50 µl of these mixtures is then transferred to the ELISA plates with the solid phase and left to react for 30 minutes at 37°C on an orbital shaker.

iii) *Detector, conjugate and substrate:* The same steps described for the IS-ELISA are performed using monovalent antisera homologous to the test antigen, as detectors

iv) *Interpretation of the results:* 50% end-point titres are expressed in $\log_{10}$ in reference to the 50% OD of the antigen control, according to the Spearmann–Kärber method. Titres of >1.0 (1/10) are considered to be positive.

2.2. Competitive enzyme-linked immunosorbent assay

A C-ELISA for detection of antibodies has also been developed. The procedure described here is based on a procedure described by Afshar *et al.* (1993). It uses vesicular stomatitis VSNJV and VSIV recombinant antigens as described by Katz *et al.* (1995).

2.2.1. Test procedure

i) *Solid phase:* Antigens are diluted in carbonate/bicarbonate buffer, pH 9.6, and 75 µl is added to each well of a 96-well ELISA plate. The plates are incubated overnight at 4°C; coated plates can be frozen, with antigen *in situ*, at –70°C for up to 30 days. The plates are thawed, antigen is decanted, and 100 µl of blocking solution (5% nonfat dry milk powder solution in PBS [for example, 5 g dry milk powder dissolved in 95 ml PBS) is added. The plates are then incubated at 25°C for 15–30 minutes and blocking solution is decanted. The plates are washed three times with PBS/0.05% Tween 20 solution.

ii) *Liquid phase:* 50 µl of serum diluted 1/8 in 1% nonfat dry milk in PBS is added to each of the duplicate wells for each sample. A positive and negative control serum for each virus species should be included on each ELISA plate. The plates are incubated at 37°C for 30 minutes. Without washing, 50 µl of bioreactor fluid is added to each well and plates are incubated at 37°C for 30 minutes.

iii) *Detector:* The plates are washed three times, and 50 µl of goat anti-mouse horseradish-peroxidase conjugate diluted in 1% nonfat dry milk with 10% normal goat serum is added to each well. The plates are incubated at 37°C for 30 minutes, washed three times, and 50 µl of tetramethyl-benzidine (TMB) substrate solution is added to each well. The plates are incubated at 25°C for 5–10 minutes and then 50 µl of 0.05 M sulphuric acid is added to each well. The plates are read at 450 nm and the optical density of the diluent control wells must be > 1.0.

iv) *Interpretation of the results:* A sample is positive if the absorbance is ≤50% of the absorbance of the diluent control. Note that horses naturally infected with VSNJV virus have been known to test positive by this assay for at least 8 years following infection.

2.3. Virus neutralisation test

Virus and cells: VSIV, COCV, VSAV and VSNJV are propagated in BHK, IB-RS-2 or Vero cell monolayers and stored in liquid nitrogen or frozen at –70°C (Allende *et al.,* 1992).

2.3.1. Test procedure

i) The test sera are heat-inactivated, including control standard sera, for 30 minutes at 56°C.

ii) Starting from 1/8 dilution, sera are diluted twofold or fourfold in cell culture medium, and the dilution series is continued in a cell-culture grade flat-bottomed 96-well microtitre plate using at least two rows of wells, preferably four rows (depending on the degree of precision required) and a volume of 50 µl per well. An extra well with 1/8 dilution test serum is used for toxicity control of sera. Dilutions of control sera with known titres (positive, weak positive and negative) are also included in the test.

iii) Add 50 µl per well of the VSV strain (VSIV, COCV, VSAV and VSNJV) stock at a dilution in culture medium calculated to provide 100 $TCID_{50}$ (50% tissue culture infective dose) per well. In the toxicity control wells, add 50 µl of culture medium in place of virus. Add 100 µl of culture medium to one row of empty wells as cell controls. A back titration of virus stock is also undertaken, at least four wells per dilution, to check the potency of the virus (dose acceptance limits 32–320 $TCID_{50}$ per well). An alternative protocol can be a viral dose of 1000 $TCID_{50}$ per well (tolerance range between 750 and 1270 $TCID_{50}$) to increase the specificity of the test.

iv) Incubate the plates for 60 minutes at 37°C in a 3–5% CO_2 atmosphere to allow viral neutralisation.

v) Add 100 µl per well of the BHK, IB-RS-2 or Vero cell suspension at 3×10^5 ml, containing 10% FBS for cell growth.

vi) Incubate the plates for 48–72 hours at 37°C, either in a 3–5% CO_2 atmosphere or seed the plate with pressure-sensitive tape and incubate.

vii) Check the cell cultures for the onset of CPE and read the results: positive monolayer wells – where the virus has been neutralised, have no CPE and the cells remain intact (blue-stained cells sheets); negative wells – where virus has not been neutralised, have a CPE (empty cavity – no staining). If staining is used fix the cells with 10% formol/saline for 30 minutes. For staining, the plates are immersed in 0.1% crystal violet in 1 % ethanol and 5 % buffered formalin for 30 minutes.

vii) Validate the test by checking the back titration of the virus (which should give a value of 100 $TCID_{50}$ per well with a permissible range of 32–320 $TCID_{50}$), the control sera and the cell control wells. The positive control serum should give a titre of twofold dilution (±0.3 log_{10} units) from its target value. The weak positive serum should be positive. The negative serum should give no neutralisation. In the cell control wells, the monolayers should be intact.

iv) *Interpretation of the results:* Virus neutralising titres of serum antibody responses (titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected). This can be calculated by the Spearman–Kärber or Reed Muench methods. The 50% neutralisation titre of each serum is expressed as log 10. When only two repetitions per dilution are performed, the highest inverse of the dilution that neutralised 100% of the cavities can be considered. In general, a titre of ≥32 (1.5) or more of the final serum dilution in the serum/virus mixture is regarded as positive for VSV antibodies. Laboratories are encouraged to verify this cut-off internally, with reagents provided by a WOAH Reference Laboratory, where available. If cytotoxicity is observed in the control wells, the sample is reported to be toxic (no result) unless neutralisation of the virus without cytotoxicity is observed at higher dilutions and a titre can be read without ambiguity (Allende *et al.,* 1992).

Note: Seroconversion is considered when there is a four-fold increase in antibody titre between paired serum samples collected with a minimum interval of 7–14 days between the first (acute phase of the disease) and the second blood collection (convalescent phase).

2.4. Complement fixation test

A detailed description of this test is given in Section B.1.5. This is modified as follows. The CFT may be used for quantification of early antibodies, mostly IgM. For this purpose, twofold serum dilutions are mixed with 2 CFU_{50} of known antigen and with 5% normal bovine or calf sera included in 4 CHU_{50} of complement. The mixture is incubated for 3 hours at 37°C or overnight at 4°C. Subsequently, the haemolytic system is added followed by incubation for 30 minutes at 37°C. The serum titre is the highest

dilution in which no haemolysis is observed. Titres of 1/5 or greater are positive. This CFT has low sensitivity and is frequently affected by anticomplementary or nonspecific factors.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

VSV infections can have significant impacts on the health and production aspects of animals, resulting in considerable economic losses for producers. Reduced feed intake caused by oral lesions can result in weight loss and delays to market. Lesions on the feet can cause temporary locomotor problems affecting the ability of an animal to obtain food and water, and permanent foot problems that result in the animal being culled. Lesions of the mammary gland can impact the ability of the dam to nurse her offspring and for harvesting milk for sale. Animals may be culled if mammary or teat lesions are severe. Although vaccination is not widely practised, vaccine is used to reduce the severity of clinical signs and the economic impacts of the disease.

Attenuated virus vaccines have been tested in the field in the USA, Panama, Guatemala, Peru and Venezuela (Lauerman *et al*., 1962; Mason, 1978) with unknown efficacy. Killed vaccines for VSIV and VSNJV are manufactured in Colombia and Venezuela (2002 WOAH vaccine survey). Although a commercial vaccine combining VS and FMD antigens in a single emulsion for Andean countries has been tested in vaccination–challenge experimentation and published (House *et al*., 2003), the vaccine is not produced/applied routinely.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Identity of the seed and the source of the serum used in growth and passage of the virus should be well documented, including the source and passage history of the organism.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The purity of the seed and cells to be used for vaccine production must be demonstrated. The master seed virus (MSV) should be free from adventitious agents, bacteria, or *Mycoplasma*, using tests known to be sensitive for detection of these microorganisms. The test aliquot should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against the seed virus and the virus/antibody mixture is cultured on several types of cell line monolayers. A cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended as one of the cell lines chosen for evaluation of the MSV. Bovine viral diarrhoea virus is a potential contaminant introduced using FBS in cell culture systems. Cultures are subpassaged at 7-day intervals for a total of at least 14 days, then tested for adventitious viruses that may have infected the cells or seed during previous passages.

2.2. Method of manufacture

2.2.1. Procedure

Once the vaccine is shown to be efficacious, and the proposed conditions for production are acceptable to regulatory authorities, approval may be granted to manufacture vaccine. Virus

seed can be grown in cell culture. Selection of a cell type for culture is dependent on the degree of virus adaptation, growth in medium, and viral yield in the specific culture system. Vaccine products should be limited to the number of passages from the MSV that can be demonstrated to be effective. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. Dose of virus used to inoculate cell culture should be kept to a minimum to reduce the potential for viral defective interfering particles. When the virus has reached its appropriate titre, as determined by CPE, fluorescent antibody assay, or other approved technique, the virus is clarified, filtered, and inactivated (for killed vaccines).

2.2.2. Requirements for substrates and media

Cell cultures should be demonstrated free of adventitious viruses. All animal origin products used in the production and maintenance of cells (i.e. trypsin, FBS) and growth of virus should be free of adventitious agents, with special attention paid to the presence of bovine viral diarrhoea virus.

2.2.3. In-process controls

Cell cultures should be checked macroscopically for abnormalities or signs of contamination and discarded if unsatisfactory. Virus concentration can be assessed using antigenic mass or infectivity assays.

An inactivation kinetics study should be conducted using the approved inactivating agent (β-propiolactone or 1 ml for 100 ml of viral suspension of 0.1 M binary ethyleneimine [BEI during 24 hours]) on each viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, then inoculated on to a susceptible cell line, should indicate a linear and complete loss of titre by the end of the inactivation process.

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation (if killed vaccine) and prior to further processing.

2.2.4. Final product batch tests

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.

i) Sterility and purity

During production, tests for bacteria, *Mycoplasma*, and fungal contamination should be conducted on both inactivated and live vaccine harvest lots and confirmed on the completed product (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

ii) Safety

The use of target animal batch release safety tests or laboratory animal batch release safety tests should be avoided wherever possible.

iii) Batch potency

Potency is examined on the final formulated product. Mirroring what is done for the potency test in FMD vaccines, a vaccination–challenge test has being proposed for testing VSV vaccines (House *et al.,* 2003). The gaps in knowledge regarding the pathogenesis of VSV infection and the immune mechanism that affords protection against viral infection are limitations for development and implementation of a validated protocol for a challenge test. However, for batch release, indirect tests can also be used for practicability and animal welfare considerations, as long as correlation has been validated to protection in the target animal during efficacy tests. Frequently indirect potency tests include antibody titration after vaccination of target species. Ideally, indirect tests are carried out for each strain for one species and each formulation of vaccine to establish correlation between the indirect test results and the vaccine efficacy test results.

Relative potency could be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended.

For killed and modified live virus (MLV) vaccines product safety will be based on an absence of adverse reactions such as shock, abscesses at site of inoculation, etc. In the specific case of MLV vaccines, it would not be expected to see clinical signs. If clinical signs of vesicular stomatitis virus are observed, use of the vaccine should be reconsidered. Residual virus should be evaluated for prior to mixing the antigen with adjuvant. Initial safety is evaluated in a few animals for 21 days under close observation to assess for gross safety issues. If the vaccine passes this first safety test, the vaccine is used in the field in a larger number of animals to evaluate if subtle safety issues are present: adverse reactions/swelling, abscesses, shock, etc.

ii) Reversion-to-virulence for attenuated/live vaccines

Reversion to virulence for live viral vaccines is often demonstrated by back passage through susceptible species. Virus is isolated from the vaccinated animal and the isolated virus is then used to inoculate additional animals. Sequential passage through animals should show that animals remain clinically healthy with no demonstration of typical vesicular stomatitis lesions.

iii) Environmental consideration

Inactivated vesicular stomatitis vaccines probably present no special danger to the user, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. MLV vaccines may pose a hazard to the user depending on the level of inactivation of the virus.

Preservatives should be avoided if possible, and where not possible, should be limited to the lowest concentration possible. Vaccine bottles, syringes, and needles may pose an environmental hazard for vaccines using adjuvants or preservatives and for MLV vaccines. Instructions for disposal should be included within the vaccine packaging information and based on current environmental regulations in the country of use.

2.3.2. Efficacy requirements

The gaps in knowledge regarding the pathogenesis of VSV infection and the immune mechanism that affords protection against viral infection are limitations for the development and implementation of a validated protocol for an efficacy test. Ideally vaccine efficacy should be estimated in vaccinated animals directly by evaluating their resistance to live virus challenge. Vaccine efficacy should be established for every strain to be authorised for use in the vaccine.

Live reference VSV viruses corresponding to the virus strains circulating in the region are stored at ultralow temperatures. Each challenge virus is prepared as follows. Tongue tissue infected by VSV should be obtained from original field case of VS and received at the Reference Laboratory in glycerol buffer as described in Section B. *Diagnostic Techniques*.

The preparation of cattle challenge virus follows the process described in Chapter 3.1.8 *Foot and mouth disease*, Section B.1.1 *Virus isolation*, with the view of obtaining a sterile 10% suspension in Eagles minimal essential medium with 10% sterile FBS.

The preparation of the stock of challenge virus to be aliquoted is prepared starting from lesions collected in two cattle over 6 months of age, previously recognised to be free of VSV antibodies. These animals are tranquillised, for example using xylazine 100 mg/ml (follow instructions for use), then inoculated intradermally (i.d.) in the tongue with the suspension in about 20 sites, 0.1 ml each. The vesiculated tongue tissue is harvested at the peak of the lesions, approximately 2 days later.

A 2% suspension is prepared as above and filtered through a 0.2 μm filter, aliquoted and frozen in the gas phase of liquid nitrogen, and constitutes the stock of challenge virus. The infective titres of this stock are determined both in cell culture ($TCID_{50}$) and in two cattle (BID_{50}: 50% bovine infective dose). These two cattle that have been tranquilised using xylazine, are injected intradermally in the tongue with tenfold dilutions (1/10 through 1/10,000), using four sites per dilution (Henderson, 1949). The cattle titrations are read 2 days later. Most frequently, titres are above 10^6 $TCID_{50}$ for 0.1 ml and above 10^5 BID_{50} for 0.1 ml calculated using the Spearmann–Kärber method. The dilution for use in cattle challenge test is 10 000 DIB_{50} in a total volume of 4× 0.1 ml by intralingual injection for both the 50% protective dose (PD_{50}) test and the PGP (protection against generalised foot infection) test (House *et al.,* 2003).

i) Vaccination–challenge method

For this experimental method, a group of 12 VSV sero-negative cattle of at least 6 months of age are vaccinated with a bovine dose by the route and in the volume recommended by the manufacturer at day 0 and day 40. These animals and a control group of two non-vaccinated animals are challenged 2 weeks or more after the second vaccination. The challenge strain is a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating a total of 10,000 BID_{50} intradermally into four sites (0.1 ml per site) on the upper surface of the tongue. Animals are observed at 7–8 days after challenge.

It was proposed that vaccinated animals showing no lesion on the tongue should be considered fully protected. Vaccinated animals showing lesions at one, two, or three inoculation sites should be considered partially protected, and animals showing lesions at four sites are considered not protected (House *et al.,* 2003). Control animals must develop lesions at four sites. Vaccine should fully protect at least nine animals out of 12 vaccinated (75% protection), the remaining animals being partially or not protected. This test gives a certain measure of the protection following the injection of two commercial bovine doses of vaccine in a limited cattle population.

Although the vaccination-challenge method has been described and published (House *et al.*, 2003) data on the validation under field conditions for the efficacy of released vaccine are not available.

ii) Efficacy in other species

Efficacy tests in other target species, such as horses, are not yet described or standardised. In general, a successful test in cattle should be considered to be sufficient evidence of the quality of a VS vaccine to endorse its use in other species.

2.3.3. Duration of immunity

The duration of immunity (D.O.I) of a VS vaccine will depend on the efficacy (formulation and antigen payload). As part of the approval procedure the manufacturer should be required to demonstrate the D.O.I. of a given vaccine by either challenge or the use of a validated alternative test, such as serology at the end of the claimed period of protection.

2.3.4. Stability

The stability of all vaccines including oil emulsion vaccines should be demonstrated as part of the shelf-life determination studies for approval. Vaccines should never be frozen or stored above the target temperature.

Vaccines should be stored at 4–8°C, with minimal exposure to light. The shelf life should be determined by use of the approved potency test (Section C.2.2.4.iii) over the proposed period of viability.

i) For animal production

Virus(es) used in vaccine production should be antigenically relevant to virus(es) circulating in the field. A vaccination/challenge study in the species for which the vaccine will be used will indicate the degree of protection afforded by the vaccine. Species used in vaccination/challenge studies should be free of antibodies against vesicular stomatitis. Vaccination/challenge studies should be conducted using virus produced by the intended production method, at the maximum viral passage permitted, and using an experimental animal model. It is necessary to confirm the sensitivity, specificity, reproducibility, statistical significance and confidence level of such experimental model.

Antibody levels after vaccination measured *in vitro* could be used to assess vaccine efficacy provided a statistically significant correlation study has been made. For vaccines containing more than one virus (for example, VSNJV and VSIV), the efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different viruses exists.

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label.

If the vaccine is to be used in horses, swine, cattle, or other ruminants destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

ii) For control

The same principles apply as for animal production usage. In addition, it should be noted that antibody responses in vaccinated animals may not be differentiated from animals exposed to field virus. Therefore, vaccinated animals will need to be clearly identified if serological methods will be used in conjunction with compatible clinical signs to assess field virus exposure.

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NB: There are WOAH Reference Laboratories for vesicular stomatitis
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for vesicular stomatitis

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.1.25.

WEST NILE FEVER

SUMMARY

***Description and importance of the disease**: West Nile fever is a mosquito-borne viral disease that can affect birds, humans and horses causing inapparent infection, mild febrile illness, meningitis, encephalitis, or death. West Nile virus (WNV) is a member of the genus* Flavivirus *in the family* Flaviviridae*. The arbovirus is maintained in nature by cycling through birds and mosquitoes; numerous avian and mosquito species support virus replication. For many avian species, WNV infection causes no overt signs while other birds, such as American crows* (Corvus brachyrhynchos) *and blue jays* (Cyanocitta cristata), *often succumb to fatal systemic illness. Among mammals, clinical disease is primarily exhibited in horses and humans.*

Clinical signs of WNV infection in horses arise from viral-induced encephalitis or encephalomyelitis. Infections are dependent on mosquito transmission and are seasonal in temperate climates, peaking in the early autumn in the Northern Hemisphere. Affected horses frequently demonstrate mild to severe ataxia. Signs can range from slight incoordination to recumbency. Some horses exhibit weakness, muscle fasciculation, and cranial nerve deficits. Fever is not a consistently recognised feature of the disease in horses.

***Identification of the agent:** Bird tissues generally contain higher concentrations of virus than equine tissues. Brain and spinal cord are the preferred tissues for virus isolation from horses. In birds, kidney, heart, brain, liver or intestine can yield virus isolates. Virus can also be isolated from mosquitoes. Cell cultures are used most commonly for virus isolation. WNV is cytopathic in susceptible mammalian cell culture systems. Viral nucleic acid and viral antigens can be demonstrated in tissues of infected animals by reverse-transcriptase polymerase chain reaction (RT-PCR) and immuno-histochemistry, respectively.*

***Serological tests:** Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), haemagglutination inhibition (HI), IgG ELISA, plaque reduction neutralisation (PRN) or virus neutralisation (VN) tests. The ELISA, VN and PRN methods are most commonly used for identifying antibody against WNV in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus, Usutu virus, Japanese encephalitis virus, or tick-borne encephalitis (TBE) virus may be encountered.*

***Requirements for vaccines:** A formalin-inactivated WNV vaccine derived from tissue culture, WNV live canarypoxvirus-vectored vaccine, a WNV DNA vaccine and chimeric vaccines are licensed for use in horses.*

A. INTRODUCTION

West Nile virus (WNV) is a zoonotic mosquito-transmitted arbovirus belonging to the genus *Flavivirus* in the family *Flaviviridae* (Smithburn *et al*., 1940). The genus *Flavivirus* also includes Japanese encephalitis virus (see Chapter 3.1.10 *Japanese encephalitis*), St Louis encephalitis virus, Murray Valley encephalitis virus, Usutu virus, and Kunjin virus, among others (Burke & Monath, 2001). WNV has a wide geographical range that includes parts of Europe, Asia, Africa, Australia (Kunjin virus) and in North, Central and South America. Migratory birds are thought to be primarily responsible for virus dispersal, including reintroduction of WNV from endemic areas into regions that experience sporadic outbreaks (Burke & Monath, 2001). WNV is maintained in a mosquito–bird–mosquito transmission cycle, whereas humans and horses are considered dead end hosts. Genetic analysis of WN isolates separates strains into multiple lineages (Mackenzie & Williams, 2009; Vazquez *et al*., 2010). Lineage 1 isolates are found in northern and central Africa, the Middle East, Europe, Indian subcontinent, Australia (Kunjin virus) and in

North and Central America, and Colombia and Argentina in South America (Morales *et al*., 2006). Lineage 2 strains are endemic in central and southern Africa and Madagascar, with co-circulation of both virus lineages in central Africa (Berthet *et al*., 1997; Burt *et al*., 2002). Circulation of WNV strains of lineage 2 have been reported in Austria (Wodak *et al*., 2011), Greece (Danis *et al*., 2011), Hungary (Bakonyi *et al*., 2006), Italy (Savini *et al*., 2012), Romania (Sirbu *et al*., 2011) and Russia (Platonov *et al*., 2011). Strains from either lineage 1 or lineage 2 viruses might be implicated in either human or animal disease.

WNV was recognised as a human pathogen in Africa during the first half of the 20th century. Although several WN fever epidemics were described, encephalitis as a consequence of human WN infection was rarely encountered prior to 1996; since then, outbreaks of human WN encephalitis have been reported from France, Greece, Israel, Italy, North America, Romania, Russia and Tunisia. During the 1960s, WN viral encephalitis of horses was reported from Egypt and France (Panthier *et al*., 1966; Schmidt & El Mansoury, 1963). Since 1998, outbreaks of equine WNV encephalitis have been reported from Argentina, Canada, France, Israel, Italy, Morocco, Spain, and the United States of America. In 2011, an outbreak of equine encephalitis due to Kunjin virus, a lineage 1 subtype of WNV, was reported in Australia (Frost *et al,* 2012; Hall *et al.,* 2001). There was no evidence of disease in humans or birds caused by this virus.

The occurrence of disease in humans and animals along with bird and mosquito surveillance for WNV activity demonstrate that the virus range has dramatically expanded including North, Central and South America as well as Europe and countries facing the Mediterranean Basin.

The incubation period for equine WN encephalitis following mosquito transmission is estimated to be 3–15 days. A low titre level viraemia may precede clinical onset (Bunning *et al*., 2002; Schmidt & El Mansoury, 1963). WN viral encephalitis occurs in only a small percentage of infected horses; the majority of infected horses do not display clinical signs (Ostlund *et al*., 2000). The disease in horses is frequently characterised by mild to severe ataxia. Additionally, horses may exhibit weakness, muscle fasciculation and cranial nerve deficits (Cantile *et al*., 2000; Ostlund *et al*., 2000; 2001; Snook *et al*., 2001). Fever is an inconsistently recognised feature. Treatment is supportive and signs may resolve or progress to terminal recumbency. The mortality rate is approximately one in three clinically affected unvaccinated horses. Differential diagnoses in horses include other arboviral encephalidites (e.g. eastern, western or Venezuelan equine encephalomyelitis, Japanese encephalitis), equine protozoal myelitis (*Sarcocystis neurona*), equine herpesvirus-1, Borna disease and rabies.

Most species of birds can become infected with WNV; the clinical outcome of infection is variable. Some species appear resistant while others suffer fatal neurologic disease. Neurological disease and death have been documented in domestic geese in Israel and Canada, and in many native and exotic zoo birds in the USA during the emergence of WNV (Austin *et al*., 2004; Steele *et al*., 2000). In Europe fatal neurological disease has been reported in wild birds (Zeller & Schuffenecker, 2004). WNV has been associated with sporadic disease in small numbers of other species, including squirrels, chipmunks, bats, dogs, cats, white-tailed deer, reindeer, sheep, alpacas, alligators and harbour seals during intense periods of local viral activity. WNV was isolated from a dromedary camel indicating this species as a possible source for the viral infection (Joseph *et al.,* 2016).

There has been confirmed transmission of WNV in humans by blood transfusion, organ transplantation and breast milk, but most human infections occur by natural transmission from mosquitoes. Laboratory acquired infections have also been reported (Campbell *et al.,* 2002).

Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). No vaccines are presently available for use in humans, however there is currently a WNV vaccine being used in human clinical trials. (NIH, 2015).

Due to the occurrence of inapparent WNV infections, diagnostic criteria must include a combination of clinical assessment and laboratory tests.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of West Nile fever and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[a]						
Nested RT-PCR[b]	–	++	–	+++[c]	–	–
Real time RT-PCR[b]	–	++	–	+++[c]	–	–
Immunohisto-chemistry	–	–	–	+	–	–
Isolation in tissue culture	–	++	–	++	–	–
Detection of immune response						
IgM capture ELISA	–	–	–	++	–	–
IgG indirect and competitive ELISAs[d]	++	–	+	–	++	++
PRN	++	–	+	+	++	++
VN	++	–	+	+	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcriptase polymerase chain reaction; IgM = immunoglobulin M; ELISA = enzyme-linked immunosorbent assay; PRN = plaque reduction neutralisation; VN = virus neutralisation.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]RT-PCR methods may be used to declare domestic birds free from infection.
Restrictions to movements do not include dead-end hosts such as horses.
[c]RT-PCR positive results from horses are a rare event, thus to confirm suspect cases, serological tests such as IgM capture ELISA and seroconversion assessed by PRN or VN are recommended.
[d]Both ELISA techniques lack specificity as they cross-react with antibodies directed to other flaviviruses, thus positive samples should be confirmed by a more specific test such as PRN or VN.

1. Identification of the agent

1.1. *In-vitro* and *in-vivo* culture

Attempts to detect virus from live, clinically ill horses are not usually successful due to the fleeting viraemia. Specimens for virus isolation include brain (particularly hindbrain and medulla) and spinal cord from deceased encephalitic horses (Ostlund *et al*., 2000; 2001); a variety of bird tissues including brain, heart or liver may be used with success (Steele *et al*., 2000). WNV can also be isolated from mosquitoes. In general, virus isolates are obtained more easily from avian specimens and to a lesser extent from mosquitoes and horses.

Virus may be propagated in susceptible cell cultures, such as rabbit kidney (RK-13), African green monkey kidney (Vero), baby hamster kidney (BHK-21), or pig kidney cells. Primary isolation in embryonated chicken eggs or *Aedes albopictus* (C6/36) cell lines followed by passages in mammalian cells can also be used. More than one cell culture passage may be required to observe cytopathic effect (CPE). Confirmation of WNV isolates is achieved by indirect fluorescent antibody staining of infected cultures or nucleic acid detection methods (see below).

1.2. Molecular methods – detection of nucleic acid

Several polymerase chain reaction (PCR) methods have been described for the identification of WNV and some are available as commercial kits. Included here is a real-time reverse-transcriptase (RT)-PCR with the capacity to detect both lineage 1 and lineage 2 WNV (Eiden *et al.,* 2010). Additionally, a conventional, gel-based RT-PCR designed to detect lineage 1 North American strains is described (Johnson *et al*., 2001). Both assays have been successfully employed with field-collected samples. Lineage 1 WNV from France, Egypt, Israel, Italy, Kenya, Mexico and Russia demonstrate a highly conserved nucleotide sequence in the target region, regardless of species of origin (Lanciotti *et al*., 2000). While the laboratory practices required to avoid contamination in a nested method are stringent, there is higher sensitivity for detection of North American strains of WNV RNA with the conventional nested procedure, particularly in equine field samples. The efficiency of the conventional RT-PCR to detect other WNV lineages is not known. In view of the continued evolution and possible emergence of new WNV strains, it is important that the designs of PCR tests are constantly monitored and updated when necessary. Samples appropriate for WNV RT-PCR include mammalian brain, avian brain, kidney, heart, liver, spleen, intestine, and insect pools. In any PCR assay it is imperative to include positive and no-template controls. For the nested RT-PCR, measures must be employed to avoid cross-contamination with products of the primary RT-PCR during the transfer of the outer primer product to the nested PCR reaction tubes. For any PCR reaction to be valid, the control reactions must fall within the expected range.

1.2.1. Extraction of viral RNA

Several commercial kits are available for RNA extraction. Select a kit appropriate for the type of sample and follow the manufacturer's recommendations.

1.2.2. Real-time reverse-transcriptase PCR

The following method was developed by Eiden *et al.* (2010) for concurrent identification of lineage 1 and lineage 2 WNV. Strain identification may be achieved by sequencing of the resultant amplicon and alignment with WNV reference strains. The procedure has been slightly modified from the published method and included here are the primers and probe directed to the NS2A region of the WNV genome. The assay may be performed with a commercial kit of choice that provides the expected amplification of the controls. The cycling parameters must be adjusted to conform to the kit requirements and melting temperatures of primers and probe. Primer and probe concentrations may be adjusted to achieve optimal results. An internal control and appropriate control primers and probe may be included to confirm valid test conditions.

Primers/probe (NS2A region of genome):

Forward primer: GGG-CCT-TCT-GGT-CGT-GTT-C

Reverse primer: GAT-CTT-GGC-YGT-CCA-CCT-C

Probe: FAM-CCA-CCC-AGG-AGG-TCC-TTC-GCA-A-BHQ

Per sample, prepare 20 µl volume of the RT-PCR reagents (per kit instructions) containing a 0.9 µM concentration of each primer and a 0.25 µM probe concentration. Dispense 20 µl of the mixture into each sample PCR tube or well. Add 5.0 µl of the extracted RNA sample, seal the tube/plate, and place in the thermocycler. Run the samples under the conditions described for the kit in use, beginning with a reverse transcription incubation, followed by 45 cycles of amplification. Ct values of 37 or less are considered positive for WNV. Ct values of 37.1 through 42 are considered suspect, and should be repeated. Values higher than 42 are negative. For the

PCR to be valid, the Ct values of the positive controls should fall within the expected range. No-template controls must be negative.

1.2.3. Conventional reverse-transcriptase PCR

The following method was developed for detection of lineage 1 WNV (Johnson *et al.,* 2001). The procedure may be conducted as a one-step RT-PCR using the outer primers only, or as a nested assay. The nested assay is the most sensitive RT-PCR and is recommended for testing of mammalian brain tissues or other samples that may contain a low amount of virus. The target of this RT-PCR is the E region of the WNV genome. The assay may be performed with a commercial kit of choice that provides the expected amplification of the controls. The cycling parameters must be adjusted to conform to the kit requirements.

Outer primers:

1401F: ACC-AAC-TAC-TGT-GGA-GTC

1845R: TTC-CAT-CTT-CAC-TCT-ACA-CT

Nested primers:

1485F: GCC-TTC-ATA-CAC-ACT-AAA-G

1732R: CCA-ATG-CTA-TCA-CAG-ACT

Per sample, prepare 45 µl volume of the RT-PCR reagents containing a 0.6 µM concentration of each outer primer. Dispense 45 µl of the mixture into each sample PCR tube. Add 5.0 µl of the extracted RNA sample and place the tubes in the thermocycler. Run the samples under the conditions described for the kit in use, beginning with a reverse-transcription incubation, for 60 minutes or according to the kit's recommendation, at the temperature specified by the manufacturer for the enzyme provided, followed by sample denaturation and 35 cycles of amplification. For the nested reaction, prepare a similar reaction mixture (without reverse transcriptase) of 49 µl per sample containing the nested primers and dispense into PCR tubes. Transfer 1.0 µl of the outer primer amplification product to the nested tube and place in the thermocycler. Use caution when transferring the amplification product to avoid cross contamination of samples. Perform 35 amplification cycles per kit recommendations. Based on the melting temperatures of the primers, the annealing temperature for primary RT-PCR and nested PCR should be 46°C. Following amplification, mix 8–10 µl of each PCR product with a loading buffer containing a DNA stain. Load the mixture into a gel and analyse by agar gel electrophoresis and ultraviolet visualisation. WNV-positive samples will be identified by a 445 base-pair band (outer primer amplification only) and/or a 248 base pair band (nested amplification). For the PCR reaction to be valid, positive controls must have the bands of the expected size, and template controls should not have bands. PCR amplicons may be sequenced for identity confirmation.

1.3. Antigen detection – immunolabelling techniques

Immunohistochemical (IHC) staining of formalin-fixed avian tissues is a reliable method for identification of WNV infection in birds. Brain, heart, kidney, spleen, liver, intestine, and lung are often IHC-positive tissues in infected birds. The success rate of IHC detection in positive birds is enhanced by the examination of multiple tissues. The specificity of identification (e.g. flavivirus specific or WNV specific) depends on the selection of detector antibody. The brain and spinal cord tissues of horses with WN viral encephalitis are inconsistently positive in IHC tests; many equine encephalitis cases yield false-negative results. Failure to identify WNV antigen in equine central nervous system does not rule out infection. For further advice, consult the WOAH Reference Laboratory.

2. Serological tests

Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), haemagglutination inhibition (HI), IgG ELISA, plaque reduction neutralisation (PRN), and microtitre virus

neutralisation (VN) (Beaty *et al*., 1989; Hayes, 1989). The IgM capture ELISA described below is particularly useful for detecting equine antibodies resulting from recent natural exposure to WNV. Equine WNV-specific IgM antibodies are usually detectable from 7–10 days post-infection to 1–2 months post-infection. Most horses with WN encephalitis test positive in the IgM capture ELISA at the time that clinical signs are first observed. WNV neutralising antibodies are detectable in equine serum by 2 weeks post-infection and can persist for more than 1 year. The ELISA, HI, VN and PRN methods are most commonly used for identifying WNV antibody in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus or Japanese encephalitis virus, will be encountered. The PRN test is the most specific among WNV serological tests; when needed, serum antibody titres against related flaviviruses can be tested in parallel. Finally, WN vaccination history must be considered in interpretation of serology results, particularly in the PRN and VN tests and IgG ELISA. An IgM capture ELISA may be used to test avian or other species provided that species-specific capture antibody is available (e.g. anti-chicken IgM). The PRN test is applicable to any species, including birds.

2.1. ELISA and related methods

2.1.1. Equine IgM capture ELISA

Several kits for IgM detection from equine specimens are commercially available, alternatively WNV and negative control antigens for the IgM capture ELISA may be prepared from mouse brain (see Chapter 3.6.5 *Equine encephalomyelitis* [*Eastern, Western and Venzeuelan*]), tissue culture or recombinant cell lines (Davis *et al*., 2001). Commercial sources of WNV testing reagents are available in North America. Characterised equine control serum, although not an international standard, can be obtained from the National Veterinary Services Laboratories, Ames, Iowa, USA. Virus and negative control antigens should be prepared in parallel for use in the ELISA. Antigen preparations must be titrated with control sera to optimise sensitivity and specificity of the assay. Equine serum samples are tested at a dilution of 1/400 and equine cerebrospinal fluid samples are tested at a dilution of 1/2 in the assay, or as specified by the kit manufacturer. To ensure specificity, each serum sample is tested for reactivity with both virus antigen and control antigen.

2.1.2. Example test procedure

i) Coat flat-bottom 96-well ELISA plates with 100 µl/well anti-equine IgM diluted in 0.5 M carbonate buffer, pH 9.6, according to the manufacturer's suggested dilution for use as a capture antibody.

ii) Incubate plates overnight at 4°C in a humid chamber. Coated plates may be stored for several weeks in a dry or desiccated chamber.

iii) Prior to use, wash plates twice with 200–300 µl/well 0.01 M phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST).

iv) Block plates by adding 300 µl/well freshly prepared 5% nonfat dry milk in PBST (or as specified by the kit manufacturer) and incubate 60 minutes at room temperature. After incubation, remove blocking solution and wash plates three times with PBST.

v) Test and control sera are diluted 1/400 (cerebrospinal fluid is diluted 1/2) in PBST and 50 µl/well of each sample is added to duplicate sets of wells (total of four wells per sample) on the plate. Include control positive and negative sera prepared in the same manner as samples.

vi) Cover the plates and incubate 75 minutes at 37°C in a humid chamber.

vii) Remove serum and wash plates three times in PBST.

viii) Dilute virus and negative control antigens in PBST and add 50 µl of virus antigen to one set of wells per test and control sera and add 50 µl normal antigen to the second set of wells per test and control sera.

ix) Cover the plates and incubate overnight at 4°C in a humid chamber.

x) Remove antigens from the wells and wash the plates three times in PBST.

xi) Dilute horseradish peroxidase conjugated anti-*Flavivirus* monoclonal antibody[1] in PBST according to manufacturer's directions and add 50 µl per well.

xii) Cover the plates and incubate at 37°C for 60 minutes.

xiii) Remove conjugate and wash plates six times in PBST.

xiv) Add 50 µl/well freshly prepared ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) chromogen with hydrogen peroxide (0.1%) and incubate at room temperature for 30 minutes. Alternative chromogens may be used as indicated by the kit manufacturer.

xv) Measure absorbance at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing control antigen.

2.1.3. Indirect and competitive ELISAs

Numerous indirect and competitive commercial and in-house ELISAs have been developed and are used to detect WNV antibodies. While competitive assays are applicable for sera of all species, indirect assays require enzyme-labelled species-specific secondary antibodies. Most ELISA techniques lack specificity as they cross-react with antibodies directed to other flaviviruses especially those of the Japanese encephalitis serogroup. They are very useful for epidemiological and surveillance purposes as well as a screening method. A positive ELISA result however should be confirmed by a more specific test such as VN or PRNT. Some indirect or competitive ELISAs can detect antibodies of any immunoglobulin class (IgM, IgG, etc.). Vaccinated horses often test positive on the indirect or competitive ELISAs.

2.2. Neutralisation

2.2.1. Plaque reduction neutralisation (applicable to serum from any species)

The PRN test is performed in Vero cell cultures in either 25 cm^2 flasks or 6-well plates. The sera can be screened at a 1/10 and 1/100 final dilution or may be titrated to establish an endpoint. A description of the test as performed in 25 cm^2 flasks using 100 plaque-forming units (PFU) of virus is as follows.

Prior to testing, serum is heat inactivated at 56°C for 30 minutes and diluted (e.g. 1/5 and 1/50) in cell culture medium. Virus (200 plague-forming units per 0.1 ml) working dilution is prepared in media containing 10% guinea-pig complement. Equal volumes of virus and serum are mixed and incubated at 37°C for 75 minutes before inoculation of 0.1 ml onto confluent cell culture monolayers. The inoculum is adsorbed for 1 hour at 37°C, followed by the addition of 4.0 ml of primary overlay medium. The primary overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle's Basic Salts Solution without phenol red, 4% fetal bovine serum, 100 µg/ml gentamicin and 0.45% sodium bicarbonate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 72 hours at 37°C. A second 4.0 ml overlay prepared as above, but also containing 0.003% neutral red is applied to each flask. Following a further overnight incubation at 37°C, the number of virus plaques per flask is assessed. Endpoint titres are based on 90% reduction compared with the virus control flasks, which should have about 100 plaques.

2.2.2. Virus neutralisation – microtitre format

The microtitre VN assay is capable of identifying and quantifying antibodies against WNV present in test samples. Its performance is comparable to the PRN test (Di Gennaro *et al.,* 2014); however, it requires less sample volume than PRN and is more suitable when only small volumes of samples are available (Weingartl *et al*., 2003). Appropriate precautions are necessary to prevent human exposure when using live WNV in unsealed microtitre plates.

1 Available from the Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE, Mail Stop C21, Atlanta, Georgia, 30333, USA.

2.2.3. Test procedure

The cell line commonly used is African green monkey kidney (Vero). The VN test requires 3–5 days to be completed.

i) Twenty-five µl each of several dilutions of the test serum, starting from 1/5 to 1/640, are added to each test well of a sterile flat-bottomed microtitre plate and mixed with an equal volume of 100 $TCID_{50}$ of WNV reference virus. Plates are incubated at 37°C in 5% CO_2 in a humidified incubator.

ii) After 1 hour of incubation, approximately 10^4 Vero cells are added per well in a volume of 50 µl of Minimal Essential Medium containing antibiotics. Following incubation for 3–5 days, the test is read using an inverted microscope

iii) Wells are scored for the degree of CPE observed. A sample is considered positive when it shows more than 90% of CPE neutralisation at the lowest dilution (1:10). The serum titre represents the highest serum dilution capable of neutralising more than 90% of the CPE in the tissue culture.

A VN titre greater or equal 1/10 is usually considered specific for WNV. However it should be noted that birds and mammals may show cross reactions at this level after infection with, or vaccination against, Japanese encephalitis or St Louis encephalitis viruses. Cross-reactivity also exists between WNV and the Usutu virus however, specific antibodies can be attributed to one or the other virus as there is a neutralising titre of fourfold or higher for one virus compared with the other when independently tested.

C. REQUIREMENTS FOR VACCINES

1. Background

In 2003, the United States Department of Agriculture (USDA) issued a license for a formalin-inactivated WNV vaccine derived from tissue culture for use in horses. The European Committee for Medicinal Products for Veterinary Use (CVMP) approved this product in 2008. In 2011, the product was conditionally licensed by USDA for use in alligators. In 2004, an inactivated human cell line-derived WNV vaccine obtained a market authorisation in Israel as a veterinary vaccine for geese. Information on Biotechnology-derived vaccines has also been licensed, as detailed in section C.3 below. These vaccines have demonstrated sufficient efficacy and safety in adequately vaccinated horses. Vaccination may be helpful in preventing neurological signs associated with WNV infection.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

See chapter 1.1.8 for general requirements for master seeds and allowable passages for vaccine production.

2.1.1. Biological characteristics of the master seed (MS)

The isolate of WNV used as the master seed virus (MSV) for vaccine production must be accompanied by documentation describing its origin and passage history.

i) Inactivated vaccines

Virulent virus potentially may be used in inactivated vaccines, provided that manufacturing methods ensure complete inactivation of the MSV. The completed vaccine must be safe in host animals at the intended age of vaccination and provide protection after challenge.

ii) Live vaccines

The MSV must be safe in host animals at the most susceptible age for infection. It should not increase in virulence or undergo detectable genetic changes with repeated passage through host animals. Ideally, the MSV should not be shed from vaccinated animals into the environment; otherwise, shedding should be minimal and transient. The MSV should not adversely impact non-target species with which a vaccinated animal may have contact. The completed vaccine must provide protection after challenge.

iii) Recombinant vaccines

Recombinant vaccines may be live or inactivated, and recombinant MSVs are subject to the same guidelines as conventional MSVs. In addition, recombinant MSVs expressing foreign genes should stably produce the foreign antigens.

iv) DNA vaccines

The MS is the host organism (e.g. *Escherichia coli*) that expresses the plasmid used in the vaccine. The completed DNA vaccine is subject to the same requirements as listed above.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The MSV must be tested for purity, identity, and freedom from extraneous agents at the time before it is used in the manufacture of vaccine. The MSV must be free from bacteria, fungi and mycoplasma. It also must be free of extraneous viruses, including equine herpesvirus, equine adenovirus, equine viral arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic equine cell type.

2.1.3. Validation as a vaccine strain

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible horses against a virulent challenge isolate found in the geographical area of intended vaccine use. Ideally the challenge isolate and MSV should be heterologous. MSVs intended for use in live vaccines also must be tested for innocuity in susceptible non-target species, such as birds.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic

In the event that an emerging strain or variant of WNV results in an emergency epizootic situation that cannot be controlled by currently available vaccines, provisional acceptance of a new MSV should be considered, ideally for use in an inactivated vaccine. Such acceptance should be based on a risk analysis of potential contamination of the new MSV with extraneous agents. This risk assessment should consider the source and passage history of the MSV and characteristics of the vaccine manufacturing process, including the nature and concentration of the virus inactivant.

2.2. Method of manufacture

2.2.1. Procedure

The MSV should be propagated in cell lines known to support the growth of WNV. See chapter 1.1.8 for additional guidance on the preparation and testing of master cell stocks. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the MSV, unless further passages prove to provide protection in the host animal.

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum (FBS), may be used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with WNV working virus stock, which is generally 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Inactivated vaccines may be chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant. The duration of the inactivation period is based on demonstrated inactivation kinetics.

DNA vaccine expression cassettes are amplified in *E. coli* (or other suitable vector). Purified plasmids are formulated into a vaccine.

2.2.2. Requirements for ingredients

All ingredients used in the manufacture of WNV vaccine should be defined in approved manufacturing protocols and consistent from batch to batch. See chapter 1.18 for general guidance on ingredients of animal origin. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

2.2.3. In-process controls

Production lots of WNV must be titrated in tissue culture before inactivation to standardise the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Inactivated WNV lots must be tested for completeness of inactivation. Ideal protocols incorporate concentration and amplification steps, to enhance detection of residual live virus.

Production lots of DNA are quantified by analytical methods and characterised before standardisation and blending at the correct DNA content. Production lots must not exceed the highest acceptable level of lipopolysaccharide (LPS) content, based on safety testing.

2.2.4. Final product batch tests

i) Sterility

Inactivated and live vaccine samples are examined for bacterial and fungal contamination. The volume of medium used in these tests should be enough to nullify any bacteriostatic or fungistatic effects of the preservatives in the product. To test for bacteria, ten vessels, each containing a minimum of 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C from 10 days and observed for bacterial growth. To test for fungi, ten vessels, each containing a minimum of 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C from ten days and observed for fungal growth. Individual countries may have other requirements.

ii) Identity

Separate batch tests for identity should be conducted if the batch potency test, such as tissue-culture titrations of live virus vaccines, does not sufficiently verify the identity of the agent in the vaccine. Identity tests may include fluorescent antibody or serum neutralisation assays.

Additionally, if all-in, all-out manufacturing processes and sanitation are not used and more than one agent is propagated in a laboratory, identity tests shall demonstrate that no other vaccine strain is present.

iii) Safety

Safety tests in target animals are not required in many regions for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

Batch safety tests may be conducted in guinea pigs, mice, or host animals. The product should be administered according to label recommendations in host animals. Individual countries may require dose overage (e.g. 2×–10×). No systemic or local adverse reactions should occur after vaccination.

The requirement for an *in-vivo* batch safety test may be exempted if the safety of the product is demonstrated and approved in the registration dossier and production is consistent with that described in chapter 1.1.8 and Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*).

iv) Batch potency

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. In vitro techniques to compare a standard with the final product are also acceptable in determining the relative potency of a product (USDA, 2011). The standard should be shown to be protective in the host animal.

Live viral products are titred in cell cultures to determine the potency of the final product. Compared to the minimum protective dose established in the immunogenicity trial, the batch release potency titre should include overages for batch test variability and loss of potency over product dating. In the absence of specific data to support an alternative, overages of 0.7 $\log_{10}$ and 0.5 $\log_{10}$, respectively, are recommended.

DNA vaccines may be tested for bioactivity and DNA content using parallel-line direct quantification methods that compare a standard preparation to the final product.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of WNV vaccine, all relevant details concerning manufacture of the vaccine, in-process controls, and quality control testing (see Sections C.2.a and C.2.b) should be submitted to the authorities. Test results shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume

2.3.2. Safety requirements

The inherent safety of the vaccine strain, if it will be used in the manufacture of a live vaccine, is tested at the Master Seed level.

i) Target and non-target animal safety

The Seed should be tested in host animals of the most susceptible age. The animals should be monitored for clinical disease and virus shed/spread. The organism dose should be no less than that expected in completed vaccine. Individual countries may have overdose requirements. If the vaccine will be intended for use in specific subpopulations (e.g. pregnant animals), they also should be included in target animal safety studies. Additionally, similar studies should be conducted in susceptible non-target species (e.g. birds).

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Demonstrate that repeated *in-vivo* passage of the MSV does not increase virulence. (For additional guidance see Section: *Increase in Virulence Tests* in chapter 1.1.8.)

The final vaccine formulation (inactivated or live) should be tested in a limited number of target animals prior to a larger-scale field study. The final vaccine formulation should not cause adverse reactions.

Field safety studies should be conducted before any vaccine receives final approval. Generally, two serials should be used, in three different geographical locations under

typical animal husbandry conditions, and in a minimum of 600 animals. The vaccine should be administered according to label recommendations (including booster doses) and should contain the maximum permissible amount of WNV antigen. (If no maximum antigen content is specified, serials should be of anticipated typical post-marketing potency.) About one-third of the animals should be at the minimum age recommended for vaccination.

iii) Precautions (hazards)

Vaccine should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection. Warnings should be included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

To register a WNV vaccine, a batch produced according to the standard method and containing the minimum amount of antigen or potency value must provide protection against virulent challenge in the minimum-aged animal recommended for vaccination. Each future commercial batch shall be tested before release to ensure it has at least the same potency demonstrated by the batch used for the efficacy study.

WNV vaccine efficacy is often estimated in vaccinated horses by evaluating post-challenge viraemia and/or neurological signs (e.g. muscle fasciculation, ataxia, seizures). Efficacy is estimated in alligators by evaluating post-challenge viraemia and/or lymphohistiocytic skin (pix) lesions. Twenty-five vaccinates and ten placebo-vaccinated controls are recommended.

Horses may be challenged intrathecally or via controlled exposure to infected mosquitoes. Alligators may be challenged subcutaneously. All animals should be monitored for 14–21 days after challenge. Viraemia should be evaluated qualitatively (i.e., detectable presence or absence) by validated assays. The vaccination effect may be assessed by calculating prevented fraction with a 95% confidence interval. Individual countries may have minimum efficacy requirements, but in no case should the lower confidence interval include zero.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Vaccination is only recommended for horses and alligators in WN-positive areas. Vaccinated horses may develop a serological titre that may interfere with the ability to export the horse.

2.3.5. Duration of immunity

Studies to determine a minimum duration of immunity should be conducted before the vaccine receives final approval. Duration of immunity should be demonstrated in a manner similar to the original efficacy study, challenging animals at the end of the claimed period of protection. At a minimum, the duration should be for the length of the mosquito season in seasonally infected areas. It may be desirable to demonstrate longer immunity for animals at higher risk and in infected areas with year-round mosquito activity.

2.3.6. Stability

Live and inactivated vaccines are typically assigned an initial expiry date of 18 or 24 months, respectively. Real-time stability studies should be conducted to confirm the appropriateness of all expiration dating. Product labelling should specify proper storage conditions.

3. Vaccines based on biotechnology

In 2003, the USDA licensed a live canarypoxvirus-vectored WNV vaccine for use in horses. In 2005, the USDA issued the first fully licensed WNV DNA vaccine for animals in the USA. The vaccine contains genes for two WNV proteins, and therefore, does not contain any whole WNV, live or killed. In late 2006, a chimeric vaccine, based on a yellow fever (YF) virus vector, was licensed by USDA for use in horses. The CVMP approved a recombinant canarypox virus WNV product in 2011 and an inactivated chimeric flavivirus strain YF-WNV product in 2013. In addition to meeting requirements for efficacy, potency, purity and safety, recombinant seeds must undergo a risk

analysis. The conclusion of the risk analysis must be that the vaccine does not have significant environmental impact.

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NB: There is a WOAH Reference Laboratory for West Nile fever (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for West Nile fever.

NB: FIRST ADOPTED IN 2004 AS WEST NILE ENCEPHALITIS. MOST RECENT UPDATES ADOPTED IN 2018.

SECTION 3.2.

APINAE

INTRODUCTORY NOTE ON BEE DISEASES

Bees are insects that are closely related to ants and wasps. There are many thousands of species of bee, most of which are not social but solitary insects. The honey bee, Apis *species, lives as a colony, which is a family of social insects. A honey bee colony is a super-organism with important implications for disease epidemiology, where disease transmission at both individual and colony levels needs to be considered. There are many species and subspecies of honey bees that are adapted to their environment.*

Two species are important for bee keeping – the western honey bee Apis mellifera*, and the eastern honey bee* A. cerana*. The Western honey bee is native to the continents of Europe and Africa and is the largest of the cavity-nesting honey bees. It is found in almost every country in the world. Twenty-four subspecies of* A. mellifera *are currently recognised. At least two subspecies of* A. mellifera *are of concern to managed beekeeping. The African bee,* A.m. scutellata*, was accidentally introduced into South America and is known for its defensive behaviour. The Cape bee,* A.m. capensis*, can be a major problem to other subspecies of* A. mellifera *as it is a serious social parasite of these in a commercial beekeeping context.*

It is thought that all bees are susceptible to the known diseases of bees, but different subspecies may have varying susceptibility. The diagnosis and control of honey bee diseases at the colony level is quite difficult. More than with other animals, the possibilities and the methods for clinical observation and diagnosis applied depend on seasonal conditions. This is mainly aggravated in regions with a reduced rearing of brood at certain times of the year, normally in winter, and the temporal production of bee products. In terms of treatment with medicinal products and the application of chemical disinfection methods, honey production should always be taken into account as such treatments can contaminate bee products such as honey, wax and pollen.

When sampling a colony of bees for diagnosis of diseases, sampling of dead bees, if present, in or outside the hive, might best reflect the health status of the colony. If live bees are to be sampled, these must first be killed with diethyl ether or in a deep freezer (–20°C) overnight. Bees may also be killed by submersion in 70% ethyl alcohol, e.g. when collected for diagnosis of acariosis (Acarapis)*. Larval and pupal smears must be made when testing for brood diseases or a piece of comb containing brood showing visible signs of disease may be sent to the laboratory. Honey bees are susceptible to diseases caused by parasites, fungi, bacteria and viruses. Honey bee colonies may also be affected by various pests, predators and adverse environmental factors.*

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* *

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2013.

CHAPTER 3.2.1.

ACARAPISOSIS OF HONEY BEES (INFESTATION OF HONEY BEES WITH *ACARAPIS WOODI*)

SUMMARY

Acarapisosis is a disease of the adult honey bee Apis mellifera *L. and other* Apis *species. It is caused by the tarsonemid mite, known as the tracheal mite,* Acarapis woodi*. The adult female mite is approximately 150 μm in size, and is an internal parasite of the respiratory system, living and reproducing mainly in the large prothoracic tracheae of the bee. Sometimes they are also found in the head, thoracic and abdominal air sacs and can also be found at the base of the bee's wings. Mites feed on the haemolymph of their host.*

Pathogenic effects in infected infested bees depend on the number of mites within the trachea. The mites can cause both mechanical injuries and physiological disorders consequent to the obstruction of air ducts, lesions in the tracheal walls, and the depletion of haemolymph. As the mite population increases, the tracheal walls, normally white and translucent, become opaque and discoloured with blotchy black areas, probably due to melanin crusts.

Some strains of bees are less susceptible to A. woodi *infestation. The mortality rate in infested bees may range from moderate to high. Early manifestations of infestation normally go unnoticed, and only when infestation is heavy does it become apparent. This is usually in spring. The mite spreads by direct contact. Generally, adult bees less than 4 days post-emergence are more susceptible. Reproduction occurs within the tracheae of adult bees, where female mites may lay up to 14 eggs. There are usually more females than males, though the ratio can vary. Development takes 11–12 days for males and 14–15 days for females.*

Detection and identification of the agent: *The mites are detected only by laboratory methods either by microscopy, or molecular detection.*

For microscopy, the mites need to be observed inside the tracheae or removed from them to be observed microscopically. The thoraces of suspect bees are dissected to expose the tracheae. Each trachea is examined under a dissecting microscope (×40–60), where the mites will be seen through the transparent wall as small oval bodies.

Alternatively, larger samples of suspect bees can be ground or homogenised in water, followed by coarse filtration of the suspension, and centrifugation. The deposit is treated with undiluted lactic acid for 10 minutes. This is then mounted for microscopic examination.

The mites may be stained by histological techniques so that they can be observed within the bee trachea. The tracheae are separated out, cleared with 5–10% potassium hydroxide, and stained with 1% methylene blue. This is the best method (thoracic disc method) for large numbers of samples.

For molecular detection, both conventional and real-time polymerase chain reaction (PCR) methods that detect the cytochrome I oxidase gene of Acarapis woodi *can be used. Amplicons from the conventional PCR must be sequenced to provide confidence in the detection of* A. woodi*, as distinct from the related mites* A. dorsalis *and* A. externus*. A sample of 105 bees is obtained from a colony and their abdomens removed and discarded. Seven separate DNA extractions are performed, with 15 bees each, where bees are homogenised in lysis buffer and the extracted DNA subjected to PCR. The real-time PCR is useful when large numbers of samples are processed. False-positive detections of* A. externus *and* A. dorsalis *may be possible, and confirmatory testing by microscopy is required.*

Serological tests: *Serological tests are not available.*

Requirements for vaccines: *There are no vaccines available.*

A. INTRODUCTION

Acarapisosis (syn. Acariosis or acarine disease) is a disease of the adult honey bee *Apis mellifera* L. and other *Apis* species, caused by the microscopic tarsonemid mite *Acarapis woodi* (Rennie). The adult female mite is approximately 150 μm in size (Figure 1) and is an internal parasite of the respiratory system. These tracheal mites enter, live and reproduce mainly in the large prothoracic tracheae of all bees, feeding on the haemolymph of their host. Sometimes they are also found in the head, thoracic and abdominal air sacs (Giordani, 1965; Wilson *et al*., 1997).

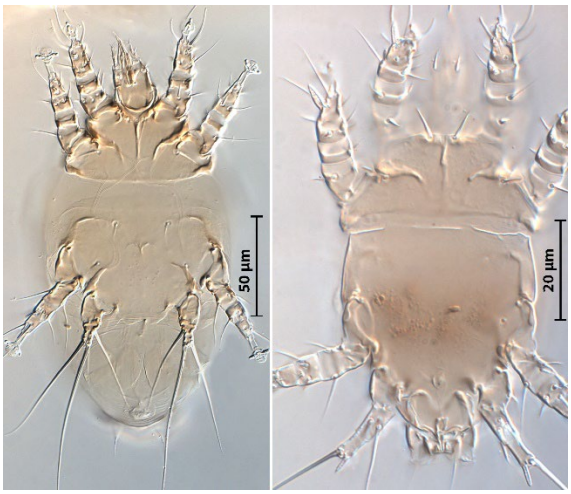

Fig. 1. **Acarapis woodi (*Rennie*).** ***Ventral views of adult female (left) and adult male.***

The pathogenic effects on individual bees depends on the numbers of mites within the tracheae and are attributable both to mechanical injuries and physiological disorders consequent to the obstruction of the air ducts, lesions in the tracheal walls, and to the depletion of haemolymph. As the parasite population increases, the tracheal walls, which are normally whitish and translucent, become opaque and discoloured with blotchy black areas, probably due to melanin crusts (Giordani, 1964).

Colony loss rates may vary, but these losses can be very high. When the mite first establishes in bee populations with no prior exposure. Early signs of infestation may go unnoticed, except for a slow dwindling in the colony size. Only when infestation is heavy does it become apparent. This is generally in spring after the winter clustering period when the mites have bred and multiplied undisturbed in the longer-living winter bees. Some races of bees, such as Buckfast bees (Brother Adam, 1968) and some hygienic strains, are less susceptible to *A. woodi* infestation. Mites spread from one bee to another by direct contact. Generally, only young adult bees (under 4 days post-emergence), are susceptible. Attempts to rear *A. woodi* artificially have had limited success (Bruce *et al.,* 1991). Controlled infestation of immature bees can be achieved (Giordani, 1970) and has allowed for the determination of mite life cycle, host preference and host resistance, and the effect of the mites on adult bees. Reproduction occurs within the tracheae of adult bees, where female mites may lay 8–20 eggs. There are 2–4 times as many females as males; development takes 11–12 days for males and 14–15 days for females.

There are no reliable clinical signs for the diagnosis of acarapisosis as the signs of infestation are not specific and the bees behave in much the same way as bees affected by other diseases or disorders. They crawl around in the front of the hive and climb blades of grass, unable to fly. Dysentery may be present. Heavy mite infestations in winter months affects the ability of colonies to regulate their cluster temperature leading to chilling, which can be a significant cause of death Otis & Scot-Dupree (1992).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available and their purpose

Method	Purpose					
	Population freedom from infestation	Individual animal freedom from infestation prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infestation – surveillance	Immune status in individual animals or populations post-vaccination
Agent detection and identification						
Microscopy – bee dissection	+++	–	+++	+++	++	–
Microscopy – bulk sample	+	–	+	++	+	–
Conventional PCR	+	–	+	++	+	–
Real-time PCR	++	–	++	++	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.

1. Detection and identification of the agent

Acarapisosis can be detected only in the laboratory using microscopic examination or by molecular detection. The number of bees sampled determines the detection threshold of the method. It has been shown that a 1 to 2% rate of infestation can be detected by sampling 50 bees. Sequential sampling data are available (Frazier *et al*., 2000; Tomasko *et al*., 1993). The best time to take bee samples is in the early spring or late autumn when *Acarapis* populations are high. Visualisation of mites is easier in older bees, which have more mites. Even though drones can be found to have a higher abundance of mites per bee (Dawicke *et al.,* 1992) the most significant caste affected by *A. woodi* is the worker bee population, which far outnumber drones and are present in the colony throughout the entire year, including the seasons that a colony is most vulnerable, being winter and early-spring.

1.1. Microscopy – dissection of individual bees

Microscopy provides the simplest and most reliable technique for the laboratory diagnosis of acarapisosis, allowing for the detection of early infestations and enabling the infestation rate to be established. Even light infestations can be detected using a dissecting microscope (40–60×). Only in very exceptional instances will it be necessary to employ higher magnifications to make a diagnosis. However, detection methods using microscopy are demanding techniques and require lots of time, especially when a large number of samples is to be processed.

A sample of 50 bees (see above) should be collected from the colony. These are mainly bees crawling and unable to fly, found within about 3 metres of the front of the hive. This is preferable to random collection from within the colony. The bees may be living, dying, or dead. Live bees must first be euthanised with ethyl alcohol or in a deep freezer (–20°C) for up to 48 hours; sampled dead bees should not have been dead for more than 2–3 days unless kept at 4°C for up to 4 weeks or –20°C several months. They may be preserved indefinitely in a preservative such as Oudeman's solution: glacial acetic acid (80 ml); glycerol (50 ml); 70% ethanol (870 ml).

1.1.1. Test procedure: direct preparation (Milne, 1948; Lorenzen & Gary, 1986)

i) Place bee under a dissection microscope on their backs and hold using forceps or insect pins, then remove the head and first pair of legs from the thorax using a blade.

ii) Remove the prothoracic sclerite (collar) using forceps.

iii) The two thoracic tracheal trunks in the mesothorax are exposed (Figure 2). Positive diagnosis consists of either the presence of melanisation of one or both tracheae or, in light infestation, of the presence of oval translucent bodies (eggs etc.) easily seen within the tracheae.

iv) For further microscopic examination, especially for confirmation of light infestation, remove the tracheae and put them onto a slide, with a drop of Hoyer's medium: distilled water (50 mm), chloral hydrate (200 g), glycerine (20 ml) and crystalline gum arabic (30 g). Under the microscope at 100× magnification the adult mites as well as their individual stages of development can be recognised.

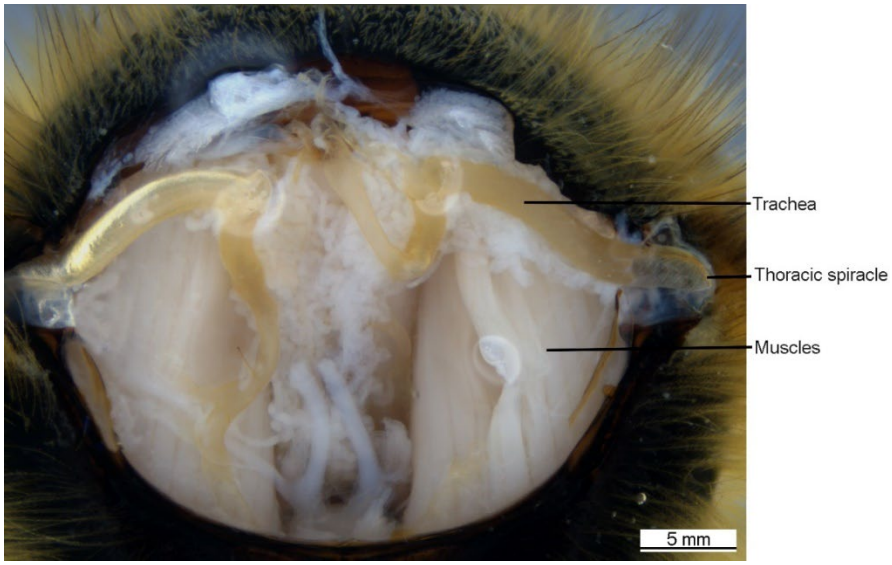

Fig. 2. The first thoracic pair of tracheae exposed in the mesothorax of a bee (Acarapis woodi are not present in this specimen).

1.1.2. Test procedure: thoracic disc method (Peng & Nasr, 1985; Sammataro *et al.*, 2013)

i) Lay bees on their backs and hold with forceps.

ii) Remove the heads and forelegs using a second pair of forceps. This exposes the tracheae in the mesothorax.

iii) Using a sharp scalpel or razorblade, cut through the thorax in front of the middle pair of legs to create a 1–1.5 mm thin thoracic section (disc). Many thoracic discs can be prepared in this way and kept chilled at 4°C, or frozen, before further preparation.

iv) Muscle is cleared from the thoracic disc by heating at 60°C in a 5–10% solution of potassium hydroxide (KOH) for 2 hours.

v) Debris is removed by rinsing the sections under running water in a fine sieve.

vi) The sections are returned to a 5–10% solution of potassium hydroxide and heated to 60°C for 1 hour. The sections will become transparent in the centre leaving only the sclerotised tergites and main tracheal trunks.

vii) The sections are washed gently under running water in a fine sieve to remove any residual debris.

viii) Sections are transferred to a petri dish containing distilled water and a few drops of 1% aqueous methylene blue; staining for 5 minutes.

ix) When staining is complete, remove the sections from the staining solution and transfer them to a dissecting microscope for visual assessment. Examine at 20–40× magnification, with illumination from below. Mites are easily seen through the transparent wall as small, oval bodies (Figure 3).

x) The sclerotised tergites can be torn away using forceps to make the trachea more visible. Trachea can then be mounted on a glass slide and transferred to a compound microscope for more detailed visualisation of mites (*ca.* 100× magnification; Figure 3C).

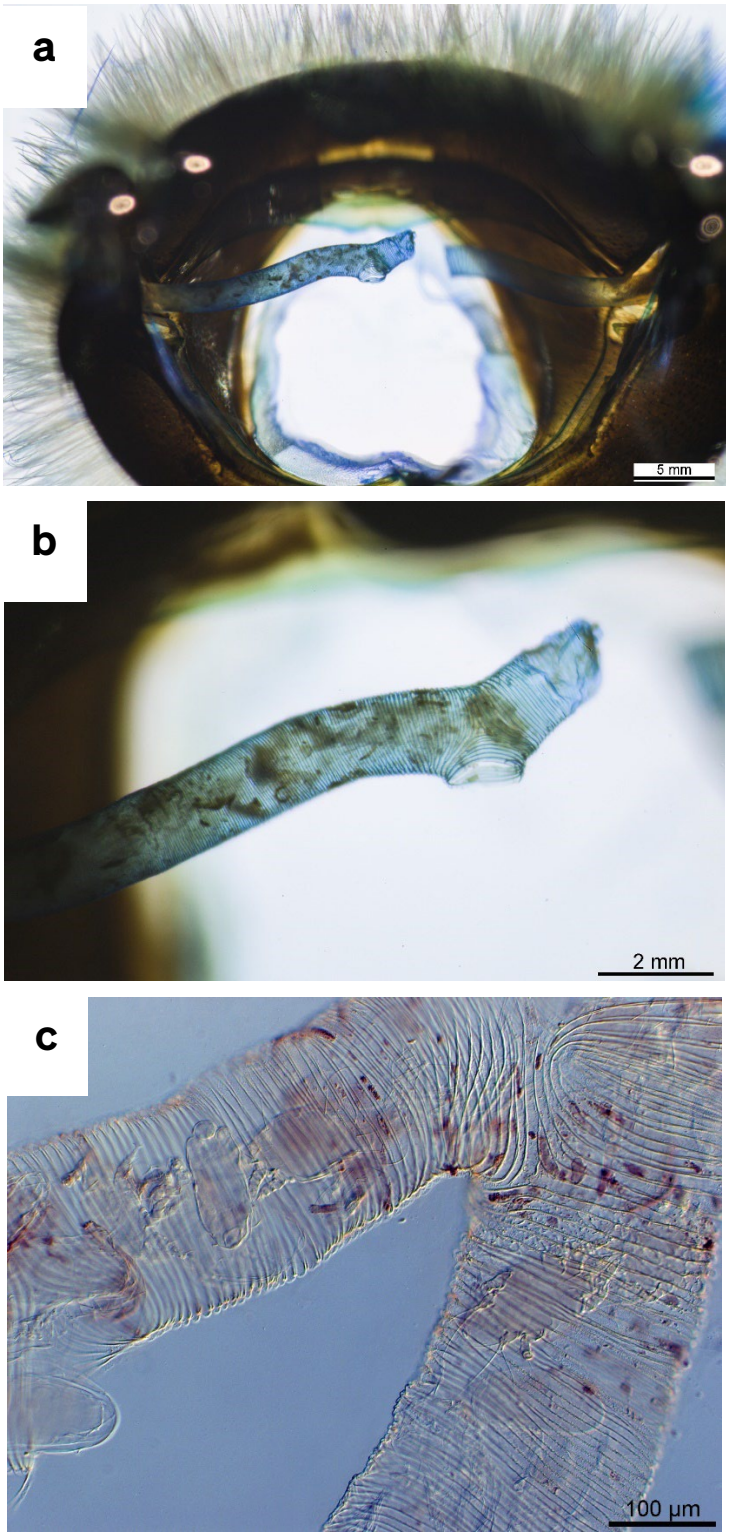

Fig. 3. **(*a*)** ***Presence of Acarapis woodi in tracheae of honey bee, revealed using the thoracic disc method and a dissecting microscope;*** **(*b*)** ***under higher magnification;*** **(*c*)** ***presence of Acarapis woodi in tracheae viewed using a compound microscope.***

1.2. Microscopy – bulk sample preparation and screening based on mite morphology (Colin *et al.*, 1979)

A sample of 200 bees is collected at random from the suspect colony. The wings and legs of each bee are removed from the thorax, and the bodies are pooled in a 100 ml container that has been one-quarter filled with water. This suspension is homogenised three times, each time for several seconds, in a homogeniser at 10,000 rpm with the addition of more water. The resulting suspension is strained through a sieve (mesh 0.8 mm) and the sieve is rinsed with water to a final volume of approximately 50 ml. The filtrate is centrifuged at 1500 ***g*** for 5 minutes and the supernatant fluid is discarded. A few drops of undiluted lactic acid solution are added to the debris of the deposit, which will contain the mites. This is left for 10 minutes to allow the muscle fibres to dissolve, and is then mounted under a cover-slip for microscopic examination. This technique is quicker than dissection, but is less accurate. External mites *A. externus* and *A. dorsalis*, both of which are morphologically similar to *A. woodi*, are often found on the neck and thorax of healthy bees and can very easily be mistaken for *A. woodi* (Delfinado-Baker *et al.*, 1982). It seems, however, that they do not cause any serious threat to bees or beekeeping. This method should therefore only be chosen if all that is required is a rough estimation of the degree of infestation in a region. It is not suitable for detection of an incursion into a region.

1.3. Molecular detection of *Acarapis woodi* infestation of *Apis mellifera*

Detection of *Acarapis woodi* infestation in bee colonies using PCR methods is faster and more efficient than microscopy, and may be more sensitive. However, caution must be exercised when interpreting the results of PCR testing due to the genomic similarities between *A. woodi* and the close-relatives *A. dorsalis* and *A. externus*, which for certain rare genotypes may lead to false-positive detections of *A. woodi*. Positive detections require confirmation by microscopy.

1.3.1. Extraction of nucleic acid from *Apis mellifera* for detection of *Acarapis woodi* (Delmiglio *et al.*, 2016)

A conservative sample size of 105 bees per colony should be used, although smaller sample sizes can be used if it is expected that high mite infestations are present. A maximum number of 15 bees can be used in a single DNA extraction, ensuring that a single bee with a low-level of infestation (<10 mites) can be detected. A minimum of seven DNA extractions are required to test a colony sample of 105 bees.

i) 105 bees are shaken in warm water on an orbital mixer for 20 minutes to dislodge external mites.

ii) The abdomen of each bee is removed using a clean scalpel and the heads and thoraxes are placed into filter-mesh grinding bags to separate exoskeleton fragments after maceration. 0.5 ml of a nucleic acid lysis buffer is added per bee; commercial nucleic acid extraction buffers that contain chaotropic salts e.g. guanidine thiocyanate, are available for DNA extraction.

iii) Bees are macerated in the lysis buffer using a grinder or paddle blender, and 600 µl of lysate is placed into a clean reaction tube with 30 µl proteinase K (concentration) and incubated at 65°C for 30 minutes with mixing

iv) The lysate is then subjected to centrifugation at 8000 ***g*** for 1 minute.

v) The resulting supernatant is aspirated and subjected to DNA extraction; commercial DNA extraction kits are available and selected kits should be validated for diagnostic purposes before use e.g. magnetic-bead particle separation methods, or affinity column-based separation.

1.3.2. Conventional PCR (Evans *et al.*, 2007; Kojima *et al.*, 2011; Navajas *et al.* 1996)

Conventional PCR approaches for the detection of *Acarapis woodi* are available but require confirmatory sequencing of the amplicons to provide confidence of detection. Nucleic acid extraction can be performed using the method described in Section B.1.3.1, but alternative DNA extraction techniques have been used for the conventional PCR approaches, including extraction of DNA from individual *Acarapis* sp. mites.

Evans *et al.* (2007) use amplification of the mitochondrial cytochrome oxidase I gene (Navajas *et al.,* 1996) to detect *Acarapis* sp., with sequencing of the amplicon to provide species-level determination of *A. woodi, A. externus* and *A. dorsalis*. Nested-PCR primers are also available that may enhance the sensitivity of detection.

Table 2. PCR primer sequences

Primer/Probe	Sequence (5' → 3')	Amplicon length*	Region
MitCOI.F	AGT-TTT-AGC-AGG-AGC-AAT-TAC-TAT	559 bp*	Cytochrome oxidase I
MitCOI.R	TAC-AGC-TCC-TAT-AGA-TAA-AA		
AcwdCOI.F	TCA-ATT-TCA-GCC-TTT-TAT-TCA-AGA	377 bp*	Cytochrome oxidase I
AcwdCOI.R	AAA-ACA-TAA-TGA-AAA-TGA-GCT-ACA-ACA		

*Inferred from primer alignment with genbank accessions, KX790788 and LC512730.

If using a commercial PCR kit, the required reagents may already be included. Check and follow the manufacturer's instructions.

PCR reactions using MitCOI primers (Evans *et al.,* 2007; Navajas *et al.,* 1996) are set up in a total volume of 25 µl, as follows:

i) 1–5 µl template DNA (see Section B.1.3.1);

ii) 0.2 µM forward (MitCOI.F) and reverse primer (MitCOI.R);

iii) 1 mM dNTPs;

iv) 2 mM $MgCl_2$;

v) 1 U of Taq polymerase in the appropriate PCR buffer

Using the following thermocycling conditions: 30 cycles of 94°C (1 minute), 52°C (1 minute), and 72°C (1 minute); and a final cycle of 72°C (5 minutes).

Subsequent nested-PCR on the amplicon using the same reaction conditions but with internal forward (AcwdCOI.F) and reverse (AcwdCOI.R) primers may provide greater sensitivity for detection, where Evans *et al.* (2007) report this nested-PCR approach can be used to determine the *Acarapis* species of a single isolated mite. The molecular weights of the amplicons can be determined by electrophoresis in a 0.8% agarose gel and staining with a DNA-intercalating dye. Amplicon size is not sufficient for *Acarapis* species-level assignment, and sequencing of the amplicon with comparison to reference sequences on genetic databases is required to be confident when assigning a detection to one of the three *Acarapis* species.

Amplicons can be purified using a commercially-available method, such as resin-binding or enzymatic-digestion of <100 bp fragments, and then amplicons sequenced using Sanger-method, or an alternative sequencing approach. Amplicon sequence should be aligned with *Acarapis* sp. sequences from genetic databases and a phylogenetic tree constructed to determine the closest relative.

An alternative series of primers is available for the detection of *Acarapis* sp. (Kojima *et al.,* 2011) but they have not been trialled against *A. dorsalis* and therefore have unknown utility in distinguishing *A. dorsalis* from *A. woodi*.

1.3.3. Real-time PCR (Delmiglio *et al.*, 2016)

Specific detection of *A. woodi* using real-time PCR can be achieved by amplification of a 113 nt single variable region within the mitochondrial cytochrome oxidase I gene (COI) (Delmiglio *et al.,* 2016). A small proportion of genetically different *A. externus* have been reported to cross-react

with this assay. Therefore, if a real-time PCR positive is recorded in a population of bees where *A. woodi* has not been reported before, then a confirmatory test using microscopy should be performed.

Table 3. Primer sequences for real-time PCR

Primer/Probe	Sequence (5'→ 3')	Amplicon length	Region
aw_F1-flap	AAT-AAA-TCA-TAA-TGA-TAT-CCC-AAT-TAT-CTG-AGT-AAT-G	113 bp	Cytochrome oxidase I
aw_R3	AAT-ATC-TGT-CAT-GAA-GAA-TAA-TGT-C		
aw_LNAprobe	6-FAM-ACC[+T]GT[+C]AA[+T]CC[+A]CCTAC-BHQ1		

*[+] locked nucleic acid bases

If using a commercial PCR kit the required reagents may already be included. Check and follow the manufacturer's instructions.

PCR reactions (modified from Delmiglio *et al.,* 2016) are set up in a total volume of 10 µl, as follows:

i) 1 µl template DNA (see Section B.1.3.1);

ii) 0.3 µM forward (aw_F1-flap) and reverse primer (aw_R3);

iii) 0.1 µM probe (aw_LNAprobe);

iv) 1 mM dNTPs;

v) 3.5 mM $MgCl_2$;

vi) 0.3 µg bovine serum albumin

vii) 1 U of Taq polymerase in the appropriate PCR buffer

Using the following thermocycling conditions: 95°C (2 minutes), 35 cycles of 95°C (10 seconds), 59°C (45 seconds).

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

ACKNOWLEDGEMENTS

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NB: At the time of publication (2022) there was no WOAH Reference Laboratory for *acarapisosis of honey bees* (infestation of honey bees with *Acarapis woodi*) (please consult the WOAH Web site for the most up-to-date list: https://www.woah.org/en/what-w e-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989 AS ACARIASIS OF HONEY BEES. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.2.2.

AMERICAN FOULBROOD OF HONEY BEES (INFECTION OF HONEY BEES WITH *PAENIBACILLUS LARVAE*)

SUMMARY

Description of the disease: *American foulbrood (AFB) affects the larval stage of the honey bee* Apis mellifera *and other* Apis *spp., and occurs throughout the world.* Paenibacillus larvae, *the causative organism, is a bacterium that can produce over one billion spores in each infected larva. The spores are extremely resistant to heat and chemical agents, and can survive for many years in scales (from diseased dead brood), hive products and equipment. Only the spores are capable of inducing the infection.*

Combs of infected colonies have a mottled appearance due to a mixture of healthy capped brood, unhealthy capped cells and uncapped cells. This is not a characteristic of AFB only. Cell cappings of a diseased larva appear moist and darkened, becoming concave and possibly punctured as infection progresses. The larval or pupal colour changes to creamy brown and then to a dark brown with a ropy appearance when drawn out. In some cases the larval remains are rather watery. The diseased brood eventually dries out to form characteristic brittle scales that adhere tightly to the lower sides of the cell. The formation of a pupal tongue is one of the most characteristic but rarely seen signs of the disease and precedes the formation of the scales. The clinical signs of AFB are very diverse and depend on the genotype involved, the stage of the disease and the strength of the bee colony (and possibly its resistance to AFB). Genotypes ERIC I–V are all pathogenic for honey bees

Detection and identification of the agent: *Diagnosis of AFB is based on identification of the pathogenic agent and the presence of clinical signs. The analyst can make use of a broad range of sample types. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. Some of the identification methods require a previous culturing step, while others can be performed directly on collected samples. Five solid culture media are recommended: PLA* (Paenibacillus larvae *agar), MYPGP agar, BHIT agar, J-agar and Columbia sheep blood agar. Two polymerase chain reaction (PCR) protocols are described in this chapter that can be used for rapid confirmation of clinical AFB and for identification of bacterial colonies after a cultivation step. The biochemical profiling of* P. larvae *is based on the catalase test, the production of acid from carbohydrates and the hydrolysis of casein depending on the genotype involved. Further, antibody-based techniques and the microscopic identification of the pathogenic agent are described.*

Serological tests: *There are no serological tests available.*

Requirements for vaccines: *No vaccines are available.*

A. INTRODUCTION

American foulbrood (AFB) is an infectious disease of the larval stage of the honey bee *Apis mellifera* and other *Apis* spp., and occurs throughout the world where such bees are kept. *Paenibacillus larvae*, the causative organism, is a Gram-positive bacterium that can produce over one billion spores in each infected larva. The bacterium is a round-ended, straight or sometimes curved rod, which varies greatly in size (0.5–0.8 μm wide by 1.5 –6 μm long), occurring singly and in chains and filaments; most strains are motile. The sporangia are often sparse in vitro, and the

ellipsoidal, central to subterminal spores, which may swell the sporangia, are often found free, measuring 0.6 × 1.3 μm (Heyndrickx *et al*., 1996).

By using repetitive element polymerase chain reaction (rep-PCR) and primers ERIC1R-ERIC2, five different genotypes (ERIC I, II, III, IV and V) are differentiated (Beims *et al.,* 2020). Genotypes ERIC I and II correspond to the former subspecies *P. l. larvae* while genotypes ERIC III and IV correspond to the former subspecies *P. l. pulvifaciens* (Genersch, 2010). Genotype ERIC V was recently isolated from a Spanish honey sample and identified as a new genotype (Beims *et al.,* 2020). All five genotypes differ in colony and spore morphology, in their metabolism of carbon sources and most importantly in virulence. Exposure bioassays revealed that members of ERIC II-V are highly virulent against larvae in terms of the time course of mortality. Almost all larvae infected with these genotypes are killed within approximately 3–7 days (Beims *et al.,* 2020; Genersch *et al.,* 2005; Rauch *et al.,* 2009). This means that only a minor proportion of the larvae die after cell capping resulting in the described clinical signs of AFB (ropy stage, foulbrood scale). In contrast, genotype ERIC I usually takes around 12 days to kill an infected larva and is therefore considered less virulent than ERIC II-V for the individual larva (Beims *et al.,* 2020; Djukic *et al.,* 2014; Genersch, 2010; Genersch *et al.,* 2005). Epidemiological studies showed that only ERIC I and ERIC II are frequently isolated from AFB-disease colonies. *Paenibacillus larvae* genotype ERIC I is the most frequent genotype while genotype ERIC II seems to be more restricted although both have been reported worldwide. Genotypes ERIC III and IV have not been identified in the field for decades, but exist as a few isolates in culture collections (de Graaf *et al*., 2013; Genersch, 2010). Genotype, ERIC V was recently isolated from a Spanish multi-flower honey sample. Because fingerprinting profiles generated via electrophoresis of rep-PCR amplified DNA are not consistently reproducible between laboratories, it is necessary to include reference *P. larvae* strains previously typed. To enhance the discrimination of strains, the analysis using ERIC primers can be complemented with the use of other primers (de Graaf *et al.,* 2013). A multilocus sequence typing (MLST) scheme revealed the distribution and biogeography of 294 samples of *P. larvae,* grouped in 34 sequence types (as of March 2022 [https://pubmlst.org/plarvae/]), across six continents (Morrissey *et al.,* 2015). Currently, whole genome sequencing (WGS) based genotyping offers unprecedented resolution in discriminating highly related strains. Thus, it is well suited for the investigation of the evolution of pathogens even over short time periods (Uelze *et al.,* 2020). Applied to the investigation of *P. larvae*, WGS-based genotyping showed a good correlation with conventional typing methods (MLST and ERIC-PCR) and proved to be efficiently used for cluster delineation in AFB outbreak epidemiological investigations (Bertolotti *et al*., 2021). The spores are extremely heat stable and resistant to chemical agents. Only spores are capable of inducing the infection. The infection can be transmitted to larvae by nurse bees or by spores remaining at the base of a brood cell. Although the larval stages of worker bees, drones and queens are susceptible to infection, infected queens and drone larvae are rarely seen under natural conditions. The susceptibility of larvae to AFB disease decreases with increasing age (de Graaf *et al.,* 2013); larvae cannot be infected later than 53 hours after the egg has hatched. The mean infective dose (LD_{50}= spore dose at which 50% of the larvae are killed) needed to initiate infection, though very variable, is 8.49 ± 1.49 spores in 24–28 hour-old bee larvae (Hansen & Brodsgaard, 1999). Exchanging combs containing the remains of diseased brood is the most common way of spreading the disease from colony to colony. In addition, feeding or robbing of spore-laden honey or bee bread, package bees and the introduction of queens from infected colonies can also spread the disease. Wax contaminated with the spores of *P. larvae*, used in the production of comb foundations, can also spread the disease if not properly treated. The early detection of AFB helps to prevent further spread.

While there is generally a low risk of human infection with AFB organisms, it should be noted that fatal bacterial septicaemia has been reported in drug users injected with honey contaminated with *P. larvae* spores (Rieg *et al.,* 2010). Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

1. Epizootology and clinical signs

Spores of *P. larvae* can survive in bee products (honey, wax, dead larvae) and in the environment for 3 to 10 years and for 35 years in dry larval scales. Purified spores can survive even more than 70 years.

The clinical signs of AFB are very diverse and depend on the genotype involved, the stage of the disease and the strength of the bee colony (and possibly its resistance to AFB) (Genersch *et al*., 2005). Larvae can be killed rapidly at an early age when they are curled at the base of uncapped brood cells. Adult worker bees will remove these dead larvae leaving only an empty cell (Brodsgaard *et al*., 2000). Other larvae will die later on in their development, when they are in an upright position, filling most of the brood cell. Larvae infected with ERIC I usually die after brood cell capping, whereas larvae infected with other types usually die before cell caping.

In severely infected colonies, the combs have a mottled appearance caused by a pattern of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. The capping of a cell that contains a diseased larva appears moist and darkened and becomes concave and punctured as the infection progresses (Figure 1a). Also, the larva or pupa changes colour, first to beige and eventually to a dark brown. The larvae can become glutinous in consistency and can be drawn out as threads when a probe is inserted into the larval remains and removed from the cell (match-stick test) (Figure 1b). This is probably the best-known technique for field diagnosis of the disease, but in some cases the larval remains are rather watery, resulting in a negative match-stick test. Finally, 1 month or more after the larva becomes ropy, the remains of the diseased brood dry out to form typical hard, dark scales that are brittle and adhere strongly to the lower sides of the cell (Figures 1.d. and 2). If death occurs in the pupal stage, the pupal tongue protrudes from the pupal head, extending to the top of the brood cell or may angle back towards the bottom of the cell. The protruding tongue is one of the most characteristic signs of the disease, although it is rarely seen (Figure 1c). The tongue may persist also on the dried scale. European foulbrood needs to be taken into consideration as a differential diagnosis.

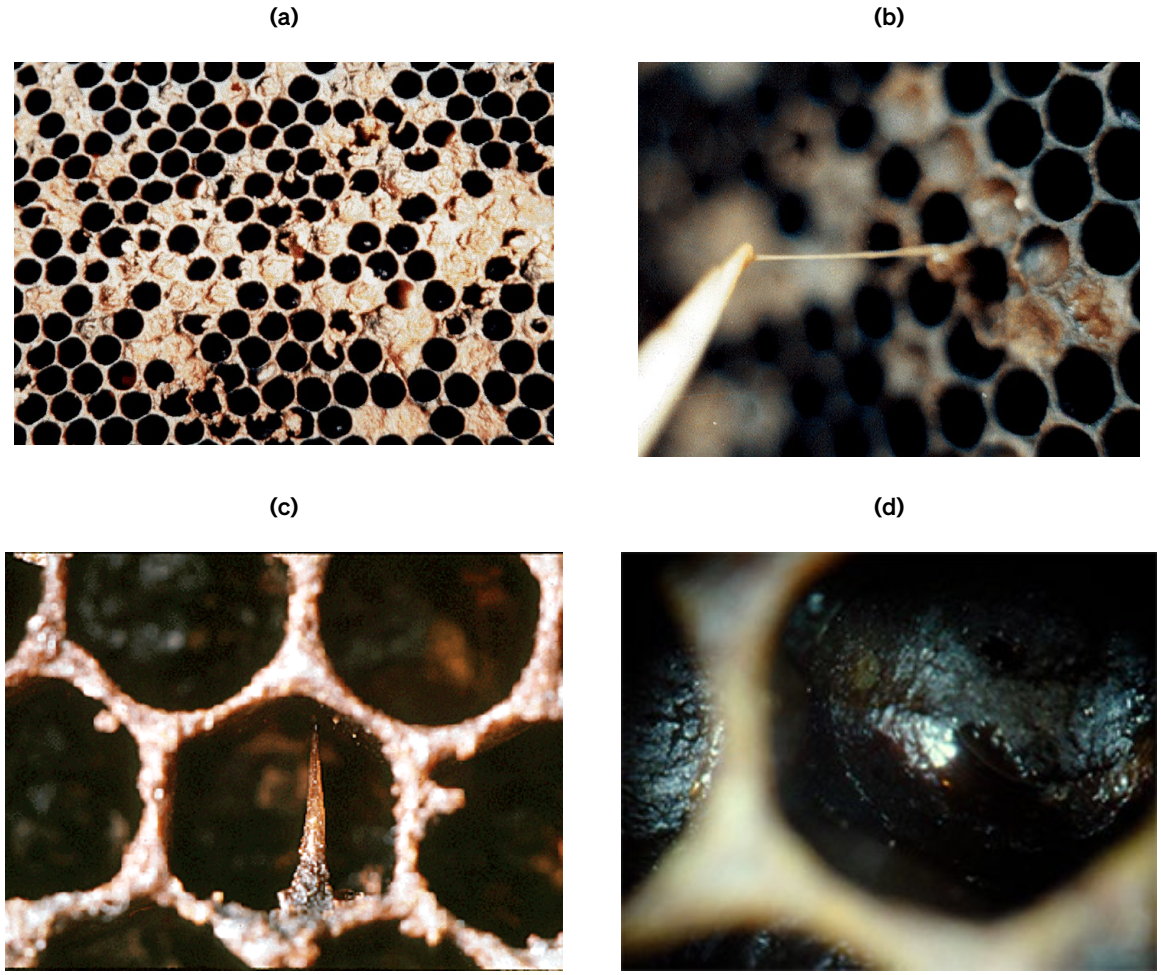

Fig. 1. Clinical American foulbrood (a–d): (a) Combs have mottled appearance. (b) A matchstick draws out the brown, semi-fluid larval remains in a ropy thread. (c) The formation of a pupal tongue is a very characteristic sign, but rarely seen. (d) Residual scale adhered to the bottom of the cell. Photos a, b and d From: A. M. Alippi; Photo (c): From: MAAREC-Mid Atlantic Apiculture and Extension Consortium (at https://agdev.anr.udel.edu/maarec/honey-bee-biology/honey-bee-parasites-pests-predators-and-diseases/diseases-of-honey-bees/nggallery/show--photocrati-nextgen_basic_thumbnails/page/1/)

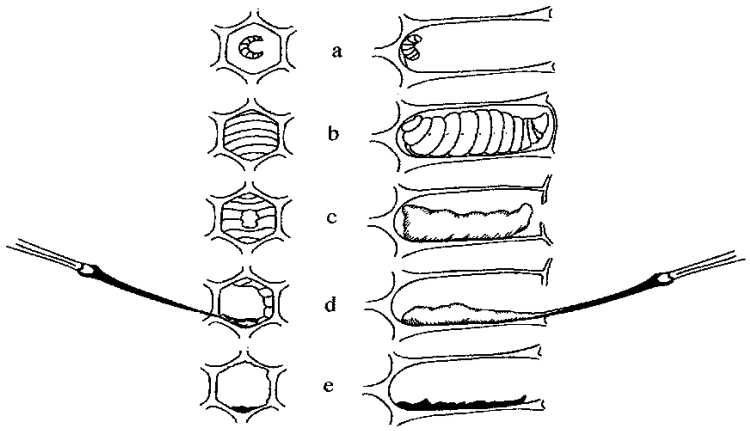

Fig. 2. Progression of the disease: (a) Point of infection. (b) Larval development to the prepupal stage. (c) Cell contents reduced and capping is drawn inwards or is punctured. (d) Cell contents become glutinous. (e) Residual scale tightly adherent to bottom of cell.

It has been demonstrated that different genotypes differ in virulence; ERIC I strains lead to 100% mortality of infected larvae within 12 days while ERIC II strains kill infected larvae in about 7 days (Djukic *et al*., 2014; Genersch, 2010; Genersch *et al*., 2005; Rauch *et al.,* 2009). The faster *P. larvae* kills infected larvae, the more infected larvae will be removed as nurse bees seem to recognise dead larvae at lower rates after cells capping (Rauch *et al*., 2009). Therefore, the proportion of larvae developing into a ropy mass under the cell cappings is higher for infections with strains of genotype ERIC I but can still be seen with other ERIC types. As veterinarians and beekeepers look for a ropy mass inside capped cells as the main sign of the disease, false negative diagnoses are likely to occur if AFB-diseased colonies are infected with strains of genotype ERIC II because only a few infected cells may be present (Rauch *et al*., 2009).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of American foulbrood and their purpose

Method	Purpose					
	Population freedom from infection	Individual hive freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[(a)]						
Bacterial isolation	+++	+++	+++	+++	+++	–
Microscopy	++	++	++	+++	+++	–
Antigen detection	++	++	++	++	++	–
Conventional PCR	+++	+++	+++	+++	+++	–
Real-time PCR	+++	+++	+++	+++	+++	–
Mass spectrometry	–	–	–	++	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection and identification of the agent

Diagnosis of AFB is based on identification of the pathogenic agent and the presence of clinical signs. The analyst can rely on a broad range of samples. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. An initial overview of clinical signs of the disease is provided in section A of this chapter. Identification techniques are microbiological characterisation, the polymerase chain reaction (PCR), biochemical profiling, antibody-based techniques and microscopy. The analyst should be aware of differences in sensitivity between the presented approaches and should select the most appropriate for a given situation.

1.1. Selection of samples

1.1.1. Collection of samples from a suspicious or diseased colony or apiary

While maintaining their colonies, beekeepers often find brood combs with signs of disease. In this case a brood sample can be collected for diagnosis. If possible, submit a whole frame to the laboratory, to avoid risk of distortion in transit. Alternatively the brood may be sampled by cutting out a piece comb of at least 20 cm^2 in size, containing as much of the dead or discoloured brood as possible. An experienced person can collect infected larval or pupal remains directly from the cells with a sterile swab, significantly reducing the sample size and facilitating packaging and sample transportation to the laboratory (see below). When microscopic examination is the method of choice, smears of the remains of diseased larvae can also be made at the apiary (de Graaf *et al.,* 2013). After air-drying the smears are packaged to be forwarded to the laboratory for microscopic examination and culture.

Every bee colony in the vicinity of such a clinical case of AFB should be considered as suspicious and a broad range of samples should be taken for confirmation. Apart from brood samples, food stores (honey, pollen and royal jelly), adult workers and wax debris can be used to detect the presence of *P. larvae* spores. Honey samples can be collected from cells close to the brood with separate disposable spoons to prevent cross-contamination between samples; however, honey may have been sitting in the comb for months at the time of sampling. Adult bees can be shaken or brushed from the combs of the brood chamber or the honey supers into a plastic bag or container. For the most reliable picture of the actual situation, bees from the brood nest (and not the honey supers) should be analysed. Wax debris can be collected at the hive bottom all year round.

1.1.2. Samples for AFB monitoring or prevention programmes

To prevent the propagation of diseased brood, honey, adult bee and debris samples can be used to detect AFB in colonies where no clinical signs are observed. Routine collection of samples from colonies or from harvested honey can be used as part of an operational or regional AFB detection programme.

Microscopic examination of smears from larvae with no clinical signs is far less sensitive at detecting spores in colonies compared with bacteriological or PCR-based methods. In fact, bacteriological and PCR-based methods will often detect spores in colonies that never develop clinical signs of AFB. High numbers of spores cultured from honey and bee samples using bacteriological methods, however, can often predict the presence of clinical AFB signs at colony, apiary and operational levels.

1.2. Packaging and transportation of samples to the laboratory

Brood comb should be wrapped in a paper bag, paper towel or newspaper and placed in a wooden or heavy cardboard box for transport; avoid any form of plastic wrapping to prevent fungal growth. Swabs with larval remains can be put into appropriate test tubes with a cap. Smears of dead larvae on microscope slides are placed into individual slide holders. These are commercially available. Adult bees can be kept frozen or submerged in 70% ethanol in leak-proof containers during transportation, although dried bees are adequate, a sample size should be at least 30 bees. Food supplies can be put into a test tube or a suitable pot, or wrapped in a plastic bag together with the spoon. Leaking and cross-

contamination of the samples must be prevented. If possible, fresh material for laboratory tests should be sent refrigerated.

Hive debris and wax should be wrapped in a paper bag, plastic pots covered with a paper lid or in paper tubes covered with a plastic lid. Secondary packaging consists of a plastic bag as a protection against cross-contamination. In the case of bulk samples, samples may be stored even in tertiary packaging (large cardboard boxes) that protects samples from mechanical damage.

The minimum amount of honey for detection of viable spores of *P. larvae* is 50 g and should be placed in a leak-proof plastic container (one per sample with an identification label).

1.3. Culture

1.3.1. Sample preparation

i) Samples for cultivation

In general, an aqueous solution containing *P. larvae* spores should be prepared for further analysis. This spore suspension is heat-shocked at 80°C for 10 minutes or 95–96°C for 3–5 minutes in order to kill vegetative forms of other microorganisms, including other spore formers. Different genotypes of *P. larvae* show variation in germination ability and their response to heat treatment is variable (Forsgren *et al.,* 2008). Direct plating of larval remains for cultivation on agar without heat treatment is possible.

Larval/pupal remains from brood comb are collected with a sterile swab and suspended in 2–10 ml of sterile water or physiological solution (0.01 M phosphate-buffered saline [PBS] or 0.9% NaCl) in a test tube. For larval or pupal remains submitted on a glass slide, add 2–3 drops of sterile water. Emulsify with an orange stick or sterile loop. Place a loopful of emulsified material onto a suitable agar plate and streak with a sterile loop to obtain isolated colonies. Plates are incubated in 5–7% CO_2 and examined daily for up to seven days. Colonies are visible from day 2 onwards.

Each spore-suspension sample should be divided and treated as follows:

a) without heat treatment;

b) with heat treatment at 80°C for 10 minutes;

and

c) with heat treatment at 95°C for 3 minutes.

Steps b) and c) are for killing vegetative forms of other microorganisms. The heat step will significantly reduce the risk that *P. larvae* colonies will become masked by these competitors. Nevertheless, bacteria of the genera *Bacillus, Paenibacillus* and *Brevibacillus* may continue to swarm over the plates which necessitates the use of semi-selective media through addition of the antibiotics nalidixic acid and pipemidic acid. In step a) (no heat treatment), as well as both antibiotics, amphotericin B at a final concentration of 16.8 µg/ml of culture medium should be used to avoid fungal contamination in the isolation plates. Incubate the plates at 37±1°C for 2–4 days.

For smears prepared from dead larvae, add 1–2 drops of sterile water and mix on the slide. Use a wire loop to prepare a new smear for Gram stain and microscopic examination for spores. A second loop of reconstituted material is used to culture onto a suitable agar plate.

Honey samples to be examined for spores are heated to 45–50°C and shaken to distribute any spores that may be present, then each honey sample should be diluted (1/1) in 0.01 M PBS pH 7.2 or 0.9% NaCl, transferred to a centrifuge tube and centrifuged at 6000 ***g*** for 40 minutes. The supernatant is discarded leaving approximately 3 ml per tube that is then vortex-mixed for 1 minute to re-suspend the pellet, and treated as described for larval remains. The samples are high speed vortex-mixed again for 2 minutes and 100–200 µl of the sediment-fluid mixture poured on suitable culture media with the addition of antibiotics and incubate at 37°C for 7–8 days (de Graaf *et al.,* 2013).

Direct plating of diluted honey is widely used, but its sensitivity is inferior to that of the centrifugation method as only a fraction of the total volume will be plated out. Whatever the method of choice is, when honey is analysed quantitatively and threshold values are set, the methodology that was used to establish these values should always be strictly followed.

An aqueous filtrate of pollen can be made by thoroughly dispersing 1 g of pollen in 10 ml final volume sterile distilled water or 0.01 M sodium PBS pH 7.2, and filtering it through Whatman No. 1 paper (de Graaf *et al.,* 2013).

When adult bees are dispatched in ethanol, the latter should be decanted and replaced by sterile water or physiological solution before crushing.

Hornitzky & Karlovskis (1989) developed a culture technique that provides a rapid mean of detecting *P. larvae* spores in adult bees that could act as a source of AFB infection for young larvae. Briefly, each sample of 30 nurse bees is homogenised in 20 ml sterile PBS for 30 seconds. The homogenate is filtered through Whatman No. 1 paper, centrifuged and the pellet is resuspended in PBS. The samples are heat-shocked (see spore-suspension sample treatments) and plated onto a suitable culture medium supplemented with nalidixic acid and pipemidic acid to inhibit the spread of *P. alvei* and other bacteria that can swarm over the plates.

Debris and bee wax (1.5 g) should be dissolved in an organic solvent (10 ml): toluene, chloroform or diethyl ether (de Graaf *et al.,* 2013). The liquid part (2 ml) is then diluted in physiological solution (6 ml). After shaking roughly, this suspension can immediately be plated out (no heat-shock). In another protocol, bee wax is first diluted in water (wax/water 1/10) and heated up to 90°C for 6 minutes. After cooling down, the organic solvent is added (organic solvent/water 1/9) and the mixture is shaken carefully. After 2 minutes standing time, a deposit of a watery solution containing *P. larvae* spores forms.

ii) Cultivation by Tween 80 Method

One gram of debris or 1 g of wax is put in a test tube with an airtight seal. Larger pieces of wax should be cut by sterile instruments into very small pieces (ideally up to 3 mm in size). Wax pieces contained in debris do not have to be further cut because they are usually very small. The smaller the pieces, the easier and faster is the process of homogenisation. Dry material prepared this way should be stirred thoroughly and diluted with 8.5 ml of sterile distilled water. The resulting suspension is then supplemented with 0.5 ml of Tween 80. Approximately 30 minutes before pipetting, required volume of Tween 80 should be withdrawn from the original container, put into another sterile container with airtight seal and immersed in a hot water bath (70±2°C) to reduce the viscosity of Tween 80 and facilitate its pipetting. The suspension of debris, water and Tween 80 is shaken thoroughly and the test tube is placed in a hot water bath (70±2°C) for 30 minutes. If the wax dissolves slowly or there are pieces of wax larger than 5 mm, the test tube can be left in the water bath for up to 1 hour. While warming the sealed tube in water bath, it should be thoroughly shaken in a longitudinal direction at least three times (preferably in several 5- to 30-second cycles 5–10 minutes apart). Thorough homogenisation results in development of homogenous greyish brown pulpy material, which can harden as it cools down. Afterwards, tubes are removed from the water bath and allowed to cool down to room temperature, by which time they should be stored for 2–4 hours until a sufficient amount of liquid is separated at the bottom of tubes. Then 2–5 ml of this liquid is withdrawn with a disposable balloon pipette and mixed with the same volume of distilled water in another sterile sealable tube. Again, the resulting mixture should be shaken thoroughly in a longitudinal direction for at least 5 minutes and put into a hot water bath (90±2°C). After 10 minutes, tubes are removed from the bath, allowed to cool down to room temperature and shaken again. Then the material is inoculated in 0.2 ml doses to 3–5 plates of MYPGP with nalidixic acid and at least one plate of blood agar serving as a control. Before culturing, the plates should be dried in a thermostat at 37±1°C. Drying time is selected according to the humidity of the culture medium surface (approximately 30 minutes). Petri dishes must be labelled accurately with the sample identity. The liquid is spread over the plates using a bent sterile plastic/glass stick or the tip of the pipette. The liquid is allowed to dry and the plates are inverted and incubated at 37±1°C for 5–8 days.

1.3.2. Stock solutions (de Graaf *et al.,* 2013)

i) Nalidixic acid stock solution

Prepare by dissolving 0.1 g in 2 ml of 0.1 N NaOH and diluting to 100 ml with 0.01 M phosphate buffer (pH 7.2), (stock concentration 1000 μg/ml). Filter sterilise. Final concentration: 10 μg/ml for larval samples and 20 μg/ml for honey samples.

ii) Pipemidic acid stock

Prepare by dissolving 0.2 g in 2 ml of 0.1 N NaOH and then diluting to 100 ml with 0.01 M phosphate buffer (pH 7.2), (stock concentration 2000 μg/ml). Filter sterilise. Final concentration: 10 μg/ml for honey samples.

After autoclaving and cooling to 50°C add the antibiotics to the media at the final concentration required and pour into sterile Petri plates (20 ml per plate) (de Graaf *et al,* 2013).

1.3.3. Culture media (de Graaf *et al.,* 2013)

Several media for cultivating *P. larvae* have been described but best results were obtained with PLA (*Paenibacillus larvae* agar) (Schuch *et al*., 2001), MYPGP agar (Dingman & Stahly, 1983), BHIT agar (brain–heart infusion medium supplemented with thiamine), J-agar and CSA (Columbia sheep-blood agar) (Hornitzky & Karlovskis, 1989). The formulations of the media are as follows:

i) PLA (*Paenibacillus larvae* agar)

This selective medium combines three different media to constitute the base, to which antibiotics and egg yolk supplements are added (Schuch *et al*., 2001). Equal quantities (100 ml) of sterile, molten *Bacillus cereus* selective agar base, trypticase soy agar and supplemented nutrient agar (SNA) are combined and mixed. SNA is composed of (per litre): nutrient agar 23 g, yeast extract 6 g, meat extract 3 g, NaCl 10 g, Na_2HPO_4 2 g; final pH is 7.4 ± 0.2. All solid media are sterilised at 121°C/15 minutes. After the three molten media are combined, 3 ml of stock nalidixic acid, 3 ml of stock pipemidic acid, and 30 ml of 50% egg-yolk suspension are added to form the PLA medium (final concentration 9 μg/ml nalidixic acid and 18 μg/ml pipemidic acid). The PLA medium is poured (20 ml) into sterile Petri dishes and plates are dried before use (45–50°C for 15 minutes).

ii) MYPGP agar (The abbreviation refers to its constituents: Mueller-Hinton broth, yeast extract, potassium phosphate, glucose and pyruvate)

MYPGP agar is composed of (per litre): Mueller-Hinton broth 10 g, yeast extract 15 g, K_2HPO_4 3 g, glucose 2 g, Na-pyruvate 1 g and agar 20 g (pH 7.1) (Dingman & Stahly, 1983). Addition of nalidixic acid and pipemidic acid is as above.

iii) J-agar

J-agar is composed of (per litre): tryptone 5 g, yeast extract 15 g, K_2HPO_4 3 g, glucose 2 g, agar 20 g (pH 7.3–7.5). Addition of nalidixic acid and pipemidic acid is as above.

iv) CSA (Columbia sheep-blood agar)

CSA is composed of (per litre): 39 g Columbia blood agar base (pH 7.3). After autoclaving and cooling to 50°C, add 5% sterile defibrinated blood (Hornitzky & Karlovskis, 1989). Addition of nalidixic acid and pipemidic acid is as above. Cultivation on CSA slants induces sporulation and allows microscopic detection of the flagellar bundles.

v) BHIT (brain–heart infusion medium supplemented with thiamine) agar

BHIT agar is composed of (per litre): 47 g brain–heart infusion agar (adjusted to pH 6.6 with HCl). After autoclaving and cooling to 50°C, a sterile solution of thiamine hydrochloride is added to obtain a final concentration of 1 mg per litre.

MYPGP agar is routinely used to cultivate *P. larvae* for AFB diagnosis and yielded the highest percentage of spore recovery, while J-agar, BHI and CSA proved to be less efficient in this respect. PLA medium also shows superior plating efficacy and inhibits the majority of micro-organisms normally present in hive and bee products (Schuch *et al.,* 2001).

If cultivation of *P. larvae* is hampered by the occurrence of fungi, the addition of 16.8 µg/ml medium of amphotericin B works very well.

A sterile cotton swab is used to transfer a portion of the sample on to the surface of the solid medium. For a quantitative evaluation, it is recommended to spread a fixed volume of the suspension on the solid agar with a sterile scraper or pipette rather than using cotton swabs.

Inoculated plates are best incubated at 37+1°C for 2–4 days in an atmosphere of 5–10% CO_2 in air, although aerobic incubation can also be used. Honey samples should be incubated longer – for at least 6 days and up to 15 days – and checked for suspect colonies at 3 and 6 days.

1.3.4. Identification

i) Colony morphology

Samples from clinically diseased larvae will produce confluent growth on plates after 2–4 days, leading to a subculturing step in order to isolate single colonies.

On PLA, colonies of *P. larvae* are small, pale green to yellow (= the same colour as the medium), with a slightly opaque and rough surface; sometimes the centre is raised.

(a) **(b)**

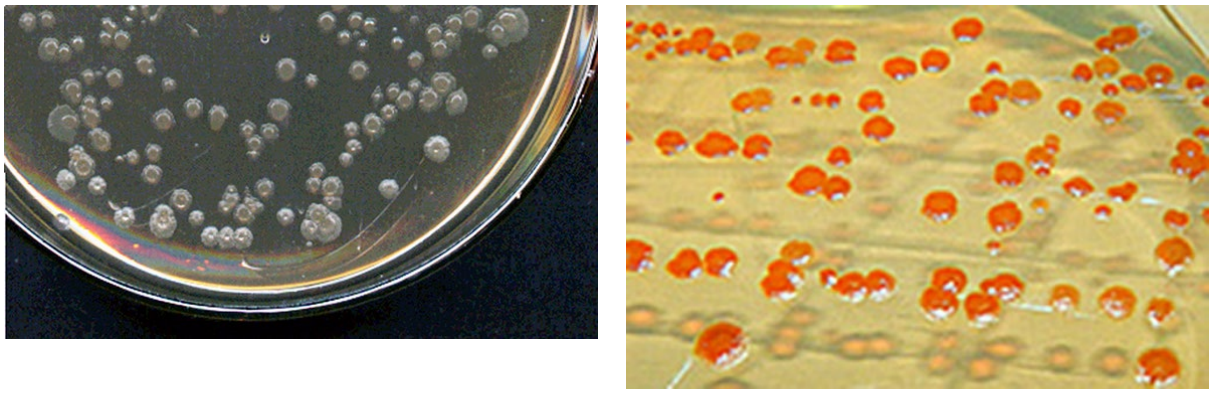

Fig.3: Appearance of* Paenibacillus larvae *colonies cultivated on MYPGP agar plates: (a) Reference strain ATCC 9545^T (ERIC I) and (b) Strain PL SAG m290 (ERIC II). Photos: A.M. Alippi

On MYPGP and J-agar, colonies are small, regular, mostly rough, flat or raised and whitish to beige coloured (Figure 3a)

On CSA agar, colonies are small, regular, rough, butyrous and greyish, somewhat transparent and slightly glistening appearance (Figure 4a).

Paenibacillus larvae colonies with orange to red pigmentation have been described, i.e. ERIC II and III genotypes that produce reddish and/or orange colonies on MYPGP, J-agar and CSA (Table 2) (Figures 3b and 4b) (Genersch *et al*., 2005; Neuendorf *et al*., 2004). According to Genersch *et al.* (2005) only genotypes ERIC II and III are pigmented, but pigmented and non-pigmented strains of ERIC IV has been reported (Dingman, 2015). It is important to point out that pigmented phenotypes can be lost under successive sub-culturing.

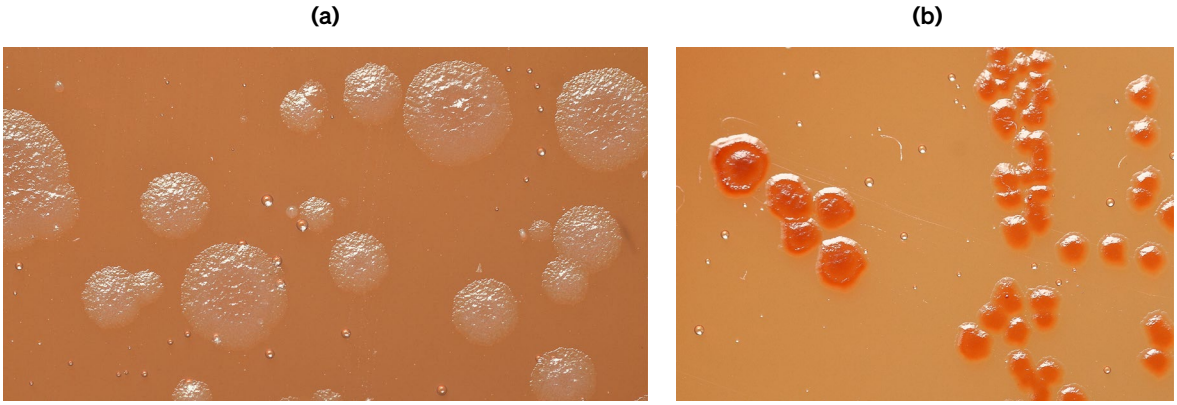

Fig.4: Characteristic colony morphology of* Paenibacillus larvae *cultivated on Columbia sheep blood agar plates: (a) Reference strain ATCC 9545^T (ERIC I) and (b) Strain PL SAG m290 (ERIC II). Photos: A.M. Alippi.

It is advised to run *P. larvae* reference strains in parallel, for instance LMG 9820 (other designation: ATCC 9545, DSM 7030, NRRL B-2605, LMG 15969) for the non-pigmented variant and DSM 16115 or DSM 16116 for the pigmented genotype. (For a complete list of *P. larvae* reference strains see de Graaf *et al.,* 2013). The new ERIC V genotype is referenced as strain DSM 106052.

Colony morphology is not conclusive but might serve to select the bacterial colonies for further identification.

Table 2. Phenotypic characteristics of* Paenibacillus larvae *ERIC genotypes. All genotypes have ellipsoidal spores (data based on Beims* et al., *2020 and Genersch* et al., *2005)

Characteristic	ERIC I	ERIC II	ERIC III	ERIC IV	ERIC V
Pigmented colonies	–	+	+	Variable	–
Spore surface (as seen by SEM)	Smooth	Convoluted	With ridges	With ridges	With ridges
Growth in nutrient broth	–	+	+	+	+
Fermentation of mannitol	–	+	+	+	NT
Fermentation of salicin	+	–	–	–	NT
Alkaline phosphatase	+	–	+	+	NT
Acid phosphatase	+	–	+	+	NT
Catalase	–	–	Weak, delayed +	Weak, delayed +	NT

NT = not tested

ii) Polymerase chain reaction

PCR may be used to identify bacterial colonies (= cell/spore suspension) after a cultivation step. DNA can be extracted as follows: one colony is suspended in 50 µl of distilled water and heated to 95°C for 15 minutes. Following centrifugation at 5000 ***g*** for 5 minutes, 1–5 µl of the supernatant is used as template DNA in a PCR 50 µl mixture (Dobbelaere *et al*., 2001). Commercial DNA extraction kits can also be used, following the manufacturer's instructions for Gram-positive bacteria. See section 1.4.2 below for the PCR method.

iii) Mass spectrometry

Using a toothpick, a bacterial colony is smeared to two wells of the target plate and allowed to dry at room temperature before 1 µl of matrix (α-cyano-4-hydroxycinnamic acid) is added to each spot and prior to placing the target plate into the machine. MALDI-TOF MS measurement is then performed. The device detects specific mass spectra of bacterial ribosomal peptides. After comparison of detected spectra with the database of known spectra, the bacterial strain is identified (Schafer *et al.,* 2014).

iv) Biochemical tests

Paenibacillus larvae can also be identified by its biochemical profile. The bacteria are catalase negative or weak delayed positive, they have a typical carbohydrate fermentation profile with acid production from glucose and trehalose, but not from arabinose and xylose, and they can hydrolyse casein or milk. The results of some tests vary within the genotypes, e.g. fermentation of mannitol and salicin (Table 2).

a) Catalase test

A drop of 3% hydrogen peroxide is placed on an actively growing culture on solid medium. Most aerobic bacteria break down the peroxide to water and oxygen, producing a bubbly foam, but *P. larvae* is negative or weak delayed positive for this reaction, depending on genotype (Table 2) (Haynes, 1972). Organisms can lose their catalase activity with age, resulting in a false negative reaction. When Colombia sheep blood agar is used for cultivation, the test cannot be done on the solid medium, as the presence of sheep blood will cause a false-positive reaction. In this case, colonies should be transferred to a clean microscope slide for the execution of the test. Here the evaluation of the test occurs as above with the naked eye.

b) Production of acid from carbohydrates

Bacteria are grown in J-broth (per litre: yeast extract 15 g, tryptone 5 g and K_2HPO_4 3 g) in which 0.5% of the test substrate, separately sterilised in aqueous solution, is substituted for the glucose (de Graaf *et al.,* 2013). The carbohydrates used are L (+)-arabinose, D (+)-glucose, D (+)-xylose and D (+)-trehalose. The cultures are tested at 14 days by aseptically removing one ml or less to a spot plate, mixing the sample with a drop of 0.04% alcoholic bromocresol purple, and observing the colour of the indicator. *Paenibacillus larvae* produces acid aerobically from glucose and trehalose. No acid is produced from arabinose and xylose (Alippi, 1992) and variable results are obtained with mannitol and salicin according to the isolate tested (Genersch *et al.,* 2005) (Table 2).

Commercial kits are also available for the biochemical characterisation of *P. larvae* (Neuendorf *et al*., 2004).

c) Hydrolysis of casein (Schuch *et al.,* 2001)

Casein hydrolysis is assayed using milk agar plus thiamine (per litre: agar 20 g, yeast extract 10 g; sterilised at 121°C/15 minutes). Add to each 70 ml cooled medium 30 ml of UHT (ultra-heat treated) skimmed milk and 1.5 ml filter sterilised 0.1% thiamine solution. Plates are streaked and examined after 5 days of incubation at 36 ± 1°C. *Paenibacillus larvae* hydrolyses casein, hence zones of clearing are observed around bacterial colonies.

v) Antibody-based techniques (for details refer to de Graaf *et al.,* 2013)

Different antibody-based techniques have been developed for the diagnosis of AFB. Most methods rely on polyclonal rabbit serum developed against pure cultures of *P. larvae* and are used for identification of isolated bacterial colonies or for direct examination of suspicious larval remains.

In an immunodiffusion test the antibodies interact with the bacterial antigen during a double diffusion process, leaving precipitation marks behind. In the fluorescent antibody technique these antibodies are conjugated with a fluorochrome dye. The resulting fluorescent antibody reacts with a bacterial smear on a slide. Any excess antiserum is washed off and the smear is examined by fluorescence microscopy. *Paenibacillus larvae* stains can be recognised specifically as brightly fluorescing bacteria on a dark background. An enzyme-linked immunosorbent assay using a monoclonal antibody specific to *P. larvae* exists. A lateral flow device for rapid confirmation of AFB has been commercialised.

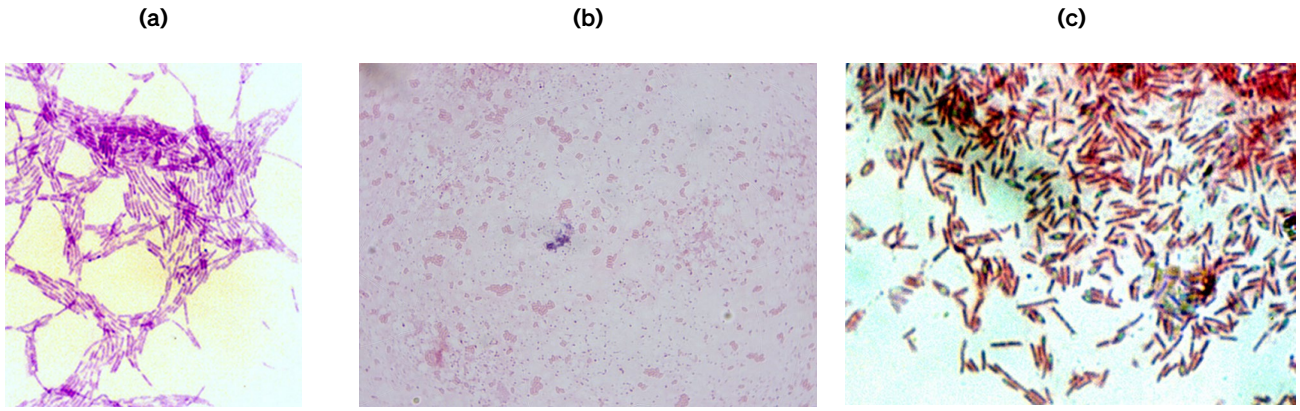

Fig. 5. Microscopic examination of Paenibacillus larvae*, the causative organism of American foulbrood: a) Gram stain reveals Gram-positive rods, occurring singly and in chains (magnification: ×1000); b) Gram stain on diseased larvae reveals pink coloured spores occurring singly or in grapes (magnification: ×1000);* c) *Schaeffer & Fulton's spore stain reveals that sporangia (stained red) are swollen by the spores (stained green), which are central to terminal (magnification: ×1000). Photos: A.M. Alippi (a and c); J.C. Thomarat & K. Sidi-Boumedine (b).*

vi) Microscopy

Microscopy and staining are useful for examination of AFB-derived clinical samples or initial bacterial culture. Several methods (including among others Gram staining, etc.) give an indication of the bacteria's structural properties, shape, morphological features, such as cell wall type and presence or absence of endospores.

The most used microscopic techniques are:

a) Gram staining

Gram staining is often done on smears of bacteria from isolated bacterial colonies. *Paenibacillus larvae* appears as Gram-positive rods with slightly rounded ends (Figure 5a).

When the Gram stain is realised on symptomatic larvae smears (Figure 5b), *P. larvae* appears as optically empty pink-coloured ellipsoidal spores (measuring about 0.6 × 1.3 µm). In some cases (recent affection of the larvae) Gram-positive rods can also be observed, either isolated or in more or less long chains.

b) Carbol fuchsin staining of larval smears

When stained with carbol fuchsin, the spores of *P. larvae* (about 0.6 × 1.3 µm) appear ellipsoidal and thick rimmed. Their colour is reddish purple while their centres remain clear.

Note: If the infection appeared in larvae less than 10 days old, long vegetative rods with coalesced flagella will be present. These flagellar bundles are characteristic of the pathogen.

c) Schaeffer & Fulton method for staining endospores

Using this method, the spores appear stained green and the vegetative cells red (Figure 5c). *Paenibacillus larvae* spores are ellipsoidal, central to terminal, swelling the sporangium and thick rimmed, measuring about 0.6 × 1.3 µm.

d) Nigrosin negative staining

A drop of bacterial suspension when mixed with nigrosine solution (5%) leads to a background stained in black whereas *P. larvae* spores appear as bright purplish-red and the vegetative forms as greyish.

As for the other laboratory analyses it is highly important to run appropriate controls, in parallel to the test samples. The different reagents, for bacterial staining, are easily available as ready to use solutions.

1.4. Polymerase chain reaction

1.4.1. Samples for PCR

Cell/spore suspensions and suspensions containing only spores have to be differentiated, the latter requiring a more complex DNA extraction step.

For rapid confirmation of clinical AFB, the samples should be prepared as follows: the remains of two diseased honey bee larvae (= cell/spore suspension) are emulsified in 1 ml of sterile distilled water and mixed thoroughly. 100 µl of this suspension is diluted with 900 µl distilled water. This dilution is vortexed and 100 µl of it is used to extract DNA by heating and centrifugation (see above) (Dobbelaere *et al*., 2001). Extraction of DNA can also be done using commercial kits according to the manufacturer´s instructions.

All aqueous solutions resulting from the sampling of adult bees, debris, bee wax, pollen and royal jelly should be considered as a spore suspension. Here, the extraction of DNA demands another approach. Indeed, spore suspensions are centrifuged at 6000 ***g*** and 4°C for 30 minutes. Next, the pellet is subjected to microwave treatment for 5 minutes at maximum power to break the spore coat, and the released DNA is suspended in 30 µl of 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA.

When spores are to be detected from honey, DNA is serially diluted with sterile distilled water to eliminate PCR inhibition caused by honey. Another DNA extraction method, based on lysozyme and proteinase K treatment, has been described (Bakonyi *et al*., 2003). Commercial DNA extraction kits can also be used according to the manufacturer's instructions.

Good results can also be obtained by incubating a pelleted spore suspension in MYPGP broth at 37°C for 2–24 hours. Thereafter, the suspension is centrifuged at 14,500 ***g*** for 5 minutes, washed with sterile distilled water and resuspended in 200 µl of sterile distilled water. This short incubation step causes spores to germinate, making them sensitive for DNA preparation by heat treatment again (see above) (Lauro *et al.,* 2003).

Positive and negative controls should be run in parallel with the test samples as well as inhibition control. This latter is necessary to detect possible inhibitors of the PCR reaction and thus to avoid a decreased PCR sensitivity or false negative results.

1.4.2. Polymerase chain reaction method

Several conventional PCR protocols have been described to identify *P. larvae* (reviewed by de Graaf *et al.,* 2006 and de Graaf *et al.,* 2013), but two of them, based on the 16S rRNA gene, have proven robustness and are described as follows:

Note: if using commercial PCR mixes the required ingredients may already be included. Check and follow the manufacturer's instructions

PCR reactions (modified from Dobbelaere *et al*., 2001) are set up as 50 µl mixtures containing:

i) 1–5 µl template DNA (see sample preparation);

ii) 50 pmol forward (AFB-F) and reverse primer (AFB-R);

iii) 10 nmol of each dNTP;

iv) 2 mM $MgCl_2$;

v) 1–2.5 U of *Taq* polymerase in the appropriate PCR buffer containing 2 mM $MgCl_2$.

Reducing the volume of the PCR mixtures to 25 µl is possible.

Use the following PCR conditions: a 95°C (1–15 minutes) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes).

PCR reactions by using PL1 and PL2 primers (Govan *et al.,* 1999) are set up as 50 µl mixtures containing:

i) 1–5 µl template DNA (see sample preparation);

ii) 2 mM $MgCl_2$;

iii) 50 pmol of forward PL1) and reverse (PL2) primer;

iv) 25 mM concentration of each dNTP;

v) 1 U of Taq polymerase per ml.

Reducing the volume of the PCR mixtures to 25 µl is possible.

Use the following PCR conditions: a 95°C (1 minute) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes). The molecular weights of the PCR products are determined by electrophoresis in a 0.8% agarose gel and staining with a suitable DNA dye. The PCR products can also be analysed using microfluidics or capillary electrophoresis devices.

Ref.	Name	Sequence (5' → 3')	PCR-product size	Specificity level
(Dobbelaere *et al.,* 2001)	AFB-F AFB-R	CTT-GTG-TTT-CTT-TCG-GGA-GAC-GCC-A TCT-TAG-AGT-GCC-CAC-CTC-TGC-G	1096 bp	species
(Govan *et al.,* 1999)	PL1 PL 2	AAG-TCG-AGC-GGA-CCT-TGT-GTT-TC TCT-ATC-TCA-AAA-CCG-GTC-AGA-GG	973 bp	species

In recent years, real-time PCR methods have been extensively developed as culture-independent approaches to detecting *P. larvae* from various honey and hive samples including larvae and adult honey bees. Compared with conventional PCR, these methods offer the possibility of detecting a target template with its quantification (if needed/necessary) in a robust, highly reproducible, sensitive manner and without post-PCR analysis steps, which are time-consuming and prone to contamination (reviewed by Okamoto *et al.,* 2022).

As in conventional PCR, it is highly important to run appropriate controls (positive, negative as well as inhibition controls) in parallel to the test samples. The use of commercial DNA extraction kits is also possible (to be used according to the manufacturer's instructions).

Regarding the choice of a PCR test for the detection of *P. larvae* samples, users should consider its fitness for purpose. Further advice can be obtained from the WOAH Reference Laboratories[1].

2. Serological tests

No serological tests are available.

C. REQUIREMENTS FOR VACCINES

No vaccines are available.

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Illustrations by Karl Weiss, extracted from Bienen-Pathologie, 1984, are reproduced with the kind permission of the author and Ehrenwirth-Verlag, Munich (Germany). Photographs are from the Animal and Plant Health Agency (APHA) York (UK) and the Informatiecentrum voor Bijenteelt, Ghent (Belgium) and published with kind permission

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

of respectively Ruth Waite and Frans J. Jacobs. Figures 3, 4 and 5 are reproduced by kind permission of Dr A.M. Alippi, National University of La Plata (Argentina).

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NB: There are WOAH Reference Laboratories for American foulbrood (infection of honey bees with *Paenibacillus larvae*) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases

NB: FIRST ADOPTED IN 1989 AS AMERICAN FOULBROOD DISEASE. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.2.3.

EUROPEAN FOULBROOD OF HONEY BEES (INFECTION OF HONEY BEES WITH *MELISSOCOCCUS PLUTONIUS*)

SUMMARY

Description of the disease: *The causal organism of European foulbrood (EFB) of honey bees is the bacterium* Melissococcus plutonius*. The identification of its presence by the observation of signs of disease in the field is unreliable without specialist training. The most usual and obvious sign is the death of larvae shortly before they are due to be sealed in their cells, but this may be for reasons other than EFB. Most infected colonies display few visible signs. Infection remains enzootic within individual colonies because of mechanical contamination of the honeycombs by the durable organism. Recurrences of disease can therefore be expected in subsequent years. The disease is widely distributed worldwide and is an increasing problem in some areas.*

Detection of the agent: *Examination, by high-power microscopy, of suitable preparations of larval remains for the presence of lanceolate cocci is adequate for most practical purposes, especially when it is done by experienced individuals.*

Traditionally the diagnosis of EFB is done by isolating and identifying the causative organism, which can be differentiated quite readily from all other bacteria associated with bees by its fastidious cultural requirements. However, non-fastidious atypical strains have also been reported recently.

The isolated bacterium can be identified and differentiated by means of simple tube agglutination tests. A single-step conventional polymerase chain reaction (PCR) and a hemi-nested PCR are available. Real-time PCRs have also been developed. These methods permit direct analysis of larvae, adult bees and honey bee products.

Serological tests: *No tests for detecting antibodies in bees are available.*

Requirements for vaccines: *There are no vaccines available.*

A. INTRODUCTION

Bee larvae usually die of European foulbrood (EFB) 1–2 days before being sealed in their cells, or sometimes shortly afterwards, and always before transformation to pupae. The disease is caused by *Melissococcus plutonius* and occurs mostly during the period when colonies are growing quickly. Most sick larvae become displaced from the coiled position in the bottom of their cells before they die. Many are quickly detected and removed by nurse bees, leaving empty cells scattered randomly among the remaining brood. Some infected larvae survive, successfully pupate and emerge as adults. These surviving larvae are able to defecate and their infected-faeces contribute to the continued propagation of the disease (Bailey, 1960).

Infected larvae that escape detection by adult bees and then die, first become flaccid and turn a light yellow colour that becomes increasingly brown, and at the same time they dissolve into a semi-liquid mass. They then become dry and form a dark brown scale that can easily be removed from the cells. Severely affected brood may have a very stale or sour odour, sometimes acidic, like vinegar, but often there is no smell.

Signs of disease can disappear spontaneously from infected colonies before the end of the active season, but are likely to return in subsequent years (Bailey & Ball, 1991; Forsgren *et al.,* 2013). Geographically, the disease appears to vary in severity from being relatively benign in some areas but increasingly severe in others (Forsgren *et al.,* 2013).

Generally, EFB has always been considered less serious than American foulbrood (AFB) because rates of recovery from EFB are higher than those from AFB, and it can often clear up with little or no intervention. However, it remains a disease of national and global concern. Indeed, more aggressive forms of the bacterium have been described in different countries (Arai *et al.,* 2012; de Leon-Door *et al*., 2018; Nakamura *et al*., 2020). Furthermore, cases of a so-called atypical EFB (Gaggia *et al*., 2015; Roy & Franco, 2021) are frequently reported. Atypical EFB is reported to be responsible for more serious, harder to clear up, recurrent outbreaks.

1. Epizootiology and clinical signs

The general signs observed in a colony are irregular capping of the brood, capped and uncapped cells irregularly distributed over the brood frame (Figure 1). EFB usually affects young larvae, which die while still coiled before they are sealed. The younger larvae affected cover the bottom of the cell and are almost transparent, with visible trachea. The colour of the larvae change from pearly white to yellow, and then brown (Figure 2). Larvae die at the age of 4 to 5 days rarely in capped cells. Infected larvae assume unnatural positions in the cells, twisted around the walls. The larvae eventually decay to a point where they form dry rubbery scales that can easily be removed from the cells. Severely affected brood may have a very stale or sour odour, sometimes acidic, like vinegar, but there may be no smell, depending on the presence of saprophytes. A late infestation with *Varroa*, before colony collapse, can produce a similar brood appearance and is an important differential diagnosis.

Fig. 1. Clinical European foulbrood: irregular capping of the brood.

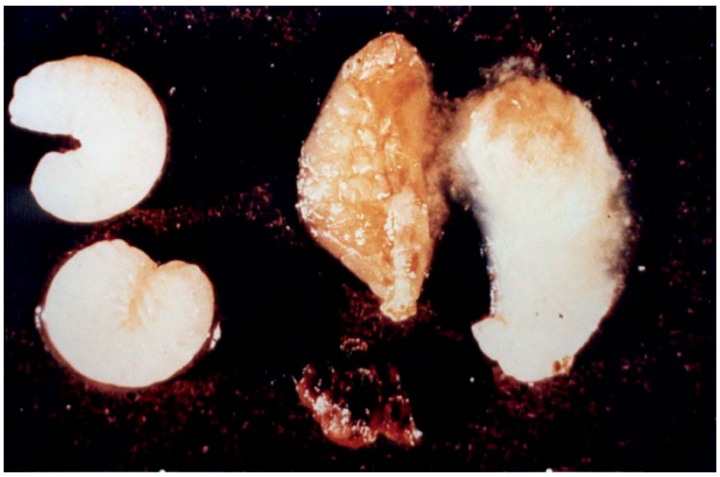

Fig. 2. The infected larvae become flaccid and turn from yellow to brown and finally transform into a dark scale. Photo by A.M. Alippi.

Signs of EFB often appear for the first time in spring and summer, and incidence may be higher when colonies are experiencing stress. However, atypical EFB cases differ from the classic descriptions of the disease: recurrent more severe signs from one year to the next that persist over time, testifying to a particular virulence. These cases could complicate field diagnosis in terms of colony outcome and visual signs (Milbrath *et al*., 2021; SVA report, 2020) particularly as they can be confused with AFB when the capped brood is affected and the dead larvae take on a "sticky" consistency that may resemble the viscous 'roping' state of AFB dead larvae (Roy & Franco, 2021). Overall,

this highlights the value of laboratory diagnosis and the establishment of surveillance to prevent future epidemics and newly emerging strains.

Atypical strains of *M. plutonius*, showing different phenotypic, biochemical and molecular characteristics, were described in Japan (Arai *et al*., 2012). Contrary to typical strains, atypical strains were shown to be non-fastidious, able to grow in aerobic conditions and on media without potassium salt supplementation. First considered to be restricted to Japan, their widespread distribution has been reported in Europe and the Americas (de Leon-Door *et al*., 2018; Wood *et al.,* 2020). To date, *M. plutonius* strains have been grouped into three clonal complexes (CC3, CC12, and CC13) by multilocus sequence typing (MLST) (as of January 2022 [https://pubmlst.org/mplutonius/]). Typical strains with fastidious characteristics in culture belonged to CC3 and CC13, whereas atypical strains with non-fastidious characteristics belonged to CC12 (de Leon-Door *et al*., 2018; Takamatsu *et al*., 2014). Furthermore, whole genome sequencing (WGS) analyses suggest that the virulence factors differ between typical and atypical strains but also within the typical strains (as strains belonging to CC13 lack some putative virulence factors). The atypical strains may also have the advantage of combining faster nutrient consumption (increased metabolic capabilities with respect to usage of different nutrient sources) along with the presence of virulence factors to lead to an accelerated death of the honey bee larvae (Djukic *et al.,* 2018). Nevertheless, further studies are needed to fully understand the virulence properties of *M. plutonius* strains and enhance our knowledge of the pathogenesis of typical and atypical EFB.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of European foulbrood and their purpose

Method	Purpose					
	Population freedom from infection	Individual hive freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[(a)]						
Bacterial isolation	+++	+++	++	+++	+++	–
Antigen detection	++	++	++	++	++	–
Microscopy	++	++	++	+++	+++	–
PCR	+++	+++	+++	+++	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection and identification of the agent

Diagnosis of EFB is based on the identification of the pathogenic agent and the presence of clinical signs. An initial overview of clinical signs of the disease is provided in this chapter, followed by identification methods that require a previous culturing step, or that can be performed directly on collected samples. The techniques involved are microbiological characterisation, the polymerase chain reaction (PCR), antibody-based techniques and microscopy. The analyst should be aware of differences in sensitivity between the presented approaches and should select the most appropriate for a given situation.

1.1. Microscopy

Freshly dead larvae are best for diagnosis. Preferably before any decomposition occurs, diseased larvae can be smeared on a microscope slide or pulled apart by pinching the cuticle about the centre of the

body with two pairs of forceps, which are then pulled apart. The mid gut contents are left exposed on the slide, still within the gelatinous, transparent peritrophic membrane. This is partially or almost completely filled with bacteria, which are easily seen as opaque chalk-white clumps. The contents of the mid-guts of healthy larvae, which are less easily dissected, have a golden-brown colour. Apparently healthy larvae may contain a mixture of bacteria and pollen. The mid-gut of healthy larvae that contain much light-coloured pollen may resemble those that are filled with bacteria.

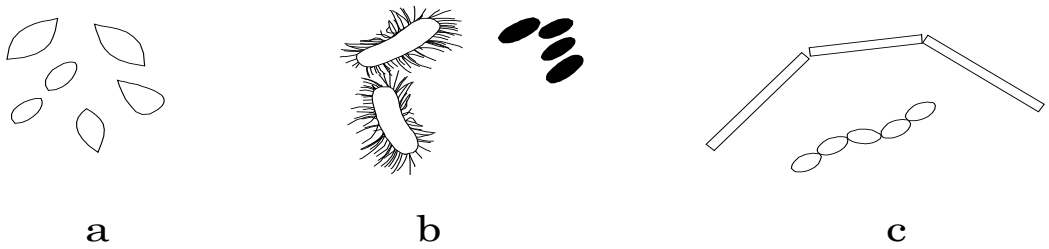

Fig. 3. Bacteria associated with European foulbrood.
(*a*) Melissococcus plutonius: ***the cause of European foulbrood occurs singly, in longitudinal chains or in clusters. Morphologically resembles*** **Enterococcus faecalis,** ***a common secondary invader.***
(*b*) Paenibacillus alvei: ***vegetative rods 2.0–7.0 × 0.8–1.2 μm with flagella; sporulating with spores lying adjacently. Both rods and spores are larger than those of*** **Paenibacillus larvae (*see American foulbrood*).**
(*c*) Achromobacter eurydice: ***slender, square-ended rods*** **in vivo** ***but can form chains of cocci*** **in vitro** ***in certain media.***

For a bacteriological investigation, a loopful of a dilute aqueous suspension of the midgut contents is transferred to a clean microscope slide and mixed with a loopful of 5% aqueous nigrosin. This is spread over one or two square centimetres, dried gently over a flame, and examined directly by high-power microscopy. The presence of numerous lanceolate cocci, about 0.5 × 1.0 μm in size, occurring either singly or in clusters, and arranged end to end in pairs or short chains, is almost certainly diagnostic of EFB. Some very slender square-ended rod-like bacteria are also usually present (Figure 3). Similar preparations made from aqueous suspensions of whole dead or decomposing larvae are likely to present a confusing array of bacteria in which *M. plutonius* will be difficult to distinguish. Alternatively, diseased larvae can be smeared on a microscope slide and submitted to the laboratory (Hornitzky & Wilson, 1989). The smears are heat fixed by flaming the slide over a burner two or three times and flooded with 0.2% carbol fuchsin for 30 seconds. Wash off the stain and allow to dry or gently blot dry with absorbent paper before microscopic examination at × 1000 (Forsgren *et al.,* 2013; Hornitzky & Wilson, 1989). Organisms are considered *M. plutonius* if they are lanceolate cocci, approximately 0.5 × 1.0 μm in size, take up the stain evenly and no unstained area of the organism is detected (Figure 4). Spores are considered to be

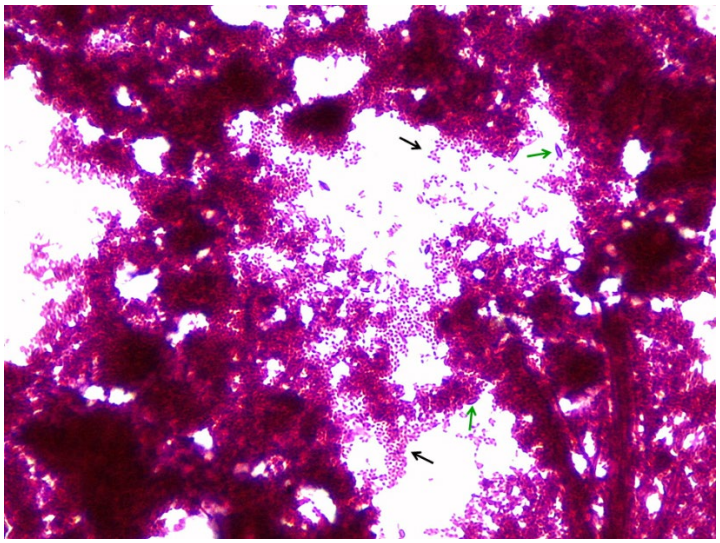

Fig. 4. Smear prepared from disease brood stained with carbol fuchsin. Black arrows indicate a mass of coccoid/lanceolate **Melissococcus plutonius** ***organism. Green arrows indicate the presence of spores of the secondary invader*** **Paenibacillus alvei*. Photo by A. M. Alippi.***

produced by the secondary invader *Paenibacillus alvei* if they are approximately 0.8 × 2.0 μm in size, and only the spore walls stain with 0.2% carbol fuchsin (Hornitzky & Wilson, 1989) (Figure 5). Alternatively, the Gram stain technique can be used on smears prepared from diseased brood. It allows verification of the Gram positive feature of *M. plutonius,* this latter appears as coccoid-shaped bacteria forming pairs or even chains (Forsgren *et al.,* 2013). Correct sampling of brood is important because even in the same brood frame, *M. plutonius* is mainly found in larvae with visual disease signs (Forsgren *et al.,* 2013).

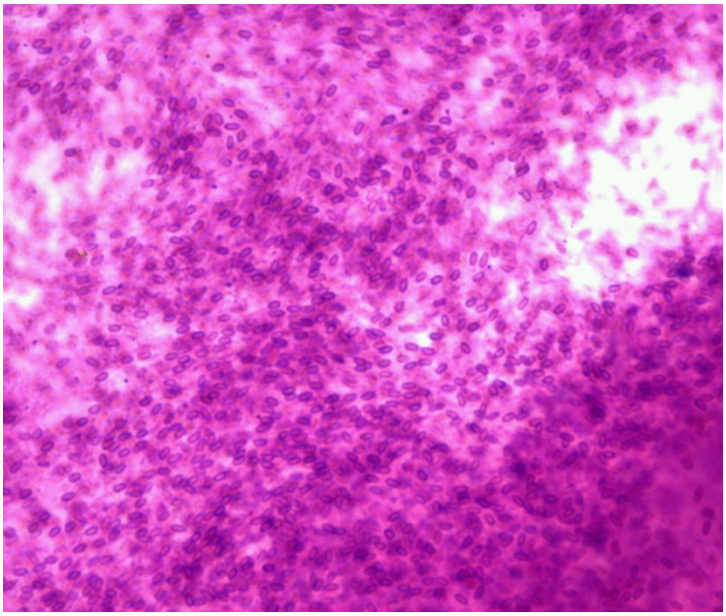

Fig. 5. **Paenibacillus alvei** ***spores in a smear prepared from disease brood stained with carbol fuchsin. Photo by A.M. Alippi***

1.2. Culture methods

During the early stages of an infection, *Melissococcus plutonius* (type strain NCIMB 702443) is the most abundant bacterium and is generally observed before the appearance of the varied microflora associated with this disease (Bailey & Collins, 1982a, 1982b). Although *M. plutonius* can be isolated from diseased brood and honey by cultivation, bacterial culture methods seem to be very insensitive detecting less than 0.2% of the bacterial cells (Djordjevic *et al.,* 1998).

1.2.1. Culture media

Melissococcus plutonius can be cultivated on a Basal medium (modified from Bailey, 1957) composed of (per litre):

i) 10 g yeast extract,

ii) 1 g L-cysteine or 1.5 g L-cysteine hydrochloride monohydrate ,

iii) 10 g glucose ,

iv) 10 g soluble starch,

v) 100 ml of 1 M KH_2PO_4 at pH 6.6

vi) 20 g agar.

M110 agar (Forsgren *et al.,* 2013) and KSBHI agar (Arai *et al.,* 2012) can also be used for isolation and cultivation of *M. plutonius*. The medium is preferably autoclaved in 100 ml lots in screw-capped bottles at 115°C for 15 minutes and poured into Petri plates immediately before use. Optional: to prevent growth of secondary bacteria, filter-sterilised nalidixic acid (dissolved in 0.1 M NaOH) may be added to a final concentration of 3 μg per ml after autoclaving (Forsgren *et al.,* 2013).

Note: for liquid cultures, replacing starch with saccharose will make the medium more transparent. This aids when checking the turbidity or the cloudiness of the cell suspension, e.g. to see if there is any bacterial growth.

All culture media should be subjected to quality control and must support the growth of *M. plutonius* from small inocula. A reference strain should also be cultured in parallel with the suspect samples to ensure that the tests are working correctly. The prepared plates are streaked with dilute aqueous suspensions of diseased larvae, or ideally, of diseased larval mid-gut. The latter can be prepared beforehand by allowing them to dry on a slide, which may then be kept for up to 18 months at 4°C or –20°C.

The preparation and storage of dried smears also eliminates most secondary organisms after a few weeks without affecting the viability of *M. plutonius*. This organism is isolated most efficiently by inoculating decimal dilutions of the aqueous suspension into agar that has been maintained molten at 45°C and which is then poured into plates. The plates must be incubated anaerobically, such as in McIntosh and Fildes jars in an atmosphere of approximately 5–10% carbon dioxide (CO_2) at 35°C for about 1 week. Small white opaque colonies of *M. plutonius* usually appear within 4 days. This bacterium is somewhat pleomorphic *in vitro*, often appearing in rod-like forms. The final pH of the medium may reach 5.5. Decreasingly fastidious strains become selected *in vitro*. Simplified or modified forms of the medium then support multiplication, especially of a serologically distinct *M. plutonius* group from Brazil (Allen & Ball, 1993) that will multiply on chemically defined media (Bailey, 1984). CO_2 remains essential. Inoculated slopes should be sealed when bacterial growth is apparent and may then be kept at 4°C for up to 6 months. Alternatively, the cultures can be suspended in a medium of 10% sucrose, 5% yeast extract and 0.1 M KH_2PO_4, pH 6.6, and then lyophilised. Isolated *M. plutonius* can also be stored by suspension in liquid media containing 10–30% glycerol and kept at –80°C.

A number of other bacteria are often associated with and may be confused with *M. plutonius*. *Achromobacter eurydice* inhabits the alimentary tract of adult bees and occurs commonly in the gut of healthy larvae in small numbers. It is more numerous in larvae infected with *M. plutonius*. The incidence of. *A. eurydice* in healthy bees is very low in winter and early spring, but it increases in summer. It forms thin square-ended rods, which can grow either singly or in chains. When grown in certain media, it sometimes resembles streptococci and has been confused with *M. plutonius*. However, its cultural characteristics closely resemble those of *Corynebacterium pyogenes* (Jones, 1975), and it multiplies poorly in the form of thin rods, under the conditions necessary for the cultivation of *M. plutonius*. The taxonomic position of *A. Eurydice* remains uncertain.

Enterococcus faecalis closely resembles *M. plutonius* morphologically and has often been confused with it, although they are both culturally and serologically distinct. Unlike *M. plutonius,* it does not remain viable for long when dried, or persist as mechanical contamination within bee colonies. It is probably brought into the hive by foraging adult bees, and is responsible for the sour smell sometimes encountered with EFB.

Enterococcus faecalis grows well *in vitro* under the conditions suitable for *M. plutonius,* but it may be readily differentiated by its ability to grow aerobically. It forms small transparent colonies within 24 hours and is a facultative anaerobe. It multiplies on a variety of the more common media with or without carbohydrates or CO_2. The final pH in the presence of glucose is 4.0. *Enterococcus faecalis* rarely exceeds the number of *M. plutonius* in bee larvae, and can usually be diluted out. When it is not diluted out it produces sufficient acid to prevent the *in-vitro* multiplication of *M. plutonius*.

Enterococcus faecalis does not multiply in bee larvae in the absence of *M. plutonius*, so its presence in large numbers can be taken as presumptive evidence of EFB.

Paenibacillus alvei is generally more common than *E. faecalis* in bee colonies affected with EFB, but it is not invariably associated with the disease and so cannot act as a reliable indicator of it; indeed, *P. alvei* has been found in colonies affected by American foulbrood as mixed bacterial spore populations on larval remains. In bee colonies, *P. alvei* multiplies only in the decomposing remains of larvae, and then its spores often predominate over all other bacteria, even to their

apparent exclusion. *Paenibacillus alvei* forms very resistant spores and becomes well established in bee colonies with enzootic EFB. It causes a characteristic stale odour. *Paenibacillus alvei* multiplies poorly under the conditions necessary for the *in-vitro* growth of *M. plutonius*. It produces a spreading growth of transparent colonies, some of which are motile and move in arcs over the surface of the agar. Cultures have the characteristic stale odour that is associated with EFB when the bacillus is present. Spores are formed rapidly.

1.3. Immunological methods

For the identification of *M. plutonius*, antisera can be prepared in rabbits against washed cultures of *M. plutonius* either by intravenous injections (Bailey & Gibbs, 1962) or by a single intramuscular injection of 1 ml of antigen suspension mixed with an equal volume of Freund's incomplete adjuvant.

Assays are made by agglutination tests in tubes containing suspensions of bacteria equivalent to 0.25 mg dry weight/ml. End-points are noted after tubes have been incubated for 4 hours at 37°C.

An enzyme-linked immunosorbent assay (ELISA) for confirmation of the presence of *M. plutonius* has been developed (Pinnock & Featherstone, 1984).

Recently, a commercially available lateral flow device for the detection of EFB using monoclonal antibodies has been developed. It provides rapid confirmatory on-site diagnosis of EFB infection in honeybee larvae in 10 minutes without the need for special equipment.

1.4. Polymerase chain reaction

Conventional PCR can be done on suspicious bacterial colonies transferred to and grown in liquid medium (Govan *et al.,* 1998). Genomic DNA is prepared according to standard methods (Wilson, 1990). Extraction of DNA using commercial kits according to the manufacturer's instructions is also suitable. Negative and positive controls should always be run in parallel with the test samples.

The DNA pellet is resuspended in 50 µl of 1 × TE buffer (10 mM Tris/HCl, pH 7.5; 1 mM EDTA [ethylene diamine tetra-acetic acid]). Approximately 1–3 µg of genomic DNA is amplified in a 50 µl reaction. The PCR reaction can also be done with larvae. Each larva is incubated individually in liquid medium overnight at 30°C in an anaerobic jar containing hydrogen plus 10% CO_2. Two millilitres of each sample is then centrifuged at 1000 ***g*** for 2 minutes, and the supernatant is centrifuged at 10,000 ***g*** for 5 minutes. The resultant pellet is resuspended in 100 µl of sterile H_2O and heated at 95°C for 15 minutes. One microlitre is amplified in a 50 µl PCR mixture. Besides template DNA this mixture also contains 2 mM $MgCl_2$, 50 pmol of forward (EFB-F) and reverse primer (EFB-R; primer sequences are given below) per µl, 25 mM (each) deoxynucleoside triphosphate and 1 U of *Taq* polymerase. Amplification of a specific DNA fragment occurs in a thermocycler under to the following PCR conditions: a 95°C (1 minute) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes).

When working with adult bees or honey samples commercial DNA extraction kits are recommended (Govan *et al.,* 1998).

A hemi-nested PCR was first developed by Djordjevic *et al.* (1998) and thereafter improved for sensitive detection of *M. plutonius* in honey, pollen, whole larvae and adult bees (McKee *et al*., 2003). Here the first 50 µl reaction mixture contains 5–30 ng genomic DNA, 3 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphate, 100 ng of the primers MP1 and MP2, 5 µl of 10 × PCR buffer (100 mM Tris/HCl, pH 8.3; 15 mM $MgCl_2$; 500 mM KCl) and 1 U of *Taq* polymerase. Conditions of amplification consist of an initial denaturation cycle at 95°C for 2 minutes followed by 40 cycles of denaturation (95°C, 30 seconds), primer annealing (61°C, 15 seconds), primer extension (72°C, 1 minute) followed by an additional extension step of 5 minutes at 72°C. The third primer MP3 is used in conjunction with MP1 to amplify a DNA fragment from 1 µl of the primary PCR product obtained in the previous reaction. PCR conditions for the hemi-nested PCR are exactly as described above except that the $MgCl_2$ concentration is lowered to 1.5 mM and the annealing temperature to 56°C.

The molecular weights of the PCR products are determined by electrophoresis in a 1.0–1.5 % agarose gel and staining with a suitable DNA stain. The PCR products can also be analysed using microfluidics or capillary electrophoresis devices.

A highly specific duplex PCR that can detect *M. plutonius* directly from diseased larvae was developed by Arai *et al.* (2014). It can differentiate strains that grow readily in culture from those with fastidious cultural requirements.

Real-time PCR methods have been developed and validated (Forsgren *et al.,* 2013; Roetschi *et al.,* 2008). They show improved sensitivity and specificity.

Ref.	Name	Sequence (5' → 3')	PCR-product size
Govan *et al.,* 1998	Primer 1 Primer 2	GAA-GAG-GAG-TTA-AAA-GGC-GC TTA-TCT-CTA-AGG-CGT-TCA-AAG-G	832 bp
Djordjevic *et al*., 1998 ; McKee *et al*., 2003	MP1 MP2 MP3	CTT-TGA-ACG-CCT-TAG-AGA ATC-ATC-TGT-CCC-ACC-TTA TTA-ACC-TCG-CGG-TCT-TGC-GTC-TCT-C	 485 bp 276 bp
Roetschi *et al.,* 2008	MelissoF MelissoR Probe	CAG-CTA-GTC-GGT-TTG-GTT-CC TTG-GCT-GTA-GAT-AGA-ATT-GAC-AAT 6'-FAM-CTT-GGT-TGG-TCG-TTG-AC-MBGNFQ	 79 bp
Budge *et al.,* 2010	EFBFor EFBRev2 Probe	TGT-TGT-TAG-AGA-AGA-ATA-GGG-GAA CGT-GGC-TTT-CTG-GTT-AGA FAM-AGA-GTA-ACT-GTT-TTC-CTC-GTG-ACG-GT-TAMRA	 69 bp

In recent years, real-time PCR methods have been extensively developed, as culture-independent approaches to detecting both *M. plutonius* and *P. larvae* from various honey and hive samples, including larvae and adult bees. Compared with conventional PCR, these methods offer the possibility of detecting a target template with its quantification (if needed/necessary) in a robust, highly reproducible, sensitive manner and without post-PCR analysis steps, which are time-consuming and prone to contamination (reviewed by Dainat *et al*., 2018; Okamoto *et al*., 2022; Riviere *et al*., 2013).

As in conventional PCR, it is highly important to run appropriate controls in parallel to the test samples. The use of commercial DNA extraction kits is possible (to be used according to the manufacturer's instructions).

Regarding the choice of a PCR test for the detection of *M. plutonius* samples, users should consider the fitness for purpose and interpretation of acquired data before adopting such methods. Further advice can be obtained from the WOAH Reference Laboratories[1].

2. Serological tests

No tests for detecting antibodies in bees are available.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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Illustrations by Karl Weiss, extracted from Bienen-Pathologie, 1984. Reproduced with the kind permission of the author and Ehrenwirth-Verlag, Munich (Germany).

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NB: There is a WOAH Reference Laboratory for European foulbrood (infection of honey bees with *Melissococcus plutonius*) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases

NB: FIRST ADOPTED IN 1989 AS EUROPEAN FOULBROOD DISEASE. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.2.4.

NOSEMOSIS OF HONEY BEES

SUMMARY

To date, two microsporidian parasites have been described from honey bees: Nosema apis (*Zander*) *and* N. ceranae. Nosema apis *is a parasite of the European honey bee* (Apis mellifera), *and* N. ceranae *of the Asian* (Apis cerana) *and the European honey bees. Both parasites are cross-infective between host species.* Nosema ceranae *has recently been detected in several geographically separated populations of European honey bees in Europe, South and North America and Asia. The pathological consequences of* N. ceranae *in* Apis mellifera *are not well known. Both types are presumed to be very similar, but* N. ceranae *seems to be more sensitive to low temperatures and to be able to reproduce even in high temperatures.* Nosema apis *and* N. ceranae *invade the epithelial cells of the ventriculus of the adult honey bee. Infections are acquired by the uptake of spores during feeding or grooming. The disease occurs throughout the world, but treatment of bees can help to prevent the spread of infection to unaffected bee colonies.*

Nosema *levels generally increase when bees are confined, such as in the autumn and winter in colder climates. The disease is transmitted among bees via the ingestion of contaminated comb material and water, and by trophallaxis; honey stores and crushed infected bees may also play a role in disease transmission. Spores are expelled with the faeces. The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood. The spores of* N. apis *are inactivated by acetic acid or by heating to 60°C for 15 minutes. To be effective, these treatments can be combined with feeding colonies with the antibiotic fumagillin to suppress infections in live bees. Many countries prohibit the use of antibiotic treatments of honey bees.*

Detection of the agent: *In some acute cases, brown faecal marks are seen on the comb and the front of the hive, with sick or dead bees in the vicinity of the hive. However, the majority of colonies show no obvious signs of infection, even when the disease is sufficient to cause significant losses in honey production and pollination efficiency. During winter, there may be an increase in bee mortality. In affected bees, the ventriculus, which is normally brown, can be white and very fragile. Microscopic examinations (×400 magnification) of homogenates of the abdominal contents of affected bees will reveal the oval spores of* Nosema *spp., which are approximately 5–7 × 3–4 µm with a dark edge* (N. ceranae *is slightly smaller, but species differentiation is difficult using light microscopy, especially as mixed infections occur). Their internal contents can be distinguished after staining with Giemsa's stain.* Nosema *spp. spores have a distinctive appearance, with a thick unstained wall and a blue-stained featureless interior. The nuclei within the spores are not visible. Staining can help to distinguish* Nosema *spp. from other microbes found in bees.*

The appearance of Nosema *spp.spores can be confused with yeast cells, fungal spores, fat and calciferous bodies or cysts of* Malpighamoeba mellificae*. The latter are similar in size to* Nosema *spp. spores, being 6–7 µm in diameter, but are completely spherical instead of oval.*

Positive identifications can be made only by observation of typical spores in the ventriculus or faeces. Very mild infections may not be demonstrable. The extent of infection is determined by counting the spores on a microscope grid and calculating the average number of spores per area and estimating from that the number of spores per bee. Identification to species level is difficult by light microscopy; polymerase chain reaction methods are preferred for this purpose.

Serological tests: *There are no applicable serological tests.*

Requirements for vaccines: *No vaccines are available.*

A. INTRODUCTION

The microsporidia *Nosema apis* (Zander, 1909) and *N. ceranae* (Fries *et al*., 1996) are parasites exclusive of the epithelial cells of the ventriculus of adult bees and both parasites occur throughout the world (Klee *et al.,* 2007). Based on molecular evidence, microsporidia are now included in the cluster Fungi (Adl *et al.,* 2005); thus, taxonomically, microsporidia are highly specialised parasitic fungi. Infection occurs by the ingestion of spores in the feed (Bailey, 1981; Webster, 1993), via trophallaxis (Webster, 1993) or perhaps after grooming of the body hairs (Bulla, 1977; Fries, 1993; Webster, 1993).

Both parasites first invade the epithelial cells in the posterior region of the ventriculus. They produce a fully developed infection throughout the epithelium within 2 weeks. The disease is transmitted among bees via the ingestion of contaminated comb material and water, and by trophallaxis; honey stores and crushed infected bees may also play a role in disease transmission. The infection mechanism of microsporidian parasites is based on mechanical injection of a polar filament protruding from the germinating spore. With physical force, the filament penetrates a host cell membrane into the host cell (ventricular epithelial cells for *N. apis* and *N. ceranae*). Through the filament, the infective sporoplasm is entered into the host cell cytoplasm where parasite replication, and later spore production is initiated (Larsson, 1986).Auto-infections can occur at the same time as new infections. Three days post-infection, mature spores start to develop in large quantities for *N. apis*, approximately a day later for *N. ceranae* (Forsgren & Fries, 2010). Both parasites have a temperature-dependent multiplication rate. *Nosema* spp. levels generally increase when bees are exposed to prolonged confinement, which increases the risk of in hive defecation. In the spring the infection levels may increase rapidly as the bees clean the combs for the expanding egg laying of the queen (Bailey, 1955) and a larger proportion of the bees are exposed to brood temperatures, where parasite replication, at least for *N. apis* is optimal (Lotmar, 1943). *Nosema ceranae* may grow better at slightly higher temperatures compared to *N. apis* (Fenoy *et al.,* 2009).

Any inherent natural defence by a bee colony against a heavy infection with the parasite depends on the colony size as well as on the prevailing weather conditions during the early part of the autumn of the previous year (Steche, 1985). If these conditions are unfavourable, the overall life expectancy of the colony is reduced. This may lead to the premature death of bees during winter or early spring. In a typical case of a colony being depleted because of a *Nosema* infection, the queen can be observed surrounded by a few bees, confusedly attending to brood that is already sealed.

In faecal droppings, spores of *N. apis* may retain their viability for more than 1 year (Bailey, 1962). Spores may also remain viable for up to 4 months after immersion in honey (White, 1919) and for up to 4.5 years in the cadavers of infected bees (Steche, 1985). The spores may lose viability after only 3 days when submerged in honey at hive temperature (Morgenthaler, 1939). Faecal contamination of wax, especially in combs used for brood rearing or other hive interior surfaces, provides sufficient inoculum for *N. apis* to be successfully transmitted to the next generation of bees and is probably the primary source of infection (Bailey, 1955). For *N. ceranae,* the durability of spores in different situations remains to be investigated, but they appear to withstand desiccation and heat better than *N.* apis spores (Fenoy *et al.,* 2009) whereas they are more sensitive to freezing temperatures (Forsgren & Fries, 2010). The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood and it seems that temperature may have a marked effect on the rates at which spores lose viability, regardless of their medium (Morgenthaler, 1939).

Spores of *N. apis* may be killed by heating hive equipment or tools to a temperature of at least 60°C for 15 minutes. Combs may be sterilised by heating to 49°C for 24 hours (Cantwell & Shimanuki, 1970). This cannot be used for *N. ceranae,* which can survive up to 60°C (Fenoy *et al*., 2009). Fumes from a solution of at least 60% acetic acid will inactivate spores of *N. apis* within a few hours, depending on the concentration; higher concentrations are even more effective and will kill spores within a few minutes (Bailey, 1957). The corresponding data are lacking for *N. ceranae*. Such procedures come under the jurisdiction of national control authorities with protocols that vary from country to country. Disinfection can be carried out, for example, by putting acetic acid solution into bowls or on to sponges that can soak up the liquid on top of a sealed stack of boxes with combs. Following disinfection after an outbreak, all combs should be well ventilated prior to use. Suppression of *Nosema* disease can also be achieved by feeding an antibiotic, fumagillin, in sugar syrup to the colony (Cantwell & Shimanuki, 1970). Use of antibiotics for honey bees is forbidden in many countries and in the European Union.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of nosemosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual hive freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Bacterial isolation						
Microscopy						
Antigen detection						
Conventional PCR						
Real-time PCR						
Mass spectrometry						

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

In acute forms of infection, especially in early spring, brown faecal marks may be noted on the comb and the front of the hive (Bailey, 1967). Lack of seasonal prevalence and symptoms such as faecal deposits have been reported for *N. ceranae* (Higes *et al.,* 2008). At the entrance to the hive, sick and dead bees may be seen, although other causes, such as pesticide poisoning and diseases of adult honey bees (such as acarapidosis should be eliminated first if this is the case. The detection of these infectious diseases requires microscopic examination. During winter, *N. apis*-infected colonies may become severely depleted of bees or die out altogether. The majority of *N. apis*-infected colonies will appear normal, with no obvious signs of disease even when the disease is sufficient to cause significant losses in honey production and pollination efficiency (Anderson & Giacon, 1992; Fries *et al.,* 1996). A proper diagnosis can be made by microscopic examination of adult bee abdomen or ventriculus, by molecular tools (polymerase chain reaction [PCR]) or by transmission electron microscopy (TEM). To diagnose a *Nosema* spp. infection using microscopy the posterior pair of abdominal segments is removed with a forceps to reveal the ventriculus, complete with the malpighian tubules, the small intestine and rectum. The ventriculus is normally brown but, following a *Nosema* spp. infection, it can become white and fragile. However, this appearance is given by other causes of intestinal disturbance, for example feeding on indigestible food stores, such as syrup containing actively growing yeast. For a reliable diagnosis, a number of bees in a sample should be examined. For example, 60 bees examined in a composite sample will detect a 5% infection level with 95% probability.

1.1. Microscopy

It is necessary to attempt to distinguish between a *Nosema* spp. infection and an infection caused by *Malpighamoeba mellificae* (Webster, 1993). There is quite often an indication of dysentery in a *N. apis* infection. In an *M. mellificae* infection, there may be diarrhoea, often of a sulphur-yellow colour and with a distinct odour. Characteristics of *M. mellificae* cysts are described later. Secondary mixed infections may occur (Morgenthaler, 1939). A simple, non-quantitative method for detecting *Nosema* spp. infection is as follows: sampled bees should be obtained from the hive entrance in order to avoid sampling young bees that are less likely to be infected. At least 60 bees should be collected in order to detect 5% of diseased bees with 95% confidence (Fries, 1993). Before sending to the laboratory, the bees should be

fixed in 4% formol, 70% ethyl alcohol or frozen in a standard freezer in order to prevent them from decomposing and to improve their reception and organisation in the laboratory. The abdomens of the bees to be examined are separated and ground up in 5 ml of water. Then water is added representing a total volume of 1 ml per bee in the sample. A drop of the suspension is placed on a slide under a cover-slip and examined microscopically at ×400 magnification, under bright-field or phase-contrast optics.

The spores are about 5–7 μm long and 3–4 μm wide (*Nosema ceranae* is slightly smaller than *Nosema apis*). They are completely oval with a dark edge. Their contents, consisting of nucleus, sporoplasm and polar tube, cannot be seen. Dyes are usually not necessary. *Nosema* spp. spores must be differentiated from yeast cells, fungal spores, fat and calciferous bodies, and from *M. mellificae* cysts, which are spherical and approximately 6–7 μm in diameter.

When air-dried, ethanol-fixed smears of infected tissue are stained with Giemsa's stain (10% in 0.02 M phosphate buffer) for 45 minutes. *Nosema* spp. spores will have a distinctive appearance, with thick unstained walls and an indistinct blue interior, without visible nuclei. Insect cells, fungal spores and other protozoa stained in this way will generally have thinner walls, blue/purple cytoplasm and magenta-coloured nuclei.

To quantify the average infection level, spore counts in a haemacytometer can be used (Cantwell, 1970) or bees can be diagnosed individually to yield the proportion of infected bees. A standardised procedure such as the following must be used.

A sample of older worker honey bees is taken from the hive entrance or from peripheral frames if weather does not permit flight conditions. The abdomens of 60 individuals are macerated in 5 ml of water using a mortar and pestle and 50 ml of water is added for a total volume of 1 ml per bee (5 ml is added later). When tissue pieces have become quite fine, the suspension is filtered through two layers of muslin (thin loosely woven cotton fabric) in a funnel leading to a graduated centrifuge tube. A second 5 ml of water is used to rinse the pestle, swirl around the inside of the mortar and pour through the subsample in the funnel. When the suspension appears to be homogenous after shaking, a sample is taken to fill the calibrated volume under the cover-slip of a haemocytometer (blood cell counting chamber). After a few minutes the spores will have settled to the bottom of the chamber. *Nosema* spp. spores appear transparent but with a very distinct dark edge and are 5–7 μm long and 3–4 μm wide. They are best seen using a magnification of ×400 and bright-field or phase-contrast optics. The number of spores in each square is counted. Where a spore lies over the edge of a square, count only those spores that straddle the left and upper edges of the square, not the right and bottom edges. The size of these chambers can vary with manufacturer but they mostly consist of two separate chambers, each with a defined volume ($0.1 mm^3$) containing a marked counting grid with an area of $1 mm^2$. The whole grid consist of 3 × 3 large squares, separated by triple lines. Each large square is further subdivided into 16 smaller squares subdivided by double lines, in total 144 squares. The spores are counted in the smaller squares with the area of $1/25 mm^2$. When the counting is completed, the number of spores per bee in the sample can be calculated according to the formula:

$$Z = \alpha/\beta \times \delta \times 250{,}000$$

Where

Z = spore numbers per bee
α = total number of spores counted
β = number of squares counted
δ = dilution factor

The number 250,000 is used because the volume in each counted square is 1/250 000 ml and the equation uses the average number of spores per counted square. If no spores are seen, the result should be designated 'not detected', but that does not mean that the bees are not infected. Regulatory agencies will decide on the level of infection useful for their purposes.

A laboratory method for the simultaneous detection of *Nosema* spp. spores and *M. mellificae* cysts consists of the individual examination of the colonies using 60 bees per colony. A suspension of the abdomens of dead bees is prepared by grinding with 5–10 ml water; the volume of water depending on the number and condition of the bees. The suspension must be filtered to remove debris that would

interfere with the examination, first through a 100 μm and then a 40 μm filter. Parts of the malpighian tubules pass through the 100 μm filter, but are collected on the 40 μm filter. They are placed on a slide or bacterial counting chamber and examined at ×400 magnification. Only a few tubules are filled with cysts after an *M. mellificae* infection. The normal structure of malpighian tubules is not visible in this case. Only cysts inside the malpighian tubules can be taken as a positive result, because *M. mellificae* cysts are often confused with fungal spores and yeast cells.

1.2. Culture

Several lepidopteran cell lines have been shown to be susceptible to both *N. apis* and *N. ceranae* infection. Susceptibility was recently demonstrated for the following cell lines (Gisder *et al.,* 2010): MB-L2 (*Mamestra brassicae*), Sf-158 and Sf-21 (*Spodoptera frugiperda*), SPC-BM-36 (*Bombyx mori*), IPL-LD-65Y (*Lymantria dispar*), and BTI-Tn-5B1-4 (*Trichoplusia ni*). All these cell lines can be obtained through national cell culture collections together with protocols on how to maintain and passage the cell lines. However, the available protocols do not yet allow the continuous propagation of *Nosema* spp. in cell culture. Thus, it is not yet possible to replace infection of bees for the production of spore suspensions.

1.3. Polymerase chain reaction (PCR)

Different methods have been developed to distinguish *N. apis* from *N. ceranae*. A multiplex PCR is described below with which both pathogen types along with *Nosema bombi* can be clearly identified at the same time (Fries *et al.,* 2013).

1.3.1. Sample preparation for PCR

Place a maximum of 30 bees in a filter grinding bag. Add 0.5 ml (DNAase/RNAase free) ddH_2O per bee and homogenise the mixture using a homogeniser. Flash-freezing in liquid nitrogen is possible prior to homogenisation to aid in mechanically breaking open cells. Without access to a robot, a pestle can be used to crush the bee tissue (frozen tissue if flash-frozen) to generate a homogeneous homogenate. Transfer 100 μl of the liquid homogenate into a microcentrifuge tube and centrifuge for 3 minutes at 16,100 ***g*** to precipitate the microsporidia and other cellular material. Discard the supernatant. Freeze the pellet by using liquid nitrogen and crush using a pestle until pulverized (in order to break open *Nosema* spore walls), and repeat 2–3 times so that *Nosema* DNA goes into solution. The DNA extraction can be easily carried out using routine procedures or commercial kits. Complete the final elution step in 100 μl AE buffer.

1.3.2. Multiplex PCR

For multiplex PCR amplification of partial 16S rRNA (=SSU rRNA) gene fragments, the following primer combination can be used. Primers were designed based on alignment of all available sequence data in GeneBank of the 16S rRNA gene from *N. apis, N. bombi* and *N. ceranae*.

Mnceranae-F	forward primer: 5'-CGT-TAA-AGT-GTA-GAT-AAG-ATG-TT-3'
Mnapis-F	forward primer: 5'-GCA-TGT-CTT-TGA-CGT-ACT-ATG-3'
Mnbombi-F	forward primer: 5'-TTT-ATT-TTA-TGT-RYA-CMG-CAG-3'
Muniv-R:	reverse primer: 5'-GAC-TTA-GTA-GCC-GTC-TCT-C-3'

Note that the Mnbombi-F primer contains variable sites to account for the sequence diversity observed for this species.

PCR product size:

for *N. ceranae:*	143 bp
for *N. bombi:*	171 bp
for *N. apis:*	224 bp

PCR conditions:

1 μl of DNA (ca. 1 ng)
0.5 U of Taq polymerase
2× Taq reaction buffer (3 mM $MgCl_2$)

0.3 mM of each dNTP (dNTP mix)
0.4 µM of Mnceranae F
0.4 µM of MnapisF
0.5 µM of Mnbombi-F
0.5 µM of Muniv-R
in 10 µl total volume

Amplification is carried out in a thermocycler under the following conditions: Initial denaturation step of 95°C for 2 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds, with a final extension step of 72°C for 5 minutes. Visualisation of the amplification products is made using standard procedures.

2. Serological tests

There are no serological tests available.

C. REQUIREMENTS FOR VACCINES

No biological products are available.

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NB: There is a WOAH Reference Laboratory for nosemosis of honey bees (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for nosemosis of honey bees

NB: FIRST ADOPTED IN 1989 AS BEE NOSEMATOSIS. MOST RECENT UPDATES ADOPTED IN 2013.

CHAPTER 3.2.5.

INFESTATION OF HONEY BEES WITH *AETHINA TUMIDA* (SMALL HIVE BEETLE)

SUMMARY

***Description and importance of the disease**: The small hive beetle,* Aethina tumida (*Murray 1867*) (Coleoptera: Nitidulidae), *is a parasite and predator of honey bees. Adults and larvae of small hive beetles feed on honey bee brood, honey and pollen. While feeding on food stores the remaining honey is fermenting and the comb is destroyed. The beetles can promote structural collapse of the nest and cause the adult honey bees to abscond from severely infested colonies. The extent of beetle-associated damage depends on climate, colony strength and other conditions. Small hive beetles tend to be more problematic in areas with warm temperatures and high humidity. The small hive beetle can be a serious problem in honey-extracting facilities where stored comb, honey and wax cappings are potential feeding and breeding areas. Beetle development from egg to adult requires 3–12 weeks, depending on humidity, temperature and food availability. The flying adult beetles actively infest honey bee colonies of all strengths and sizes.*

***Identification of the agent:** An infestation by the small hive beetle can be recognised either indirectly via colony-wide damage associated with the beetle or directly via eggs, larvae and adults. An early diagnosis can be made after opening the colony and finding adult beetles under the colony lid, on the bottom board, or hiding in the combs (especially peripheral combs). Definitive diagnosis at the laboratory is based on morphological examination under a stereomicroscope. Confirmatory testing can be done by real-time polymerase chain reaction.*

***Serological tests:** Serological tests are not applicable.*

***Requirements for vaccines:** No vaccines are available.*

A. INTRODUCTION

The small hive beetle (hereafter referred to as "beetle"), *Aethina tumida*, order *Coleoptera,* family *Nitidulidae* (Murray, 1867), is native to sub-Saharan Africa (Hepburn & Radloff, 1998) but has been found in various regions of the world over the past few decades. *Aethina tumida* was first detected in the United States of America in 1996. Since then it has spread to Canada and a number of countries in South and Central America. *Aethina tumida* has also been found in Australia, Egypt, Italy, Korea and the Philippines (WOAH WAHIS Interface, database accessed on 20/06/2017; Lee *et al.,* 2017).

1. Life cycle

The infesting small hive beetle adults mate in the colony and the female beetles oviposit several eggs in typical clutches in small cracks or within capped brood cells (Cuthbertson *et al.,* 2013; Ellis, 2005; Lundie, 1940). In some situations, more than 1000 adult beetles may occur within a colony (Elzen *et al.,* 1999). Adult beetles can survive up to 12 months (records indicate up to 16 month in laboratories; Somerville, 2003), but females die quickly when ovipositing on a daily basis (Neumann *et al.,* 2016). Females can oviposit about 1000 eggs in their lifetime (Lundie, 1940), though Hood (2004) suggested the upper limit may be 2000 eggs. Successful egg emergence is correlated with relative humidity, with fewer eggs hatching at a relative humidity of <50%. The larvae emerge from the eggs after 1–6 days (most within 3 days) and feed on pollen, honey and bee brood (Lundie, 1940; Schmolke, 1974). Adult beetles can be fed by worker bees via trophallaxis, especially while confined in bee-guarded "prisons" (Ellis, 2005). Larval development usually takes about 2 weeks (8–29 days depending on food availability and temperature; de Guzman & Frake, 2007; Ellis *et al.,* 2002b; Lundie, 1940; Schmolke, 1974). Following this, the larvae reach the

wandering phase and leave the colony to pupate in the soil surrounding the colony (Lundie, 1940). Pupation takes about 2–12 weeks depending on temperature and soil moisture (Ellis et al., 2004). Emerging adults leave the soil and can fly to search for new host colonies, thereby completing their life cycle. In laboratory conditions, the small hive beetle can survive and reproduce on ripe or rotten fruits (Buchholz *et al.*, 2008).

2. Impact of the pest

Small hive beetle is seldom a serious problem in Sub-Saharan Africa. The reasons for the apparent difference in its impact on colonies within its native range and those in its new ranges are not well understood (Ellis & Hepburn, 2006). They may include quantitative behavioural differences between African and European honey bee subspecies, different beekeeping techniques, climatic differences, or escape from natural enemies, among other plausible hypotheses (Hood, 2004; Neumann & Elzen, 2004).

While bee colony damage due to adult beetles is relatively minor, the adults can cause colonies to abscond (i.e. the adult bees completely abandon the nest; Ellis *et al.,* 2003). If not prevented, larval feeding behaviour is often associated with fermentation of stored honey, causes severe damage to combs and often results in the full structural collapse of the nest (Lundie, 1940). Economic losses also can be associated with beetle infestations in the honey-extracting facility. Environmental conditions generally associated with extracting facilities, such as high temperatures and humidity, provide optimal conditions for beetle development. Cryptic low-level reproduction may also occur either in the debris or underneath hive inserts without any signs of colony damage (Spiewok & Neumann, 2006).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of infestation with Aethina tumida and their purpose

Method	Purpose					
	Population freedom from infestation	Individual animal or bee nest freedom from infestation prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infestation – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Morphology	+++	+++	+++	+++ (adults) + (larvae)	+++	–
Real-time PCR	++	++	++	++	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction,

1. Field detection

1.1. Adult beetles

The first sign of an infestation by the small hive beetle is the occurrence of adult beetles (Figure 8). Adult beetles are ~5 mm long and ~3 mm wide, with females being slightly longer than males (Ellis *et al.,* 2001). The adults are dark brown to black (lighter shortly after eclosion). During inspections, adults avoid sunlight, hide, and can be observed while running for cover into corners or similarly over the combs. Adults can be confused with other nitidulid beetles, which can also be associated with colonies (see Section 2.2.3 below for details; also Ellis *et al.,* 2008, Marini *et al.,* 2013 and Neumann & Ritter, 2004).

1.2. Beetle eggs, larvae and pupae

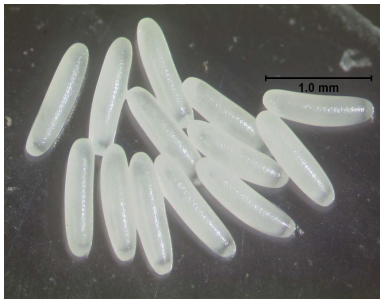

Fig. 1. Small hive beetle eggs. Photograph by Josephine Ratikan, University of Florida.

Small hive beetle eggs (Figure 1) are white, ~1.4 × 0.26 mm (length × width); ~2/3 of the size of a honey bee egg, and are deposited in clutches in cracks, on the bottom board, on the combs and underneath the cappings of sealed brood cells. Larvae (Figure 2) are whitish, up to ~1 cm long (wandering phase), have three pairs of legs, and have dorsal spikes. Larvae can be found mining in the wax combs (Lundie, 1940) or in colony debris (Spiewok & Neumann, 2006). Larval infestations are typically associated with a rotten smell due to death of honey bee brood or fermentation of the stored honey. Wandering larvae often leave smear trails (or "slime") inside and outside the colony (Figure 3). Once in the ground, the larvae excavate small pupation chambers (Figure 4) 1–20 cm deep in the soil (Pettis & Shimanuki, 2000), develop into pupae (Figure 5, whitish to dark brown depending on age, ~5 mm long and 3 mm wide) and then into adults. Most larvae tunnel into soil that is <180 cm from the colony (Pettis & Shimanuki, 2000).

Fig. 2. Dorsal (left) and ventral (right) view of a small hive beetle larva. Photographs by Josephine Ratikan, University of Florida.

Fig. 3. Comb damage attributed to the feeding/crawling habits of small hive beetle larvae. Notice the "slime" on the frame (i.e. the wax comb looks "wet" and it "glistens"). This is caused by the fermentation of honey, which is moved around the comb by crawling larvae. Beetle larvae can be seen in cells in the centre of the comb, where brood was present originally. Photograph credit, University of Georgia.

Fig. 4. Small hive beetle larvae that has tunnelled into the soil and hollowed out a chamber in which to pupate. Photograph credit, University of Georgia.

Fig. 5. Small hive beetle pupa (ventral view). Photograph by Lyle Buss, University of Florida.

It is difficult to find beetle eggs in a colony, especially at low levels of infestation. Cracks or crevices around the nest should be investigated or capped brood cells that have small holes in the cappings,

possibly indicating that a female beetle has punctured the capping and oviposited within the cell. Small hive beetle pupae can be found by sifting the soil around the colony and looking for the pupal chambers or the pupae themselves.

1.3. Visual inspection of colonies

When monitoring honey bee colonies for the presence of small hive beetles, an examination of the hive may provide an early indication of infestation. In countries still free of *A. tumida*, it is recommended to monitor sentinel apiaries in zones at risk of an introduction, to detect an infestation at an early enough stage to eradicate it (Chauzat *et al.,* 2016). Furthermore, if the intent is to eradicate *A. tumida,* sentinel colonies should be placed in the location of infested apiaries that have been previously sanitised. Such sentinel colonies act as a bait to attract free-flying beetles and must be in place immediately after all colonies are destroyed, as adult beetles staying outside colonies might survive the eradication and spread further to other hosts nearby.

It is important to adapt the method of visual inspection to limit the expected spread of *A. tumida*, as colony manipulation might induce disorder and robbing (where bees steal honey from other colonies) or adult small hive beetles may occasionally leave the colonies during manipulation. The currently used visual inspection methods in infested areas are most feasible in the field and easy to carry out for anyone trained to manipulate a beehive (EFSA, 2015; Neumann *et al.,* 2016). Colony manipulation requires a certain minimum training and awareness of small hive beetle biology and morphology, to correctly inspect field-colonies and to quickly detect and recognise damage caused by the different life stages of *A. tumida*. A colony inspection begins right at the entrance of the hive and relies on the rapid but meticulous examination of the lid, the inner cover, the frames and the bottom board. The following recommendations are from the EFSA scientific opinion (EFSA, 2015).

1.3.1. Colony inspection method (EFSA, 2015)

i) Remove the lid and check for the presence of adult beetles running away.

ii) Remove the inner cover and check both sides. Check also the top of the frames for running adults.

iii) Remove the frames from the hive one by one. Each side of the frame should be quickly observed to check the presence of adult beetles, larvae, eggs and damage. The first frame can be left outside the body of the hive to make it easier to handle the other frames. Subsequent frames should be put back into the body or super (the part of the hive in which bees store honey) to prevent robbing in the apiary during the examination.

iv) Beetles can hide inside the cells of combs. It is also important to examine the lid, the bottom board, the side faces, corners, interstices of the hive and hive components.

If robbing is unlikely, the super can be examined by placing it on the inverted lid of the hive in a sunny spot. Adults will escape from the sunlight and retreat down into the lid. After about 10 minutes, the presence of adult beetles in the lid can be checked by lifting the super (Zawislak, 2014). If there is a risk of robbing, the super should be inspected in the same way as the body of the hive, i.e. comb by comb, by replacing each frame in the box after its examination. During the examination of the body, the super can be placed on a reversed lid, so that no bees or beetles can escape (Spiewok *et al.,* 2007).

To improve the sensitivity of the visual inspection, the hive can first be removed from its original position, then opened and replaced by an empty hive (Neumann & Hoffmann, 2008; Spiewok *et al.,* 2007). Each frame is then removed and examined for beetles for the first time. The bees are then shaken into an empty box and the comb is inspected for a second time for beetles, this time in the absence of bees, before being placed into the new hive. Once all the frames have been examined, the original hive box and bottom board are inspected. However, this method is more time-consuming, and requires additional beekeeping equipment and therefore is not suitable for routine monitoring of small hive beetle infestation in large apiaries. It is however, recommended for health certification to demonstrate the absence of *Aethina tumida* infestation in a colony.

A method, originally described in Canada, uses a white 12-litre bucket fitted with a wire-mesh screen (about 6 mm) fitted halfway down the depth of the bucket. The bottom of the bucket is covered with a thin layer of vegetable oil. The frames are shaken inside the bucket and bees are stopped unharmed by

the wire-mesh whereas beetles fall into the vegetable oil. Field data suggest that this method is more sensitive than simple visual inspection when the infestation level is low[1].

Another method for colony examination is described below. The method can be used to search for beetle adults and larvae, if larval infestations are moderate to high (Ellis *et al.,* 2002a, Ellis & Delaplane, 2006), but should not be used if eradication is planned, as it might increase the number of free-flying beetles.

Fig. 6. Inspecting a colony for adult small hive beetles. The inspector on the right has shaken the bees onto a piece of plywood. Both faces of the framed comb were then bounced onto the wood to dislodge the beetles from the cells. The inspector on the left is shifting through the adult bees and using a mouth aspirator to collect the beetles. Photograph by Keith Delaplane, University of Georgia.

Notes:

i) This procedure is best accomplished with two people, one to work the colony and the second to collect the beetles if quantification is desired. Only one person is needed if beetle detection is the sole desired outcome.

ii) Some beetles inevitably fly away or hide from view during this procedure. The number of beetles that escapes is presumed to be low (<5%).

iii) This procedure is best used for qualification of adult beetles. However, larval beetles can be found this way as well.

a) Place a sheet of opaque plastic (~2 × 2 m, preferably white or light in colour) or plywood in front of the colony which you want to inspect for beetles.

b) Lightly smoke the colony.

c) Remove the lid from the colony and bounce the lid on the plywood. This should be done to dislodge all adult bees and beetles adhering to the lid.

d) A second individual (the beetle collector) should comb through the bees (this can be done with the hand or using a small stick) and collect all adult beetles seen using an aspirator. All bees on the plywood should be inspected as beetles can easily be concealed by clusters of bees (Figure 6).

e) Remove the outermost frame in the uppermost super (i.e. the uppermost “box” containing bees) and shake the bees from the frame onto the plywood.

f) The beetle collector should repeat step d.

g) Once the bees have been shaken from the frame, the frame should be turned onto its face and bounced against the plywood to dislodge adult beetles from the comb. This step should be repeated two-to-three times for both sides of the frame.

h) The beetle collector should repeat step d.

i) The individual working the colony should repeat step g to all frames in the uppermost super and then bounce the empty super on the plywood. This step should be repeated for all supers, all frames, and the bottom board of the colony.

The latter two described colony inspection methods are time-consuming and there is a very high risk of inducing disorder and robbing in the apiary (EFSA, 2015) and a risk of making the beetles fly away.

1 http://www.omafra.gov.on.ca/english/food/inspection/bees/2011-shb-report.htm

1.4. Colony examination using traps

The use of traps for small hive beetle detection has been described in the Guidelines for the surveillance of the small hive beetle (*Aethina tumida*) infestation (updated version: April 2016) developed by the European Union reference laboratory for honey bee health (Chauzat *et al.,* 2016).

The principle of most small hive beetle traps is to offer shelter from bee aggression by providing a passage that is large enough for beetles but too small for bees to enter. In their attempt to get away from the bees that chase them, *A. tumida* adults will enter the trap in which oil or veterinary medicines may be used as a killing agent. Sometimes this principle is combined with the use of bait that can increase trap efficacy. The position of the trap inside the hive is important and has to be adjusted to the hive type and to climatic conditions as beetles may hide on the bottom boards or in the periphery of the colony if climatic conditions are warm but tend to stay within the clustering bees when temperatures are low. Therefore, traps are available for all positions in the hive and all of them should be checked regularly during apiary visits.

Traps that are placed between frame top-bars consist of small containers that are covered by a grid. These kinds of traps are usually filled with vegetable oil (diatomaceous earth was successfully tested in the laboratory; Cribb *et al.,* 2013) and they were shown to be effective in North American conditions (Bernier *et al.,* 2015). The trap is placed between the top-bars of two frames, close to the brood nest or the winter cluster. When visiting the colony, traps are examined for the detection of any beetles. If the container is transparent, this observation is easy and straightforward. It was shown using these kinds of trap that bees might seal the openings with propolis thereby reducing their efficacy (Bernier *et al.,* 2015). Care must also be taken to prevent any oil spill.

In the warm season, traps placed on the bottom boards or modified bottom boards could be used for the detection of adult small hive beetles. Modified bottom boards usually consist of an oil filled tray that is placed underneath a grid or a mesh-screen. If the tray is covering the whole bottom, the hive must be levelled, but some of these traps cover only parts of the bottom board. Although these traps work well, they require hive-modification and therefore are mainly feasible for stationary beekeeping.

Many traps were invented for use on the bottom board. One example without any bait and killing agent is a 4 mm corrugated plastic strip (Figure 7). It was shown to be effective in field trials in Australia and the USA. The corrugated plastic suits the thigmotactic behaviour of *A. tumida* as it consists of square flutes, big enough for the beetle to get in, but too small for the bees to enter (approx. 4 × 4 mm). The hive does not need to be opened as traps are placed inside the hive through the entrance. It is important to properly place the trap in contact with the solid floor of the hive. If not, beetles can seek refuge in the space located between the trap and the floor. For optimum use, traps should be left in hives for a minimum of 48 hours before they are checked. The trap should preferably be made of a transparent material so that beetles can quickly be detected (Schaefer *et al.,* 2008). Other bottom board traps bring the beetle in contact with killing agents inside the traps. Chemicals can have high efficacy but there is always a risk that resistant strains may develop, residues might accumulate in honey or other hive products or that the chemicals could spread to the bees leading to adverse side effects on them. An alternative to the use of chemicals is diatomaceous earth or traps that just use adhesive film.

A biomechanical way of trapping small hive beetle inside the hive is the placement of kitchen wipes or similar material on top of the frames. The bees shred this material into fibres in which the beetles become entangled. This very simple to use and economical control method has the advantage of functioning without any lethal substance, but fibres might also end up in the honey.

Fig. 7. Corrugated plastic insert used to detect adult small hive beetles. The plastic insert contains square flutes (left) in which adult beetles hide when inserted onto the bottom board of a colony, through the colony

entrance* (*right*). *The insert must be used in conjunction with a traditional solid bottom board rather than a screened bottom board. Photographs by James Ellis* (*left*) *and Stephanie Kimball* (*right*), *University of Florida.

In low infested areas it is especially recommended to always undertake a combination of visual inspections and traps to increase the sensitivity of detection. Depending on the seasonal conditions, it may be decided to use either visual observation or traps, but, whenever possible, combining them both is best. In apiaries where inspections are frequently undertaken (sentinel apiaries), surveillance traps may be used. For single inspections, visual inspections may be best because of the higher detection sensitivity and to avoid a return visit to check the trap.

For a more detailed description of different traps refer to EFSA, 2015 and Neumann *et al.,* 2016.

2. Laboratory identification

Rapid and reliable diagnosis is crucial for the implementation of sanitary measures and to avoid spread in non-infested territories. Suspect field specimens should be sent to official laboratories for confirmation of *A. tumida* identification. Morphological identification is fast and inexpensive, and does not require sophisticated equipment. Confirmatory testing can be done by molecular methods (polymerase chain reaction [PCR]), and is particularly useful for larval identification or when specimens are damaged.

2.1. Special precautions required for sample handling

The specimens to be identified are collected in or near honey bee hives (for example, in colonies, beekeeping equipment or queen cages).

Suspect specimens should be killed before submission to the laboratory e.g. in 70% ethanol. Denatured ethanol should not be used where molecular methods are to be used because of possible PCR inhibition. Alternatively, specimens can be stored overnight at –20°C to kill the specimens.

On arrival at the laboratory packages should be opened in containment conditions. If the specimens are found to be alive on arrival, the submission should be placed at –80°C for approximately 1 hour before any work can be done with them. This procedure immobilises the specimens, which can subsequently be stored in 70% ethanol.

2.2. Morphological identification of adults and larvae

The test method aims to identify *A. tumida* by examining the external appearance of adults or larvae specimens in the laboratory. It consists of the visual examination of specimens noting morphological characteristics specifically selected to differentiate *A. tumida* from other nitidulid beetles and wax moth larvae, commonly found in honey bee colonies, queen cages or beekeeping equipment.

2.2.1. Equipment and reagents

Classical entomological materials are required for the morphological identification of *A. tumida*, including a stereomicroscope (or a magnifier), entomological tweezers, evaporating dishes (glass, plastic or porcelain) or Petri dishes, capped tubes for specimen storage, 70% ethanol (not denatured ethanol).

2.2.2. Test procedure

A general observation of the specimens should be made by placing them in a dish and checking for homogeneity (using a magnifier or stereomicroscope as necessary). If they are of uniform type, the samples can be processed further. If they are not uniform (i.e. multiple species may be present) then samples should be taken of each type present for further identification. When possible, select undamaged samples for further analysis, using entomological tweezers.

Microscopic examination should be done at different magnifications to visualise the critical identification criteria (see Section 2.2.3 below). The size of the specimens should be measured. Samples can be compared with reference specimens if available. After examination, beetles are stored in 70% ethanol.

2.2.3. Guidelines for the identification of *Aethina tumida*

Differentiation should be made between *A. tumida and* other non-pest nitidulid beetles that can be found in honey bee hives, for example: *Cychramus luteus*, found in Europe, that mainly feeds on pollen (Neumann & Ritter, 2004), *Carpophilus lugubris*, found in hives in Italy (Marini *et al.,* 2013), and *Glischrochilus fasciatus, Lobiopa insularis, Carpophilus dimidiatus* and *Epuraea corticina* found in hives in the United States (Ellis *et al.,* 2008).

The larvae of *A. tumida* can also be mistaken for larvae of the lesser wax moth, *Achroia grisella,* or the honeycomb (greater wax) moth, *Galleria mellonella*. These lepidoptera are generally found in colonies and on beekeeping equipment.

2.2.3.1. Adult form

Identification of adult *A. tumida* is based on the following morphological criteria: (Figures 8 and 9)

1. Body divided in three parts: head, thorax and abdomen
2. Three pairs of legs
3. Presence of elytra
4. Dimensions: length: 5–7 mm; width: 3–4.5 mm (approximately)
5. Colour: reddish brown when newly hatched, turning dark brown to black in adulthood
   Presence of a lighter band around thorax and abdomen (optional criterion)
   Note: The colour may change with environmental conditions and conservation of the specimens
6. Club-shaped antennae
7. Sharp posterio-lateral angles of the pronotum
8. Elytra not covering the entire abdomen

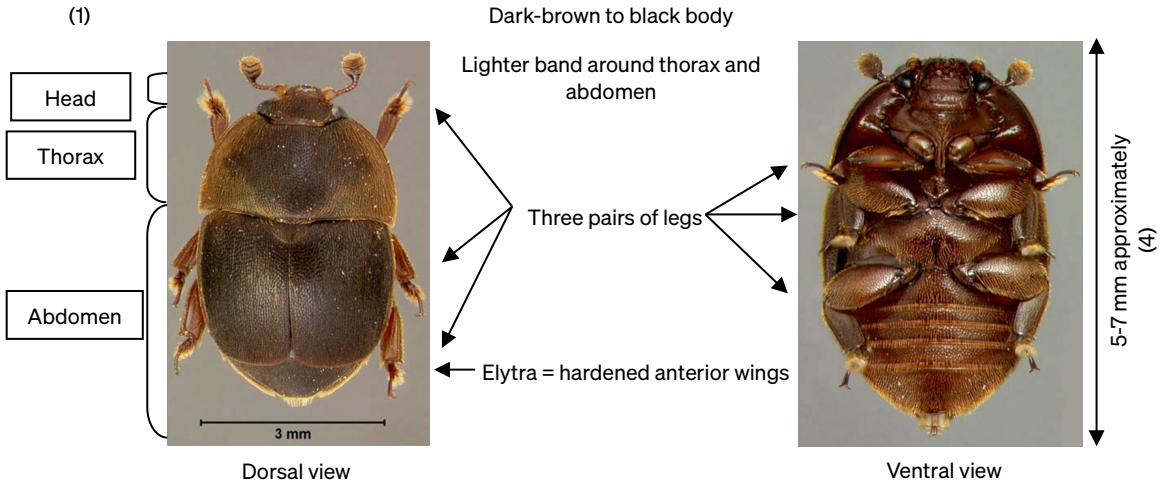

***Fig. 8. Small hive beetle,* Aethina tumida.**
***Photographs by Lyle Buss* (*left*) *and Josephine Ratikan* (*right*),**
University of Florida.

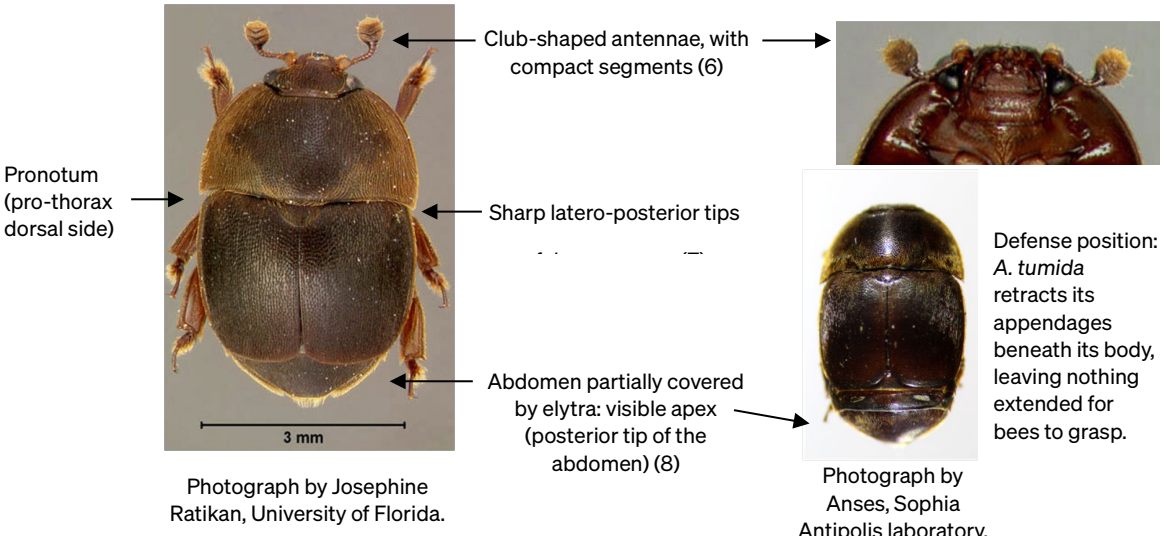

Fig. 9. Small hive beetle,* Aethina tumida *Murray.

For differential diagnosis, *Cychramus luteus* is shown below with the following features (Figure 10; Neumann & Ritter, 2004):

elytra completely covers the abdominal apex;
antennal clubs are looser with detached segments;
latero-posterior tips of the pronotum are not sharp;
colour of the body is light-brown.

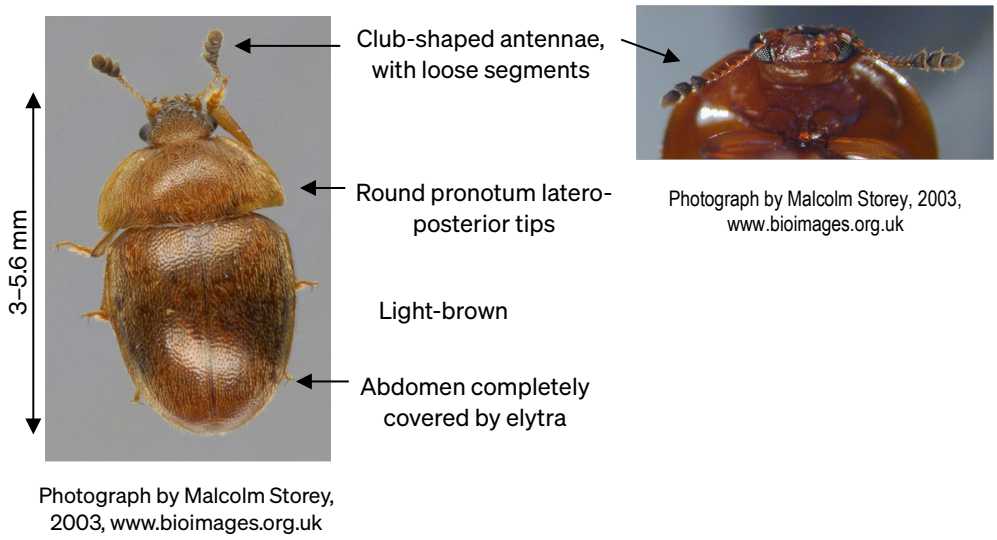

***Fig. 10.* Cychramus luteus (*Neumann & Ritter, 2004*).**

Carpophilus lugubris has the following characteristics: (Figure 11; Marini *et al.*, 2013):

body is brown;
elytra have orange regions;
legs and antennae are orange (antennal clubs are dark orange);
body length: 3.3–4.5 mm.
However, as for *A. tumida:*
elytra do not cover the entire abdomen;
club-shaped antennae have compact segments;
latero-posterior tips of the pronotum are sharp.

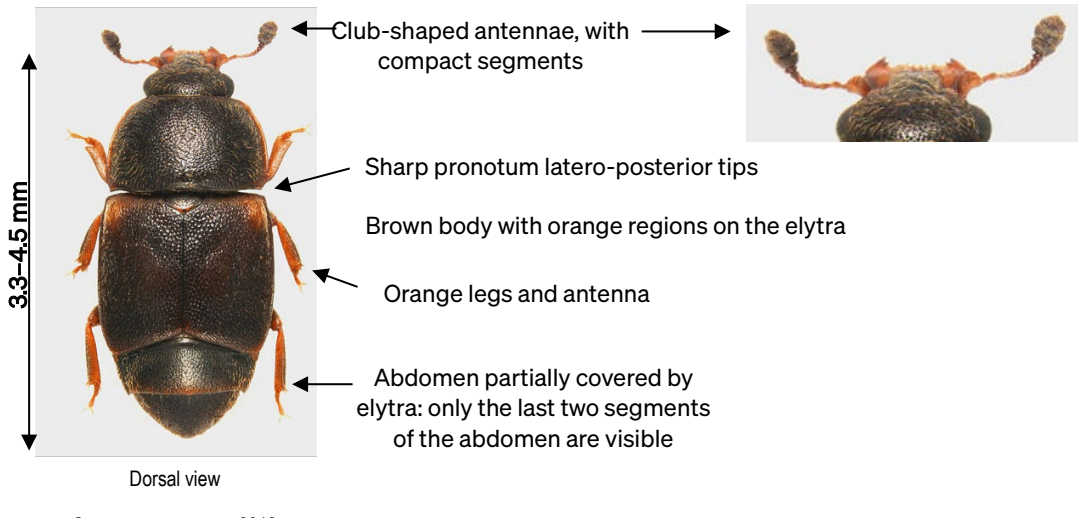

Fig. 11. Carpophilus lugubris* (*Marini* et al., *2013).

2.2.3.2. Larval form

Larvae of *A. tumida* have a light beige body. The cephalic capsule (head of the larva) is brown. The colour may change with environmental conditions and conservation of the specimens. The body length at maturity is about 1 cm (1.2 cm maximum) depending on feeding. The width is about 1.6 mm.

Larva identification is based on the following morphological criteria: (see Figure 12).

i) Three pairs of legs, one on each of the anterior (thoracic) segments

ii) Two dorsal spines on each segment (these spines are thicker on the last segment)

iii) No false legs (pseudopods or prolegs) on the ventral side of the posterior abdominal segments

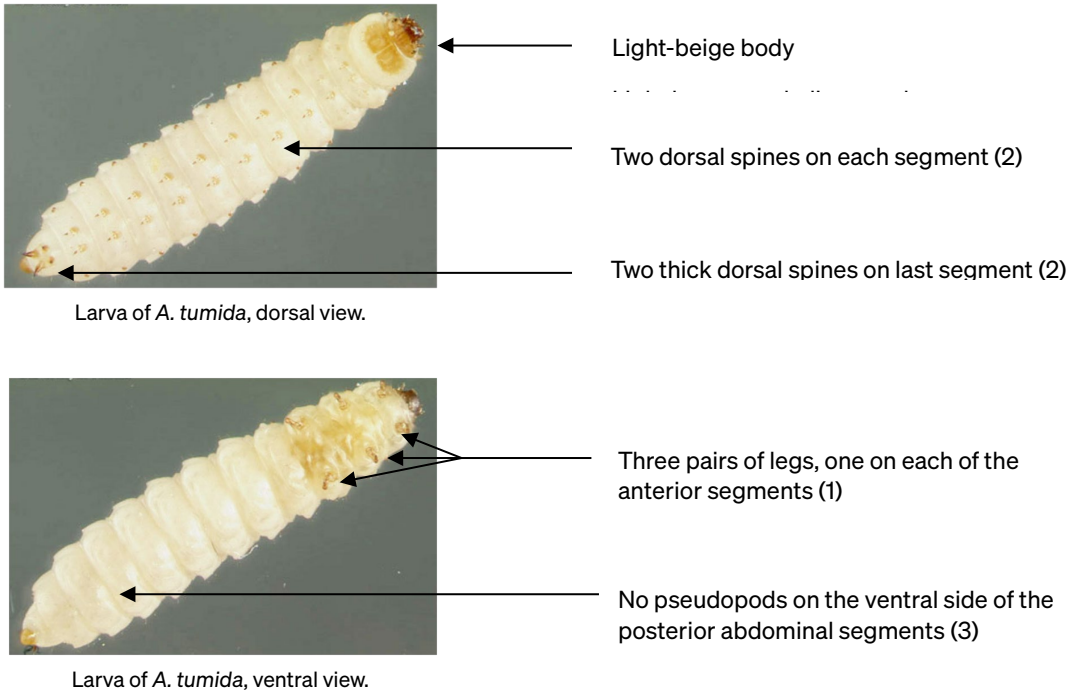

***Fig. 12. Larva of* Aethina tumida.**
Photographs by Josephine Ratikan, University of Florida.

To distinguish *A. tumida* larvae from Lepidoptera larvae (lesser wax moth, *A. grisella* and honeycomb/greater wax moth, *G. mellonella*), frequently present in honeybee hives:

The Lepidoptera larvae have pseudopods on the ventral side of the abdominal segments.

There are two bare segments between the last segment with legs and the first segment with pseudopods (Figure 13).

The Lepidoptera larvae can make a silky web, cocoons, and have dark faeces (these webs and faeces may be observed in the sample containers received by the laboratory).

2.2.4. Interpretation of results

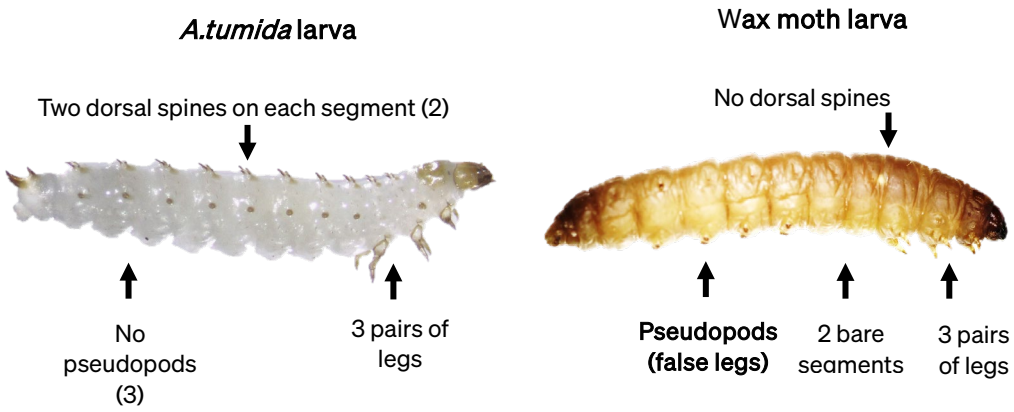

Fig. 13. Differentiation of* A. tumida *from wax moth larvae. Photographs by Nicolas Cougoule. Anses, Sophia Antipolis laboratory.

2.2.4.1. Adult form

i) If all the criteria 1 to 8 are confirmed for *A. tumida*, the result is "positive". The identification of *A. tumida* is confirmed. Confirmatory testing by PCR is advised.

ii) If certain fundamental morphological characteristics of *A. tumida* are not present (i.e. at least one out of the criteria 1 to 8), the result is "negative". The identification of *A. tumida* is not confirmed.

iii) Where definitive morphological criteria cannot be determined (e.g. damaged sample), the result is "inconclusive". Molecular identification is essential for confirmation.

2.2.4.2. Larval form

i) If all the criteria 1 to 3 are confirmed, the result is "*A. tumida* suspected". PCR testing is essential for final confirmation and confidence in the diagnosis.

ii) If at least one out of the criteria 1 to 3 is not confirmed, the result is "negative". The suspicion of *A. tumida* is not confirmed.

iii) Where definitive morphological criteria cannot be determined (e.g. damaged sample), the result is "inconclusive". Molecular identification is essential for confirmation.

2.3. Molecular identification

The morphological identification of the small hive beetle is increasingly confirmed by molecular methods using real-time PCR, especially for the examination of larvae where morphology is less clear cut. The method of real-time PCR described below has been developed by Ward *et al.* (2007) and is based on the amplification of a partial sequence of the mitochondrial gene of *A. tumida* that encodes cytochrome oxidase I (COI). The primers SHB207F and SHB315R can amplify a fragment of 109 base pairs, specific to *A. tumida*. This fragment is visualised in real-time due to a 5'-labelled probe. To take into account the two haplotypes identified by bioinformatic analysis by Ward *et al.* (2007), the SHB207F primer includes a degenerate base in the position 228 (A/G) (Genbank No. AF227645). The method was validated in accordance with Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*.

2.3.1. Sample preparation, equipment and reagents

The samples are typically adults or larvae kept in >90% non-denatured alcohol, or kept dry. Alcohol-preserved specimens should be left on tissue paper allowing the alcohol to evaporate or rinsed three times in a large volume of phosphate buffer (50 ml tube, for example). The specimen is then transferred to a 1.5 ml microtube where it is ground manually using a disposable pellet pestle. The volume depends on the size of the sample (for example: one adult beetle in 1 ml; one larva: in 200 µl). Samples can be stored at ≤ –16°C.

A real-time PCR detection system and the associated data analysis software are required to perform the test. Several proprietary systems for real-time PCR are available. The method described below uses one such system, but the precise parameters of the method should be validated according to the system in use in a particular laboratory. Because of the high sensitivity of the method, appropriate measures are required to avoid DNA contamination. All materials and methods used for the test should comply with the standards set out in the Chapter 2.1.2 *Biotechnology in the diagnosis of infectious diseases*, including measures to prevent contamination of DNA in the specimen.

2.3.2. Preparation of reagents

The real-time PCR reaction mixture is usually provided as a ready to use 2× concentration. The manufacturer's instructions should be followed for use and storage. Working stock solutions for the primers and probe are prepared with nuclease-free TE buffer at the concentration of 20 µM and 50 µM respectively. The stock solutions are stored at –20°C and the probe should be protected from light. Single-use aliquots can be prepared to reduce the number of freeze–thawing cycles and to increase the shelf life of the primers and probes.

2.3.3. Real-time PCR test procedure

Primer/probe name	Sequence
SHB207F	5'-TCT-AAA-TAC-TAC-TTT-CTT-CGA-CCC-ATC-(A/G)-3'
SHB315R	5'-TCC-TGG-TAG-AAT-TAA-AAT-ATA-AAC-TTC-TGG-3'
SHB245T probe	5'-(6-FAM)-ATC-CAA-TCC-TAT-ACC-AAC-ACT-TAT-TTT-GAT-TCT-TCG-GAC-(TAMRA)-3'

Positive and negative extraction controls, as well as reagent controls, should be included in each PCR test. To minimise the risk of contamination by the positive control, a dilution resulting in a Ct value of about 30 should be used. A suitable control would be crushed *A. tumida* beetles diluted to 10 times the detection limit of the method (LD_{method}). Alternatively a plasmid containing the target sequence may be added diluted to 10 times the detection limit of the PCR (LD_{PCR}). For the negative extraction control, it is recommended to use the buffer used for crushing the specimens. An internal positive control (IPC) is highly recommended to check the absence of PCR inhibitor in the extract analysed.

Appropriate thermocycler conditions should be determined and validated for the equipment and reagents in use in the particular laboratory.

PCR reagent mixtures are added in a clean room (no pathogens or amplification products should be handled), for example:

	Final concentration	Volume for one tube (µl)
Nuclease free H_2O	/	4.1
Real-time PCR reaction mixture (2×)	1×	12.5
SHB207F (20 µM)	320 nM	0.4
SHB315R (20 µM)	320 nM	0.4
245 probe (50 µM)	100 nM	0.05
10× IPC Mix	1×	2.5
50× IPC DNA	0.1×	0.05
Mix total volume		20

Add 5 µl of the DNA template (unknown sample or plasmid DNA) or positive or negative control to the reagent mixture to a final volume of 25 µl. DNA samples are prepared and added to the PCR mix in a separate area.

The thermocycler programme will depend on the equipment used and the real-time PCR reaction mixture, for example:

Step	Cycle	Temperature (°C)	Time (minutes)
Polymerase activation	1	95	3:00
PCR	40	95	0:10
		60	0:30

2.3.4. Interpretation of results

The threshold for the analysis of the amplification curves (determined by the background noise associated with the detection system) is usually set according to the manufacturer's instructions for the software used. It can be performed on confirmed negative specimens (e.g. larvae of wax moth *Galleria melonella* or adult beetles of the genus *Meligethes*).

A result identifying *A. tumida* by real-time PCR is considered valid only if the positive extraction and PCR controls are positive (Ct ≤ 35) and if the negative extraction and PCR controls are negative (Ct = N/A).

A positive result is recorded for any sample with a Ct value <35. Negative results are for any sample with a Ct value >35 or which presents no Ct value. Samples giving negative results should be checked for the absence of PCR inhibitor in the extract analysed through the result of the IPC. PCR inhibitors can lead to false negative results. Inhibition may be overcome by dilution of the sample for example to 1/10.

3. Serological tests

Serological tests are not appropriate or relevant to bee colony infestations.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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NB: There are WOAH Reference Laboratories for infestation with *Aethina tumida* (small hive beetle) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for infestation with *Aethina tumida* (small hive beetle)

NB: FIRST ADOPTED IN 2008. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.2.6.

INFESTATION OF HONEY BEES WITH *TROPILAELAPS* SPP.

SUMMARY

***Description and importance of the disease**: The mites in the genus* Tropilaelaps *are parasites of honey bee brood. Feeding on bee larvae and pupae causes brood malformation, death of bees and subsequent colony decline or absconding. Development requires about 1 week, and the mites are dispersed on bees. There are at least four species in the genus* Tropilaelaps. *Each species tends to be associated with a particular giant honey bee in Asia. Two species* (T. clareae *and* T. mercedesae) *are damaging pests of* Apis mellifera. *The other two species* (T. koenigerum *and* T. thaii) *appear to be harmless to* A. mellifera.

***Identification of the agent:** Molecular and morphological methods are available for identifying each species. An infestation by* Tropilaelaps *can be recognised either visually on bees or by examining hive debris. Irregular brood pattern, dead or malformed immatures, bees with malformed wings that crawl at the hive's entrance, and especially the presence of fast-running, red-brown, elongated mites on the combs, are diagnostic for the presence of* T. clareae *and/or* T. mercedesae. *An early diagnosis can be made after opening brood cells and finding immature and adult mites therein. The hive* (*colony*) *may be treated with various chemicals that cause the mites to drop off combs and bees. Sticky boards on the bottom of the colony can be used to examine hive debris and mites. Alternatively the "bump test" can be used for rapid screening. Definitive diagnosis at the laboratory is based on morphological examination under a microscope. Confirmatory testing can be done by conventional polymerase chain reaction and sequencing.*

***Serological tests:** Serological tests are not applicable.*

***Requirements for vaccines:** No vaccines are available.*

A. INTRODUCTION

Tropilaelaps spp. mites belong to the class *Arachnida*, subclass *Acari*, superorder *Parasitiformes*, order *Mesostigmata* and family *Laelapidae* (Anderson & Roberts, 2013). They should not be confused with the mite *Varroa destructor*, a parasite that is well-established in Europe. *Tropilaelaps clareae* occurs in Asia where it is a parasite of the native honey bee *Apis dorsata breviligula*. It is also a parasite of the introduced honey bee species *A. mellifera* in the Philippines and the native honey bee species *A. dorsata binghami* on Sulawesi Island in Indonesia. *Tropilaelaps mercedesae*, which was formerly mistaken for *T. clareae*, together with *T. koenigerum*, are parasites of the native *A. dorsata dorsata* in mainland Asia and Indonesia (except Sulawesi Island). *Tropilaelaps mercedesae* is also a parasite of the introduced *A. mellifera* in these and surrounding regions and, with another species, *T. thaii*, also parasitises *A. laboriosa* in mountainous Himalayan regions (Anderson & Morgan, 2007).

1. Life cycle

The colonising *Tropilaelaps* female (or females; as many as a dozen may occur within a single cell) lays from one to four eggs on a mature bee larva shortly before the brood cell is capped. The drone brood is preferred by *Tropilaelaps* and may be almost 100% parasitised (Burgett *et al.*, 1983). The mite progeny, usually one male and several females feed on and seriously damage the bee brood. Development of the mite requires about 1 week. The adults, including the foundress female, emerge with the adult bee and search for new hosts (de Guzman *et al.*, 2017).

The short life-cycle, as well as a very brief stay on adult bees, explains why populations of *T. clareae* increase faster than those of *Varroa* mites. When both *T. clareae* and *Varroa destructor* infest the same colony, the former may outcompete the *Varroa* mite (Burgett *et al.,* 1983; Ritter & Schneider-Ritter, 1988). It has been reported that when both mite species are in the same cell, the reproduction of both mites declines (Rath *et al*., 1995).

Phoretic survival on bees is quite short (only 1–2 days) because *Tropilaelaps* cannot pierce the integument of adult bees. The phoretic time for *Tropilaelaps* spp. is important in understanding the life cycle, and recent research suggests the period can be as long as 5–10 days (Wilde, 2000a; 2000b). Gravid female mites will die within 2 days unless they deposit their eggs (Woyke, 1987).

Like *Varroa*, *Tropilaelaps* can act as a potential vector for honey bee viruses, such as deformed wing virus (DWV) (Forsgren *et al.*, 2009). DWV has been reported to replicate in *T. mercedesae,* suggesting that the mite may act as a biological vector of DWV (Dainat *et al*., 2009). The impact of the mite–virus complex is not fully understood. Some data indicate that the major impact of *Tropilaelaps* infestation could be caused by the mite itself, reducing bee host immune responses (Khongphinitbunjong *et al*., 2015).

Infestation by *Tropilaelaps* causes the death of many bee larvae (up to 50%), resulting in an irregular brood pattern and of which the cadavers that may partially protrude from the cells. Many malformed bees occur, with distorted abdomens, stubby wings and deformed or missing legs, probably resulting from DWV associated infection. Some of the affected bees crawl at the hive's entrance (Atwal & Goyal, 1971). In addition, perforated cappings are seen, the result of sanitation activities by the worker bees, which evict the infested bee pupae or young adults. Some infested colonies abscond, carrying the mites to a new location.

The behavioral responses of honey bees to *T. mercedesae* depend on the *Apis* species. *A. cerana* and *A. dorsata* (the natural host of *T. mercedesae*) showed a higher behavioral resistance than *A. mellifera* (Khongphinitbunjong *et al*., 2012). In *A. mellifera*, *T. mercedesae* infestation significantly reduced honey bee lifespan and emergence weight; it also promoted DWV levels and associated clinical signs (Khongphinitbunjong *et al*., 2016) and could cause severe damage for colonies.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of infestation of honey bees with Tropilaelaps *spp. and their purpose*

Method	Purpose					
	Population freedom from infestation	Individual animal freedom from infestation prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infestation – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Morphology	+++	+++	+++	+++	+++	–
Conventional PCR	++	++	++	++	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction

1. Field detection of the mite

The first sign of an infestation by *Tropilaelaps* species is often the occurrence of red-brown, elongated mites on the combs or on adult bees (Figs. 2 and 3).

The body length depends on the species and varies between the male and the female. *Tropilaelaps koenigerum* is the smallest member of the genus with a body length of < 0.7 mm for females and ~0.575 mm for males. Female *T. mercedesae*, *T. clareae* and *T. thaii* are much longer at ~0.95–0.99 mm, ~0.87–0.885 mm and ~0.89 mm respectively, while the body lengths of male *T. mercedesae* and *T. clareae* are slightly smaller than their respective

females at 0.907–0.927 mm and 0.852–0.858 mm, respectively. Males of *T. thaii* have yet to be discovered (Anderson & Roberts, 2013).

Tropilaelaps can easily be separated from the *Varroa* mite using a ×10 magnifying glass. The body of the *Varroa* mite is wider than it is long and it moves slowly, whereas the body of *Tropilaelaps* is elongated (Fig. 1), and it is a fast-moving mite.

Tropilaelaps should also not be confused with other honey bee ectoparasites such as *Braula* flies, or other *Laelapidae* mites living in debris of honey bee hives such as *Mellitiphis alvearius* (Cook & Bowman, 1983) (Fig. 4) or the Ameroseiidae mite *Neocypholaelaps apicola* (Delfinado-Baker & Baker, 1983; Kontschán *et al.,* 2015).

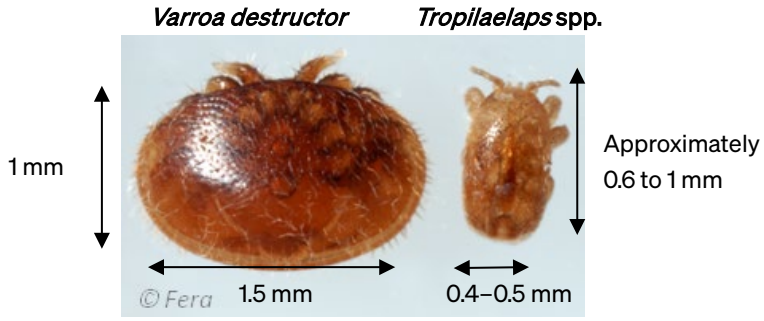


Fig. 1. **Varroa destructor** ***and*** **Tropilaelaps** ***spp.*** **(*dorsal view*).**
Photo supplied by APHA Bee Unit, York. UK Crown Copyright.

Fig. 2. **Tropilaelaps** ***on*** **Apis dorsata** ***larvae. Photo by D. Anderson.***

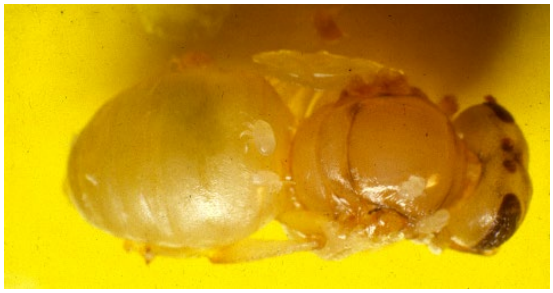


Fig. 3. **Tropilaelaps** ***offspring on*** **Apis mellifera** ***pupae. Photo by W. Ritter.***

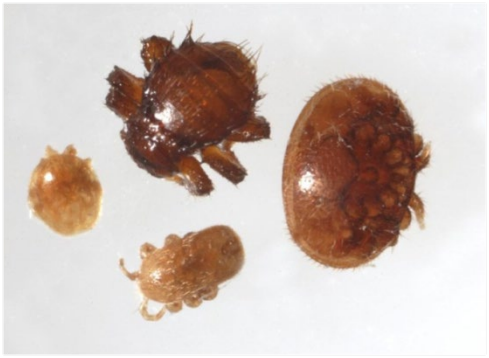


Fig. 4. **Braula coeca (*above*), Varroa destructor (*right*), Tropilaelaps *spp.* (*below centre) and* Melittiphis alvearius (*left*) (*dorsal view*).**
Photo supplied by APHA Bee Unit, York. UK Crown Copyright.

1.1. Mite collection

Methods to collect mites include an ether or sugar roll (Ritter & Schneider-Ritter, 1988). Collect approximately 100–200 bees in a wide-mouthed jar with lid. Scrape the bees into the jar or use a modified vacuum to suck them in. Knock the bees to the bottom of the jar with a sharp blow; there should be about a 2.5–5 cm layer of bees on the bottom. Remove the lid and spray a 2-second burst with ether starter fluid. Alternatively, use enough 70% alcohol or soapy water to cover the bees; or add around 25 g powdered sugar (or flour). If using ether replace the lid and agitate or roll the jar for about 10 seconds; mites should stick to walls. If using soap or alcohol, agitate and then strain out the bees with a coarse hardware cloth or mesh strainer; mites will be in the liquid. If using sugar or other powder, put screening material (such as hardware cloth) on top of the jar and shake the mites on to white paper to count; repeat every 2 minutes. For a more accurate count, finish with an alcohol or soapy water wash to collect all the mites.

1.2. Colony and brood examination

When monitoring honey bee colonies for the presence of *Tropilaelaps* (or *Varroa*), an examination of both drone and worker brood may provide an early indication of infestation. Mites can be observed inside capped bee brood by using a honey scratcher (with fork-like tines) to pull up capped pupae. The mites are clearly visible. The younger mite stages are whitish and may be almost motionless while feeding on their hosts' bodies, as their mouthparts and front legs are fixed to the cuticle of the bee host (Ritter & Schneider-Ritter, 1988). The extent of parasitisation can be estimated by opening a predetermined number of brood cells; infestation rates are then calculated as per cent of capped brood containing live mites (Burgett & Kitprasert, 1990).

1.3. Bump test

Another rapid and simple technique is the "bump test". The method consists of firmly rapping a honey bee brood frame over a collecting pan. First, all adult bees are removed from one comb containing capped brood by shaking the frame over the colony. Once adult bees are cleared away, frames are firmly bumped over a white metal pan by hitting one end of the frame on the side of the pan, turning the frame, re-bumping the frame, and repeating the process once more for a total of four bumps. This process dislodges mites on the surface of the comb, which then can be counted (Pettis *et al.*, 2013).

1.4. Sticky board examination

A precise diagnosis can be made using a sticky board covered with a mesh, such as fly screen, that prevents the bees from removing the dislodged mites. The mesh must be large enough for mites to pass through. Make a sticky board with poster board, cardboard or other white, stiff paper coated with petroleum jelly or other sticky substance (Koeniger *et al.,* 2002; Ostiguy & Sammataro, 2000; Sammataro *et al.,* 2000), or use a sheet of sticky shelf paper. Cut the paper to fit the bottom board of a hive. Cut a piece of hardware cloth or screen to fit on top of the sticky board. To keep the bees from cleaning off the board, fold under the outside edges of the screen to raise it off the board, and staple or tape in place. Leave the board in the colony for up to 3 days, collecting and examining the debris for mites. Acaricides are sometimes used to knock mites off bees and will appear on the sticky boards.

2. Laboratory identification of the mite

Rapid and reliable diagnosis is crucial to enable implementation of sanitary measures and to avoid spread in non-infested territories. In case of field suspicion, specimens suspected to belong to the genus *Tropilaelaps* should be sent to official laboratories to confirm the diagnosis. Morphological identification should be carried out for primary diagnosis. This method is fast and cheap, not requiring sophisticated equipment. Confirmatory testing may be done using polymerase chain reaction for molecular identification of *Tropilaelaps* species.

2.1. Special precautions required for sampling

The specimens to be identified are collected in honey bee hives, for example, in colonies, in batches of bees, on queen bees) or on bumble bees.

Suspect specimens should be killed before submission to the laboratory e.g. in 70% ethanol. Denatured ethanol should not be used where molecular methods are to be used because of possible inhibition of the polymerase chain reaction (PCR). Alternatively, samples can be stored overnight at –20°C to kill the specimens.

On arrival at the laboratory, packages should be opened in containment conditions. If the specimens are found to be alive on arrival, the submission should be placed at –80°C for approximately one hour before opening fully. This procedure immobilises the specimens, which can subsequently be stored in 70% ethanol.

2.2. Morphological identification of *Tropilaelaps* spp.

The method is based on the visual examination of adult mites only, taking into account the morphological characteristics of the adult *Tropilaelaps* mite compared with those of other mite genera commonly found in bee colonies (particularly *V. destructor*). The visual examination described is not sufficient to differentiate amongst the four species of *Tropilaelaps* as they are morphologically very similar (Anderson & Morgan, 2007; Tangjingjai *et al*., 2003).

2.2.1. Equipment and reagents

Classical entomological equipment and materials are required:

- Stereomicroscope
- Compound microscope (1000×)
- Hot plate
- Dishes: glass Petri dishes, porcelain ceramic dishes, watch glass or similar
- Micro-dissecting needle holders equipped with minutien pins and with pins made of fishing line (with the extremity crushed in order to obtain a spoon-like shape)
- Fine-tipped tweezers
- Glass microscope slides (classic and concave) and cover slips
- Hermetically sealed vials
- Lactic acid
- Mounting medium (e.g. Hoyer's medium) and clear nail polish for the long-term conservation of microscopic slides
- Ethanol 70% (avoid denatured ethanol).

2.2.2. Test procedure

All the specimens are placed in a dish and checked for homogeneity using a stereomicroscope. If the specimens are not homogeneous, then each type present is examined separately. Samples for examination should be selected from undamaged mites. Samples are taken using fine-tipped tweezers or needle holders and placed in a dish for further study.

Under the stereomicroscope, the mites are checked for the three primary identification criteria of *Tropilaelaps* spp. (see Table 2 below). If none of the three criteria are met, then further microscopic examination is not pursued.

For microscopic examination, the soft tissues must be cleared to reveal the morphological characteristics. Deposit a few drops of lactic acid on a microscope slide (using concave slides for larger specimens). Place the selected specimens on the slide in lactic acid with the needle holders

(fishing line equipped) (or with extra-fine tweezers). Using two holders (minutien pin equipped), position the specimens so as to have a ventral view. Place a cover glass over the microscope slide without crushing the mite, avoiding the formation of air bubbles. If possible, carefully press on the cover glass with a tweezer in order to spread open the legs, which are usually curled up beneath the body. Place the slide on a heating plate at approximately 50°C) and wait for the lactic acid to have effect (approximately 30 minutes). NB: the liquid should not boil on the slide as this would destroy the specimen.

Examine the slide(s) under the compound microscope at 100×, 200×, and then 400× magnification in order to observe fully the various diagnostic criteria as detailed in Table 2. Comparative observations should be carried out with reference slides if available. The depth of field viewed may need to vary according to the thickness of the mite's body.

Specimens can be stored at room temperature in a hermetically sealed vial with 70% ethanol. Slides may be kept long term by mounting the mites in Hoyer's medium, allowing to dry for 2 weeks at 50°C, then sealing the cover slip with clear nail varnish. For further information on storage and mounting of mites see Dietemann *et al.* (2013).

2.2.3. Identification criteria for adult *Tropilaelaps* spp.

Tropilaelaps spp. are visible to the naked eye. It is approximately between 0.6 mm and 1.0 mm long and between 0.4 to 0.5 mm wide. *Tropilaelaps* is smaller than *V. destructor* (Figs. 1 and 4). If all the morphological characteristics of the adult mite are confirmed (criteria 1 to 9 in Table 2), the result is "positive" confirmation of the identification of *Tropilaelaps* genus. If one or more of the fundamental morphological characteristics (criteria 1 to 9) of *Tropilaelaps* spp. are not present the result is "negative" and the identification of *Tropilaelaps* genus is not confirmed. If the presence or absence of all nine criteria cannot be determined (e.g. due to a damaged sample), the result is "inconclusive" and molecular methods should be used for confirmation.

Table 2. Criteria for recognising* Tropilaelaps *spp.
(*Anderson & Roberts 2013; Delfinado & Baker 1961; Smiley 1991; University of Michigan 2014*).

The following features should be examined:

a) The stigmata are tracheal openings;

b) The coxa is the first leg segment and connects the leg and the body;

c) The peritremes are tubular structures running on from stigmata. They could have a role in respiration;

d) The tritosternum is a bristle-like Y-shaped sensory organ located caudally to the gnathosoma (the gnathosoma is the body part of *Acari* that includes the mouthparts and oral aperture);

e) Reticulated means that it has broken eggshell or fish scale pattern.

	Stereomicroscope	*Compound microscope*
1. *Tropilaelaps* has four pairs of legs. The first pair is vertically aligned, resembling antennae (Fig. 5). → Class *Arachnida*	X	
2. The body is unsegmented, with a single visible region, due to the fusion of the prosoma (the equivalent of the cephalothorax) and the opisthosoma (or abdomen) into a single mass (Fig. 5). → Subclass *Acari*	X	
3. The body is longer than wide (as opposed to *V. destructor*) (Figs. 1 and 4). The ratio of length to width is greater than 1.3.	X	
4. It has a pair of latero-ventral stigmata between coxa III and IV (Figs. 7). → Order Parasitiforms		400×
5. Presence of elongated peritremes (Fig.7). Presence of a tritosternum (Fig.7) (optional criterion, difficult to observe). → Suborder *Mesostigmata*		200×
6. Elongated epigynial plate, posteriorly rounded or sharp. Triangular-shaped ventrianal plate (Figs. 5 and 6). → *Laelapidae*		100×

		Stereomicroscope	*Compound microscope*
7.	Elongated epigynial plate, at least twice as long as the ventrianal plate (Figs. 5 and 6).		100× or 200×
8.	Reticulated sternal plate (Fig. 7).		400×
9.	Opisthosoma with coarse bristles, thick at the base, on the apical half of the ventral side (Figs. 5 and 6).		200×
Note: Criteria for distinguishing between males and females: the mobile digit of the male's chelicerae is filiform (spermodactyls) (Fig. 8). The epigynial plate is shorter in the male than in the female (Fig. 8). (Anderson & Morgan, 2007)			200×

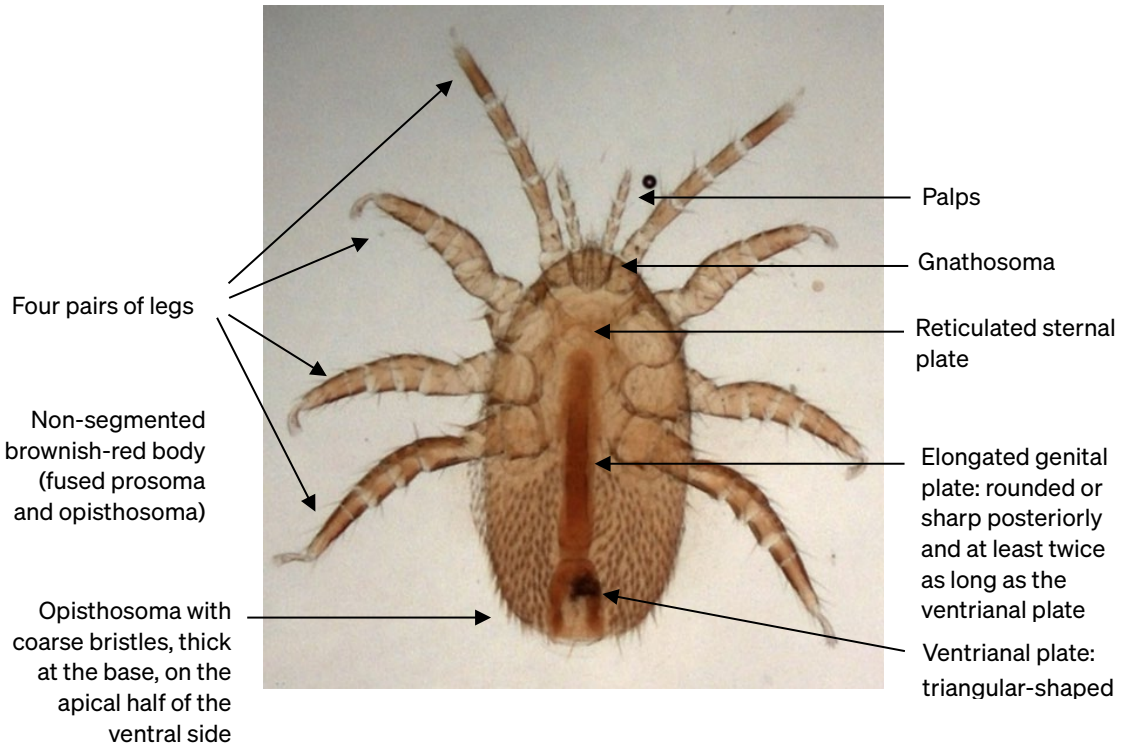
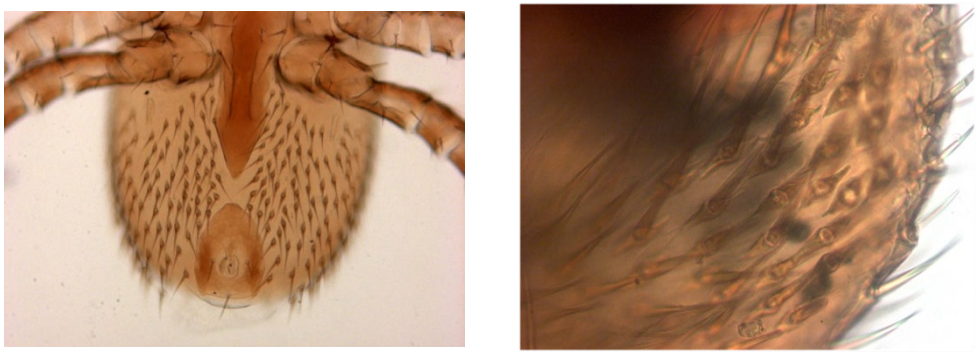


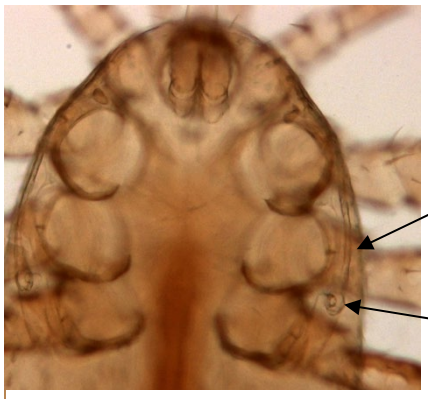
Fig. 5. **Tropilaelaps clareae,** ***female*** **(*ventral view*).**
Photo by S. Franco, Anses, Sophia Antipolis laboratory.



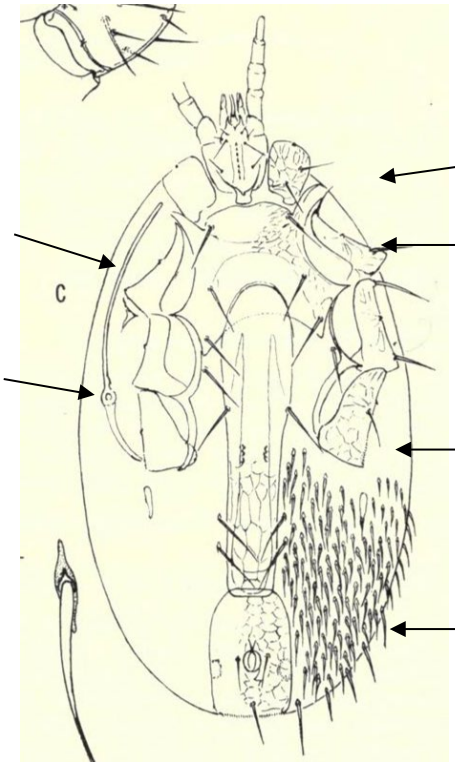
Magnification 100×

Magnification 400×

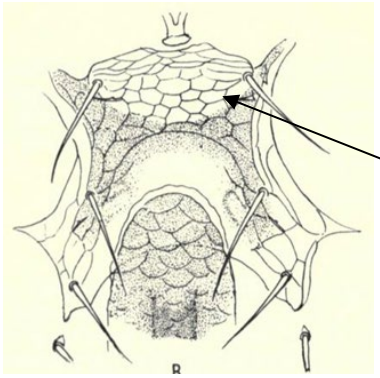
Fig. 6. Tropilaelaps sp. **(*ventral view*).** ***Opisthosoma, coarse apical bristles, thick at their base.***
Photos by S. Franco, Anses, Sophia Antipolis laboratory.



T. clareae – Magnification 100×

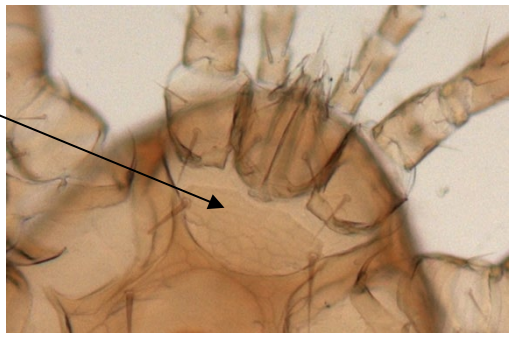


T. clareae, female (ventral view)



Sternal plate and epigynial plate (ventral view)

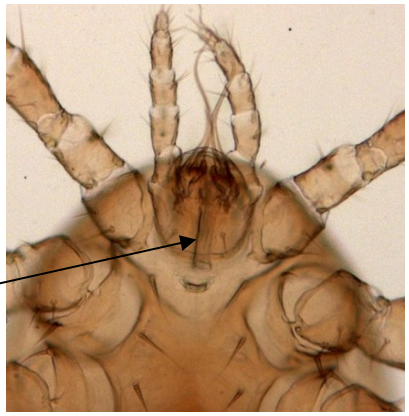
Reticulated sternal plate



T. clareae – Magnification 200×

Gnathosoma (ventral view)

Tritosternum



T. clareae – Magnification 200×

Fig. 7. **Tropilaelaps clareae,** ***anatomy.***
Source of the figures: Delfinado & Baker, 1961. Photos by S. Franco, Anses, Sophia Antipolis laboratory.

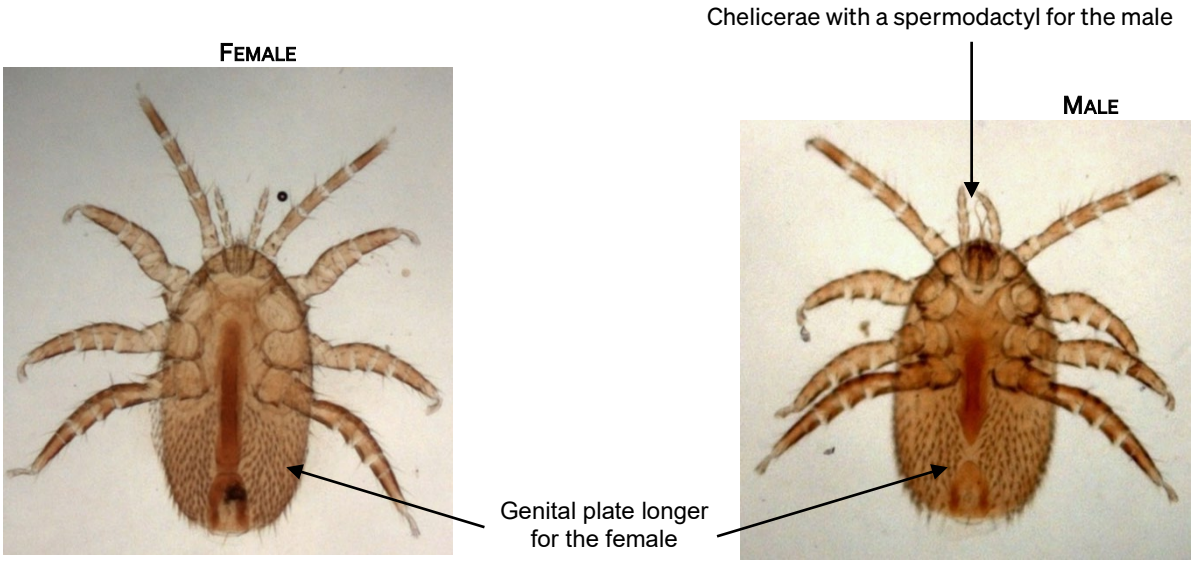


Fig. 8. **Tropilaelaps clareae***, male and female* **(***ventral view***).**
Photos by S. Franco, Anses, Sophia Antipolis laboratory.

2.3. Molecular identification

The morphological identification of *Tropilaelaps* spp. is complicated because of their resemblance to other mites that may be found in hives. PCR methods are increasingly used to confirm the suspicion of infestation. The conventional PCR method described below is based on the amplification of a partial sequence of the mitochondrial gene of *Tropilaelaps* spp. which encodes cytochrome oxidase I (COI) (Anderson & Morgan, 2007). The primers COI-TCF1 and COI-TCR2 amplify a fragment of 580 base pairs. The size of PCR products is determined by agarose gel electrophoresis in comparison with a DNA ladder (molecular weight marker). The primers are not specific to *Tropilaelaps* spp. and amplification of the COI gene from other parasites can occur, so it is necessary to sequence the DNA when a PCR product of the expected size is found.

2.3.1. Sample preparation, equipment and reagents

The samples tested are typically about 10 adult mites, kept in > 95% non-denatured alcohol or kept dry. If stored in alcohol they should first be rinsed three times in a large volume of phosphate buffer (50 ml/tube) or simply dry for several minutes on tissue paper before the DNA extraction step. The mites are then transferred to a 1.5 ml microtube. This step is important to avoid inhibition. The mites are ground in 200 µl phosphate buffer using a disposable pellet pestle in the 1.5 ml microtube. Ground samples can be stored frozen at ≤ –16°C.

A conventional PCR detection and analysis system is required. Any suitable methods or kits can be applied for the extraction of DNA, amplification in a thermocycler, followed by electrophoresis on agarose gel. All equipment and materials should be validated for use in the individual laboratory. The usual measures are required to avoid DNA contamination, and procedures should follow the standards set out in Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases.* Positive and negative controls must be used for all stages.

2.3.2. PCR procedure

The primers developed by Anderson & Morgan (2007) are as follows:

Name	Sequence
COI-TCF1	5'-CTATCCTCAATTATTGAAATAGGAAC-3'
COI-TCR2	5'-TAGCGGCTGTGAAATAGGCTCG-3'

The PCR reaction mixture should follow the manufacturer's instructions for use and storage. Working stock solutions for the primers are prepared with nuclease-free low ethylene diamine tetra-acetic acid (EDTA) TE buffer at the concentration of 20 µM. The stock solutions are stored at –20°C.

The PCR reaction mixtures are prepared in a separate laboratory room. All reagents, except the DNA samples, are mixed prior to distribution in each reaction tube. In each PCR test, appropriate controls must be included, including at least a template control (NTC, reagents only), negative controls (i.e. 1 per 10 samples tested) and a positive control (plasmid DNA solution including the sequence to be amplified, added to diluted 10 times the detection limit of the PCR [LD_{PCR}]). The amplifications are carried out in a total volume of 20 µl.

PCR reagent mixtures are added in a clean room (no pathogens or amplification products should be handled). The conditions above have been defined in the PCR validation steps. Other conditions could be applied after an optimisation.

	Final concentration	**Volume for one tube (µl)**
Nuclease free H_2O	/	12.6
Taq DNA pol (5 U/µl)	0.5 U/µl	0.2
Taq DNA pol buffer (10×)	1×	2.0
$MgCl_2$ (50 mM)	3.5 mM	1.3
dNTP mix (10 mM)	450 µM	0.9
COI-TCF1 (20 µM)	500 nM	0.5
COI-TCR2 (20 µM)	500 nM	0.5
Mix total volume		18

Add 2 µl of the DNA template (unknown sample or plasmid DNA) or negative control to the reagent mixture to a final volume of 20 µl. DNA samples are prepared and added to the PCR mix in a separate area.

An example thermocycler programme is as follows:

Step	**Cycle**	**Temperature (°C)**	**Time (minutes)**
Initial denaturation	1	95	5:00
PCR	35	94	0:30
		58	0:30
		72	0:45
Final extension	1	72	7
Hold		10	∞

Optimisation of PCR should be carried out according to the Mastermix and PCR machine used, especially testing with different annealing temperatures.

Detection of amplified products:

i) Prepare a 1.2% agarose gel in 1× TAE (Tris-acetate-EDTA) with the appropriate number of wells.

ii) 2 µl of 6× loading buffer are added to 10 µl of PCR products.

iii) Load 10 µl of the samples into the wells.

iv) To control the size of the amplified products, a 100 bp ladder is recommended.

v) Run the gel.

vi) Analyse by UV illumination after staining with a suitable DNA stain.

The interpretation of the results is based on the presence or absence of the amplified product: the size of the expected PCR product is 580 bp including the two primers. However, the presence of a PCR product of the right size is not sufficient to identify the *Tropilaelaps* genus and species. A sequencing step is required.

2.3.3. Sequencing of PCR products

If a 580 bp band is detected the PCR product must be sequenced. The method is not described here, and can be outsourced. A panel of COI sequences available on Genbank (EF025423 to EF025468 and HQ533148 to HQ533159 [Luo *et al.,* 2011]) is included in the analysis to construct the phylogenetic three and to identify the species of *Tropilaelaps*. An outgroup COI sequence from *Varroa* (EF025469, 253947435) is included.

3. Serological tests

Serological tests are not appropriate or relevant to bee colony infestations.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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*
* *

NB: There is a WOAH Reference Laboratory for infestation of honey bees with *Tropilaelaps* spp.
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratory for any further information on
diagnostic tests and reagents for infestation of honey bees with *Tropilaelaps* spp.

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.2.7.

VARROOSIS OF HONEY BEES (INFESTATION OF HONEY BEES WITH *VARROA* SPP.)

SUMMARY

The mite, Varroa destructor (*formerly identified as* Varroa jacobsoni), *is a parasite of honey bees. It feeds on the preimaginal host stages within the sealed brood cells and penetrates the intersegmental skin between the abdominal sclera of adult bees to ingest haemolymph and fat body tissues. While feeding, V.* destructor *transmits viruses – deformed wing virus, acute bee paralysis virus, Israeli acute paralysis virus and Kashmir bee virus, among others. Without treatment of the honey bee colony, the number of parasites steadily increases with the growth of the bee population and its increasing brood activity leading to the collapse of the colony within 1–4 years. The clinical signs of infestation that mainly occur late in the season, are an effect of virus infections rather than the effect of direct parasitism by the mite itself. The life span of the mite depends on temperature and humidity but, in practice, it can survive from some days to a few months.*

Detection of the agent: *There are three methods to collect* V. destructor *mites and quantify colony infestation levels: the examination of colony debris, of adult honey bees or of brood cells. Adult mites can be collected in the debris, which is all material originating from honey bee bodies or bee honeycombs that falls to the bottom of the colony where a removable board may be installed. Examination of the debris-can be undertaken after the application of a medication that kills the mites directly or that forces them to drop off the bees so that colony infestation can be measured, or it can be done without medication to quantify the natural mite mortality. Adult bees can be examined to detect and quantify the presence of mites, which provides information about the dispersal phase mite population. The examination of brood provides information about the reproductive mite population.* Varroa destructor *prefers drone to worker brood, therefore the infestation in drone brood might be higher compared with worker brood.*

Serological tests: *No serological tests are applicable.*

Requirements for vaccines: *No biological products are available.*

A. INTRODUCTION

Varroa mites are parasites of brood and adult honey bees (species of the genus *Apis*), originally infesting native Asian honey bees closely related to *Apis cerana* (Dietemann *et al*., 2013). Four obligate ectoparasitic species have been described: *Varroa jacobsoni, V. underwoodi, V. rindereri* and *V. destructor* (Figure 1). Two haplotypes of the species *V. destructor*, the Korean and the Japanese/Thailand haplotype, parasitise *Apis mellifera* (Figure 2; Dietemann *et al*., 2013). The Korean haplotype has spread worldwide, while the distribution of the Japanese/Thailand haplotype is more restricted, being only reported in Japan, Thailand and the Americas (Anderson & Trueman, 2000). In 2008, the species *V. jacobsoni* was also found for the first time parasitising *A. mellifera* in the Pacific island country of Papua New Guinea, where it has been extensively damaging honey bee colonies (Roberts *et al*., 2015). The recent host shift presents a serious threat to world apiculture. It should be mentioned that until 2000 *Varroa* mites that affect *A. mellifera* were erroneously assumed to be *V. jacobsoni* (Anderson & Trueman, 2000).

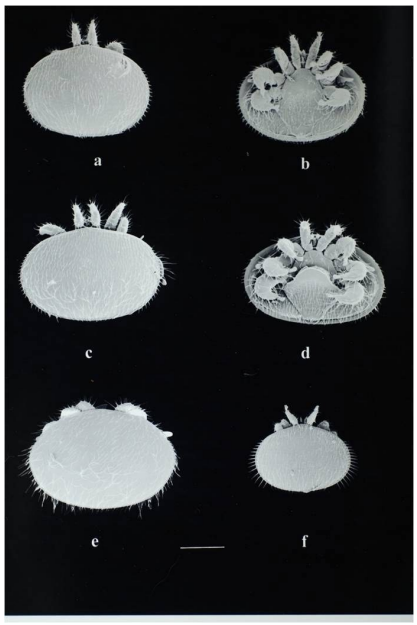

***Fig. 1. The four species of* Varroa: V. jacobsoni (*dorsal view: a, ventral view: b*); V. destructor (*dorsal view: c, ventral view: d*); V. rindereri (*e*); V. underwoodi (*f*).**
***Photograph by Denis Anderson* (*Anderson & Trueman, 2000; Dietemann et al., 2013*).**

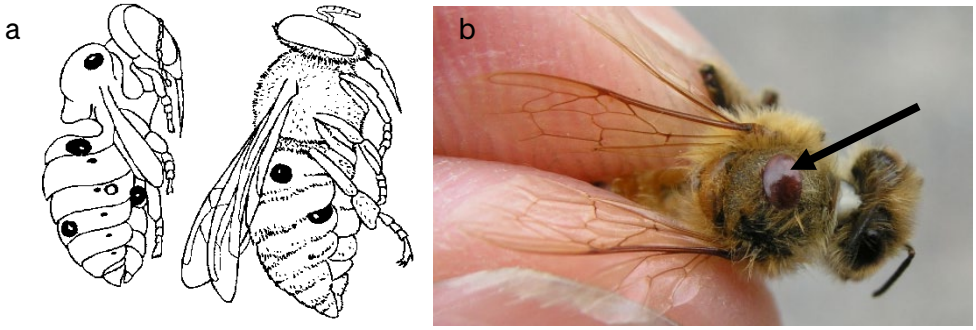

Fig. 2 a) Varroa on pupa and adult bee. Left: pupa with four Varroa female mites. Right: worker bee with two female mites.
b) Varroa (arrow) on* A. mellifera*. Photograph by Dr Rob Manning.

Varroa destructor has spread outside its native range since the 1960s, colonising other areas for the first time after it successfully shifted from the original host, *A. cerana,* to the Western honey bee, *A. mellifera* (Rosenkranz *et al*., 2010), to which it is now highly adapted. Decades later, *V. destructor* is present in most countries exploiting *A. mellifera* and only very few mite-resistant honey bee populations exist worldwide (Locke, 2016). Varroosis, also called varroatosis, is currently considered the largest threat to apiculture worldwide. Traditionally this disease has been defined as the infestation of honey bees with *Varroa* spp. However, due to the growing knowledge of viruses vectored by *V. destructor* and their confirmed important role in *Varroa*-induced colony collapse, this definition is no longer representative of the full process of the disease in *A. mellifera* (Genersch *et al*., 2010; Rosenkranz *et al*., 2010). An accurate and globally agreed definition of varroosis in *A. mellifera* is in progress among the scientific community, with efforts put into clarifying the precise role of *V. destructor* itself and the role of the different viruses carried by the mite, and thereby contextualising their relative importance in the complex of clinical signs observed. Until then, for the purposes of this *Terrestrial Manual* chapter, varroosis is linked to the detection of *Varroa* spp., regardless of the occurrence of clinical signs (see Section B. *Diagnostic techniques*).

There are more than 20 known viruses identified in honey bees, and it has been proven that *V. destructor* can act as a vector for deformed wing virus (DWV), acute bee paralysis virus (ABPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV), among others (Yañez *et al.,* 2020). Most of these honey bee viruses are positive-strand RNA viruses belonging to the Order Picornavirales (picorna-like viruses). Within this Order, DWV belongs to the Family *Iflaviridae,* and ABPV, KBV and IAPV to the Family *Dicistroviridae* (McMahon *et al*., 2018). Among all the viruses mentioned, currently the DWV is most closely associated with *Varroa* infestation (McMenamin & Genersch, 2015): it is well adapted to the life cycle of the mite (Di Prisco *et al*., 2016) and the only case where the role of *V. destructor* as

a biological vector has been proven, with effective replication of the virus in mite tissues (Yue & Genersch, 2005). Before the occurrence of *V. destructor*, honey bee viruses had been considered a minor problem to honey bee health, mainly present as subclinical infections, but since the dispersion of the mite they have been involved in large colony losses worldwide, exhibiting a markedly increased virulence (McMenamin & Genersch, 2015; Meixner *et al*., 2014). This is not surprising considering that the direct injection of the virus through mite bites is far more efficient than any of the other transmission routes, requiring fewer viral particles to establish infection, and also generating higher virus titres in affected honey bees (Brettell *et al*., 2017). At the same time, the level of infestation of *V. destructor* that causes colony damage appears to have decreased over time (fewer mites cause the same level of damage at the colony level than in the past). Another factor that points to the synergistic action of both pathogens is that the mite can trigger the replication of latent viruses already present in the honey bees, acting as an activator of endogenous viral infections (Di Prisco *et al*., 2016).

1. Life cycle, population dynamics and clinical signs

Varroa destructor lacks a free-living, bee-independent stage (Genersch, 2010). Instead, the life cycle of female mites consists of two distinct phases: the dispersal phase, where *V. destructor* parasitises adult bees, taking the opportunity to use them as a transport vehicle within the colony or between colonies, and the reproductive phase, where the mites parasitise drone or worker larvae just before cell-capping and reproduce within the sealed drone and worker brood cells (Figure 3). Male mites are short lived and can only be found inside the sealed brood cells during the reproductive phase (Rosenkranz *et al.,* 2010).

In the dispersal phase (Traynor *et al*., 2020) *V. destructor* usually inserts itself between the abdominal sternites in adult bees (Nazzi & Le Conte, 2016; Ritter, 1980) where it penetrates the intersegmental membranes in order to ingest haemolymph and fat body tissues (Ramsey *et al*., 2019). During this process the mite may acquire viral particles from covertly infected bees, and then, when it parasitises the next bee or larvae, it may directly inject these viruses into the haemocoel of the host (McMenamin & Genersch, 2015). Sometimes, *V. destructor* can also be found between the head and thorax or between the thorax and abdomen.

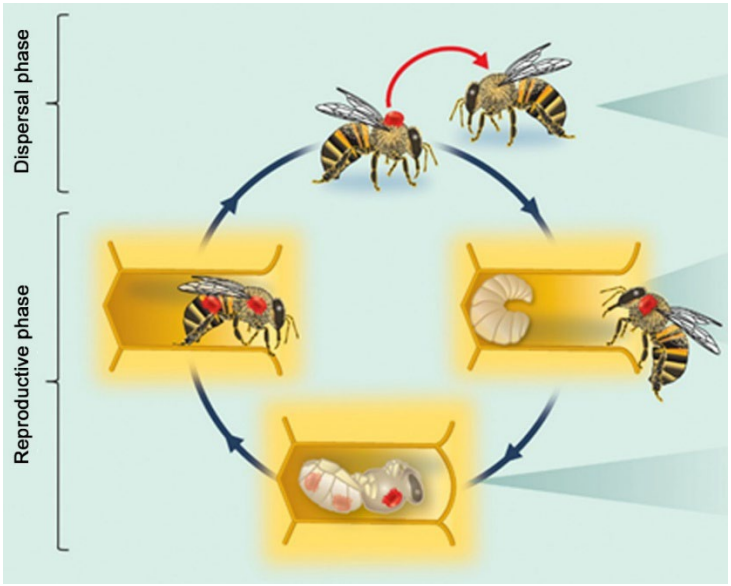

***Fig. 3. Simplified life cycle of* V. destructor*, with the dispersal and reproductive phases clearly shown (Nazzi & Le Conte, 2016*).**

The reproductive phase starts when the female mite, guided by specific cues, leaves the adult bee host to invade a suitable brood cell just before capping. *Varroa destructor* prefers drone brood over worker brood (Fuchs, 1992). Factors that support this selection are, among others: (1) the longer duration of the invasion period (Boot *et al*., 1992), (2) the more frequent and intensive tending of the drone larvae by nurse bees (Calderone & Kuenen, 2003), thereby improving incrementally the chances of *V. destructor* to reach the suitable host cell, and (3) more attractive chemical cues (Le Conte *et al*., 1989). Mites are rarely found in queen cells, which might be repellent to them (Calderone *et al*., 2002).

After the invasion of the brood cell, the female *Varroa* stay immobile at the bottom of the cell within the larval food. Once the cell has been sealed and the larvae have consumed the rest of the food, the female mite starts feeding on the bee larvae and initiates oogenesis within 26 hours for the females, 30 hours for males. Specific host signals trigger egg laying in the *Varroa* female, starting around 3 days after the capping, generally with a male egg

(unfertilised) and followed by up to five or six female eggs (fertilised) in 30-hour intervals. The offspring mites hatch a few hours after oviposition and pass through proto- and deutonymph stages until they become sexually mature: after approximately 5.8 days for females and 6.6 days for males (Rosenkranz *et al.,* 2010). During this time they repeatedly consume haemolymph and fat body tissues from the pupae developing in the cell at the same feeding site. As soon as the first female reaches sexual maturity the male mates with it, triggered by female sex pheromones, until the next female is mature. As the entire process occurs in the capped brood cell, the duration of the post-capping period is the limiting factor that determines the number of emerging and mated female mites. Two to three mated daughter mites may develop in a single infested drone brood cell, and one to two in a worker bee cell. Once the bee has completed its development and hatches, the mated daughter mites along with the mother mite, leave the cell with the emerged bee, whereas immature daughter mites and the male mite will die. The female adult mites can be transmitted between individual honey bees within the same colony or might even be spread to a new host colony through foraging and drifting workers, searching for suitable brood cells to lay eggs to start a new generation. Mites prefer nurse bees to forager bees, both for the likelihood of later being taken to a brood cell and for avoiding the risks related to the outside activities of forager bees (Figure 4; Nazzi & Le Conte, 2016; Rosenkranz *et al.,* 2010).

Under field conditions the life span of *V. destructor* mites may vary from some days to a few months, depending on temperature and humidity, and two to three reproductive circles can be accomplished. The population growth of *Varroa* mites is highly variable and depends on traits of the host, the parasite and the environment (Meixner *et al.,* 2014). Features of the mites include reproductive capacity and life span; features of the host include genotype, brood availability, presence of drone brood, colony size, behaviour (effective grooming, hygienic behaviour, swarming and absconding); and finally environmental factors such as climate, nectar flow and the density of surrounding honey bee colonies as well as their health status play an important role in population dynamics. Despite this variety in population growth, the course of the disease in *A. mellifera* is usually lethal, except in some areas, such as tropical Latin America (Rosenkranz *et al.,* 2010) and some parts of Africa (Strauss *et al.,* 2013).

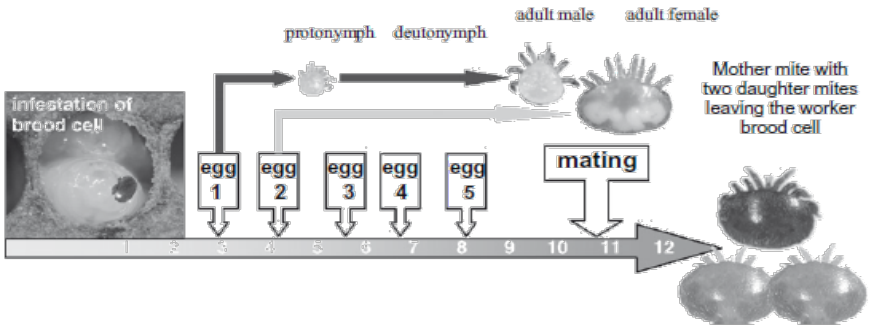

Fig. 4. Oviposition and development of* Varroa *in brood cells of worker bee (up to about day 9 in unsealed brood, up to about day 21 in sealed brood)
Reprinted from* J. Invertebr. Pathol.*, 103, Suppl. 1: S96–119, Rosenkranz P., Aumeier P. & Ziegelmann B., Biology and control of Varroa destructor, Copyright (2010), with permission from Elsevier.

Varroa destructor infested colonies of *A. mellifera* have been reported to die after 1–4 years if not systematically treated, although sometimes the process is faster and they can succumb in a few months, especially if nearby colonies are collapsing. Under temperate conditions, damage at the colony level mainly appears during autumn and winter when the host population declines, the relative parasitisation increases and consequently the long-living winter bees are damaged (Figure 5; Rosenkranz *et al*., 2010).

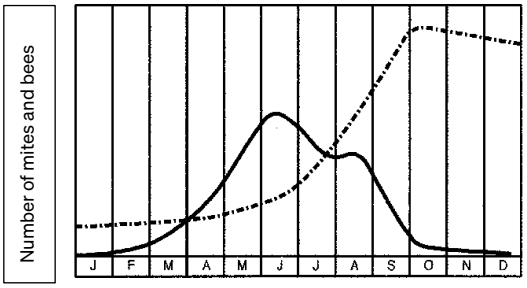

Fig. 5. Graph of populations of bees and mites over 1 year in a temperate Northern Hemisphere climate: brood numbers (solid line); mite numbers (broken line).

At the individual level, the direct pathogenic effects of the mite's feeding activities are: injuries in the cuticle, substantial depletion of haemolymph and fat body tissues and impairment of the immune system, the brood being the most sensitive host stage. Bees parasitised in the adult stage are only damaged if the infestation is severe. Parasitism during the host development results in a significant reduction in the size and weight of the hatching honey bee and increased disease susceptibility (De Jong *et al.,* 1982). Parasitised worker bees have a shorter life span, start foraging earlier and have a reduced capacity for non-associative learning, orientation and homing ability (Kralj & Fuchs, 2006). Parasitised drones have decreased flight performance and a shorter life span. Sperm production may be considerably reduced (Duay *et al.,* 2002).

At the colony level, low and moderate infestation rates often remain undetected, with clinical signs that may not be evident. Colonies have a reduced reproductive capacity however, as there is a reduced number of males available for mating, those that survive have a lower chance of succeeding, and infested colonies produce fewer swarms. There can also be a decreased growth in the honey bee population and, therefore, the honey yield can be diminished. Once a moderate infestation is reached, irreversible colony damage becomes more likely, especially in climates with a seasonal fall in temperate when the mite population still increases while the host population is decreasing (Figure 5). Brood care, social behaviour and worker bee tasks are negatively affected, leading to a weakening of the entire colony and eventually to its collapse (Le Conte *et al.,* 2010).

The clinical signs of a honey bee colony collapsing from varroosis include: high mortality at the hive entrance, rapid loss of the adult bee population, malnourished, crawling and crippled bees (with deformed wings and shortened abdomen due to the biological vectoring of DWV [figure 6]), direct visualisation of dispersal phase *Varroa* mites, scattered brood, brood cells with wax cappings fissured, sunken or partially removed, or with white patches on the cell wall (faecal accumulation site of mites), dead uncapped larvae and supersedure (replacement) of queens (Rosenkranz *et al.,* 2010).

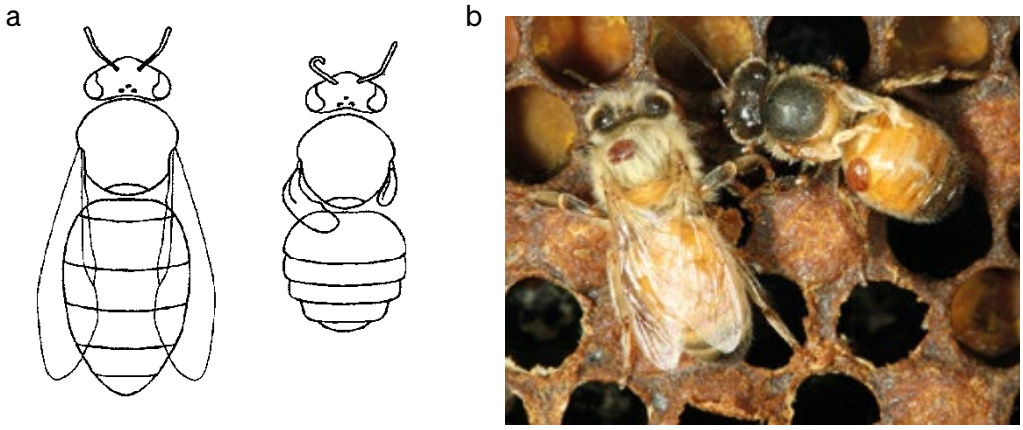

Fig. 6 a) Effect of* Varroa *on bee morphology. Left: normal bee appearance. Right: bee heavily attacked by mites. This newly emerged bee has a deformed wing and reduced abdominal volume. b) Photograph by Fanny Mondet, INRAE.

B. DIAGNOSTIC TECHNIQUES

As varroosis is established in many countries, diagnosis in these countries is not only focused on the presence or absence of *V. destructor* in honey bee colonies, but also on the mite density levels where the levels start to be damaging. Damage thresholds can be expressed in a variety of ways including (1) the infestation rate of adult bees (dispersal phase mites/100 bees), (2) daily natural mite fall and (3) total mites per colony (Dietemann *et al*., 2013). At the global level, the damage threshold of varroosis in *A. mellifera* cannot be established, and neither can a fixed infestation rate nor the number of mites per colony throughout the year. As is the case for mite population dynamics, damage thresholds are highly variable and depend on the interaction between genotype and environment together with beekeeping management practices and the time of year, resulting in substantial differences between regions (Rosenkranz *et al*., 2010). Nevertheless, in different regions of North America and Europe damage thresholds have been estimated: in the early beekeeping season it is considered to be 1–3% infestation rate of adult bees and 1–10 daily mites naturally fallen; and in the late beekeeping season the thresholds are considered to be 3–10% infestation rate of adult bees and 3000–4000 total mites per colony (Rosenkranz *et al*., 2010).

Table 1. Test methods available for the diagnosis of infestation of honey bees with Varroa spp. and their purpose

Method	Purpose					
	Population freedom from infestation	Individual animal freedom from infestation prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infestation – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Morphological identification	+++	+++	++	++	++	–
Detection methods	+++	+++	+++	+++	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.

1. Morphological identification of the agent

The predominant mite stage to be found are adult females, as they are the only ones that can survive outside the capped brood cells. The *V. destructor* female mite is a dark reddish/brown colour and has a flat, oval-shaped body approximately 1.1 mm in length × 1.5 mm in width × less than 0.5 mm in height, covered with short hairs (setae). The males are smaller than females, pear-shaped to triangular and white/light yellow in colour. Immature stages are also white/cream. All stages can be seen with the naked eye.

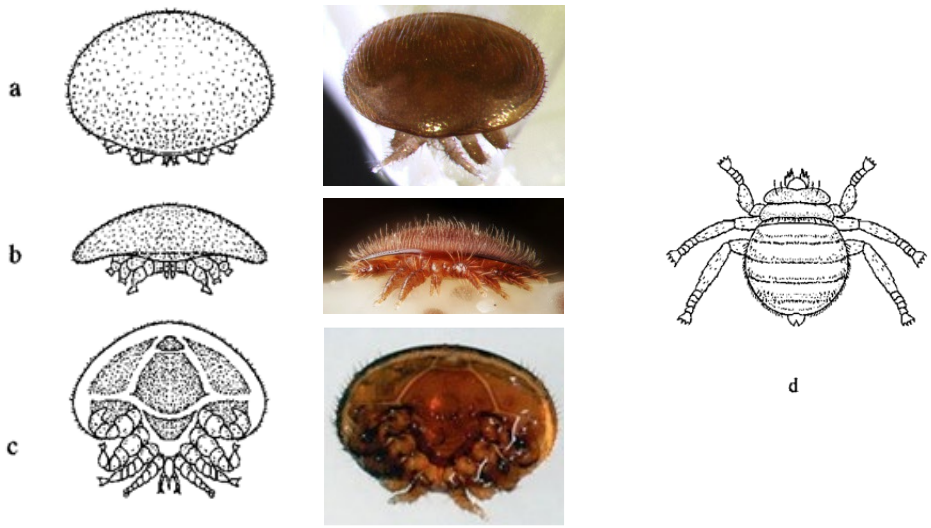

Fig. 7. Diagram of Varroa destructor (*formerly* Varroa jacobsoni *Oudemans*) (*female*).
a) Dorsal aspect. b) Anterior aspect. c) Ventral aspect. Note the flat shell-like back and four pairs of legs.
d) The bee louse (Braula coeca*, female*). *Note the lack of shell-like back and only three pairs of legs.*
First photo from M.O. Schäfer, Friedrich-Loeffler-Institut; Second photo from Gilles San Martin
(*https://aristabeeresearch.org/fr/varroa/*); *Third phot from National Bee Unit*
(*http://www.bee-craft.com/talking-about-varroa-2/*).

Varroa jacobsoni is morphologically close to *V. destructor*. It differs slightly in size, adult females of *V. destructor* being significantly larger and less spherical in shape than females of *V. jacobsoni*. However, as size criteria are difficult to appreciate, molecular techniques are advised for a confident differential diagnosis between *Varroa* species (Anderson & Fuchs, 1998).

Under some circumstances, the *Varroa* mite may be confused with the bee louse, *Braula coeca* (Figure 7). The latter is round, not oval, and being an insect, has only three pairs of legs. A number of different species of mite may be associated with *Varroa* mites on bees, but these are easily distinguished. In addition, other parasitic mites, such as those of the *Tropilaelaps* spp., are known to cause similar damage to bee colonies as the *Varroa* mites.

2. Detection of the agent

Three groups of different diagnostic techniques have been described: debris examination, bee examination and capped brood examination. Natural mite fall and adult and brood examination are only reliable for colonies with medium to high infestation rates with adequate amounts of brood (>3000 brood cells) that are not collapsing, whereas the debris examination after acaricide treatment with a product efficiency >95 % is reliable in all cases.

2.1. Debris examination

An easy method of diagnosis of varroosis is by examination of the debris generated by bees themselves, where fallen/dead mites can be found. This diagnostic technique can provide two different types of information: (1) natural mite fall and (2) mite mortality after acaricide treatment (Dietemann *et al*., 2013).

2.1.1 Natural mite fall

Apart from giving information about the natural death rate of the mite population, this non-invasive test can provide information about the grooming behaviour of the bees and, when brood is present, also about the mites detected and removed from infested brood cells by worker bees. Therefore, natural mite fall can provide information about dispersal and reproductive mite population. This technique is also recommended for monitoring activities.

i) Procedure:

Place a white or light-coloured covered insert with a screen mesh on the floor of the hive.

Considerations:

a) Arthropods such as ants must be prevented from accessing the insert, if not they will pillage the mites and thus bias the results (Dainat *et al.,* 2011). Protective measures include covering the insert with sticky material (e.g. grease, petroleum jelly, glue, gauze or impregnated absorbent paper). The sticky covering may also prevent live mites from climbing back into the hive.

b) The diameter of the screen mesh has to be small enough to avoid bees from reaching the insert and removing the mites, and big enough to allow the mites to fall through (3–4 mm).

ii) After a given time, count the mites present on the insert.

Considerations:

a) As there may be a large variety in the daily mite mortality, and given that many mites and a lot of debris makes counting difficult, the insert can be placed during 1–2 weeks to obtain a more reliable relationship between mite death rate and mite population. The counting and insert replacement frequency can be performed every 7 days or less, depending on local conditions. Consequently, the results can be expressed on a 24-hour basis.

b) A guide placed above the insert can be used for easier counting.

c) When mite fall is high, a subsample of the mites can be counted. One example to use in the field is a checker-board or sheet with a checked pattern to count mites from selected squares. In the laboratory another option is to examine large amounts of debris using a flotation procedure (see Section B.2.1.3).

2.1.2. Mortality after acaricide treatment

Procedure

i) Place a white or light-coloured insert covered with a screen mesh on the floor of the hive.

Considerations: The same as for step i) in Section B.2.1.1.

ii) Apply an acaricide with >95% efficiency.

iii) After a given time, count the mites present on the insert.

Considerations:

a) Given the rapid action of acaricides and to ease counting, mite counting frequency should be done every 3–7 days or less.

b) Depending on the acaricide applied, the length of the counting period differs:

1) If persistent acaricides unable to penetrate capped brood (i.e. most synthetic acaricides) and non-persistent acaricides capable of penetrating wax cappings (e.g. formic acid) are used, mite fall should be counted for 3 weeks. This period covers the dispersal phase mite mortality and the mortality during or after the capped stage (approximately 12–16 days) of developing bees. The results can be expressed on a 24-hour basis.

2) If non-persistent acaricides that do not penetrate capped brood are used, honey bee colonies should not have capped brood (either because of seasonal factors or because of management practices, such as caging the queen for 22 days before the treatment). As all mites are in the dispersal phase, the period of counting can be shorter. The results can be expressed on a 24-hour basis.

c) A guide and subsampling methods can also be used to facilitate counting, as well as the flotation procedure (see Section B.2.1.3).

Some countries demand the diagnostic application of certain medications to prove the absence of mites.

2.1.3. Flotation procedure

i) Dry the debris for 24 hours.

ii) Flood the debris with 70% industrial alcohol.

iii) Stir continuously for around 1 minute or, if debris contains wax or propolis particles, stir for 10–20 minutes.

iv) Identify and observe the mites that float to the surface.

2.2. Bee examination

In this second method three different effective tests can be used: alcohol wash, soapy wash and powdered sugar test. As adult bees are examined, this method provides information on the dispersal phase mite population. Samples are taken from uncapped brood frames, where bees have significantly more mites than brood-less frames (Rosenkranz *et al*., 2010).

It has to be noted that the infestation in one apiary can vary widely from other apiaries, even when all of them belong to the same beekeeper. Likewise, even in an apiary, the infestation can vary widely between colonies.

2.2.1. Alcohol wash test

This technique is invasive because bees quickly succumb when submerged in alcohol: 70–75% ethanol can be used.

Procedure:

i) Select any uncapped brood frame and check that the queen is not present. Hold the frame at approximately 10 degrees from the vertical.

ii) Take a sample of 300 bees using a jar (with a mark at the level of 100 ml of water, which is the volume occupied by 300 bees) by sliding it up and down so that the bees tumble in.

Considerations:

a) It is advised to verify that this volume (100 ml) corresponds to 300 bees for the particular subspecies of interest, as there are variations in size among *A. mellifera* subspecies.

iii) Rap the jar on a hard surface to be sure the bees are at the marked line; add or subtract bees as needed.

iv) Alcohol is added to the jar to cover the 300 bees, the lid is closed and the jar is shaken and moved in a circular motion for 1 minute to dislodge the mites.

Considerations:

a) In the laboratory a more precise method is by mechanical shaking for 30 minutes.

b) Instead of adding alcohol to the jar, the bees can be poured into a shallow dish or similar container filled with enough alcohol to cover them.

v) The content of the jar or container is then poured over a 3–4 mm sieve, which will trap the adult worker bees, into a clear plate or bowl. Check the jar or container for mites sticking to the sides.

Considerations:

a) Another option is to use a double sieve system, with the upper coarse sieve of 3–4 mm and the lower fine sieve of <0.5 mm, to collect all the mites, and then flushing the bees and mites with large amounts of warm water.

vi) The mites are collected and counted. The results can be expressed as a percentage of infestation, dividing the number of mites dislodged by the number of bees in the sample and then multiplying by 100.

2.2.2. Soapy wash test

As in the case of the alcohol wash test, this technique is invasive, killing the bees during the process. Detergents such as dish-washing soap can be used. To avoid too much foaming, which would make counting the mites difficult, low concentrated solutions are recommended, ranging from 0.2–1%.(1–5 ml of dish-washing soap in 500 ml of water). The procedure is the same as the alcohol wash test, with just two more considerations to take into account.

Procedure:

i) Select any uncapped brood frame and check that the queen is not present. Hold the frame at approximately 10 degrees from the vertical.

ii) Take a sample of 300 bees using a jar (with a mark at the level of 100 ml of water, which is the volume occupied by 300 bees) by sliding it up and down so that the bees tumble in.

Considerations:

a) It is advised to verify that this volume (100 ml) corresponds to 300 bees for the particular subspecies of interest, as there are variations in size among *A. mellifera* subspecies.

b) At the laboratory, the bees can be anaesthetised with ether, or by cooling at 4°C for 15 minutes or –18°C for 5 minutes, before undertaking the wash.

iii) Rap the jar on a hard surface to be sure the bees are at the marked line; add or subtract bees as needed.

iv) Detergent solution is added to the jar to cover the 300 bees, the lid is closed and the jar is shaken and moved in a circular motion for 1 minute to dislodge the mites.

Considerations:

a) In the laboratory a more precise method is by mechanical shaking for 30 minutes.

b) Instead of adding detergent solution to the jar, the bees can be poured into a shallow dish or similar container filled with enough detergent solution to cover them.

v) The content of the jar or container is then poured over a 3–4 mm sieve, which will trap the adult worker bees, into a clear plate or bowl. Check the jar or container for mites sticking to the sides

Considerations:

a) If there is too much soapy foam, extra water or alcohol can be added to eliminate it.

b) Another option is to use a double sieve system, with the upper coarse sieve of 3–4 mm and the lower fine sieve of <0.5 mm, to collect all the mites, and then flushing the bees and mites with large amounts of warm water.

vi) The mites are collected and counted. The results can be expressed as a percentage of infestation, dividing the number of mites dislodged by the number of bees in the sample and then multiplying by 100.

2.2.3. Powdered sugar test

This technique is not as invasive as the alcohol and soapy wash tests, and following it, bees can be placed back in their colony where they will be cleaned by the other bees. As well as its diagnostic use, this test is also recommended for monitoring activities, as is also the case for natural mite fall.

The principle of this technique is that dusting with powdered sugar will dislodge mites from their host, possibly because the sugar particles adhere to the ambulacra (distal segment of the mite legs), and thus affected mites lose their grip and fall off the bee. High humidity conditions decrease the efficacy of this test, as the powdered sugar collects moisture and clumps together. It is recommended to use fresh powdered sugar.

Procedure:

i) Select any uncapped brood frame and check that the queen is not present. Hold the frame at approximately 10 degrees from the vertical.

Considerations:

a) If greater precision ($C \leq 0.1$ or $h \leq 0.5$) is needed, sampling has to be done in three different brood frames, repeating the steps described below three times.

ii) Take a sample of 300 bees using a jar (with a mark at the level of 100 ml of water, which is the volume occupied by 300 bees) by sliding it up and down so that the bees tumble in.

Considerations:

a) It is advised to verify that this volume (100 ml) corresponds to 300 bees for the particular subspecies of interest, as there are variations in size among *A. mellifera* subspecies.

b) The lid of the jar must be modified: the centre part is replaced by a 2–3 mm hardware cloth or mesh.

c) To achieve a precision higher than $C \leq 0.1$ or $h \leq 0.5$, 900 bees in total have to be sampled.

iii) Rap the jar on a hard surface to be sure the bees are at the marked line, and add or subtract bees as needed. Once the volume is correct close the lid.

iv) Pour 1–2 table spoons (at least 7 g) of powdered sugar through the mesh or cloth, and roll the jar to cover all the bees with sugar

v) Let stand for 1 minute (in the shade in case of high temperatures so the bees do not overheat).

vi) Turn jar upside down over a clear plate or pan, and shake it for 1 minute at least, or until no more mites came out.

a) In the field, when wind may cause mites to be lost, the plate or pan can be filled with water.

vii) The mites are collected and counted. The results can be expressed as a percentage of infestation, dividing the number of mites by the number of bees in the sample and then multiplying by 100.

Considerations:

a) A drop of water can be added to the plate or pan to dissolve the powdered sugar and facilitate the counting, if it has not been previously filled with water.

viii) Reintroduce the sampled bees in the top of their colony or at the colony entrance.

2.3. Capped brood examination

For the third method, capped drone brood is examined, if available, otherwise capped worker brood is examined. As this technique is performed on the brood, it provides information about the reproductive mite population. It has to be taken into account that *V. destructor* prefers drone brood to worker brood, therefore the infestation rate in drones will be higher. This method is invasive – it will kill the sampled pupae – and is not always feasible in certain regions at certain times of year where brood may not be present (Rosenkranz *et al*., 2010).

Procedure:

i) Select 200 brood cells from the brood box.

Considerations:

a) Taking the samples from more than one brood frame will account for the spatially irregular infestation by *Varroa* mites. One option is to select four samples of 50 cells from different frames (dimensions of the cut: 25 × 25 mm: it contains approximately 50 worker cells or 40 drone cells).

ii) Examine them for the presence of mites.

Considerations: There are different methods:

a) Individual opening of each capped cell:

Examine each pupa and its cell, especially the bottom, for the presence of mites or their faeces (white spots). The results after can be expressed as:

1) Percentage of infested cells (dividing the number of infested cells by the total number of opened cells, and then multiplying by 100)

2) Total number of mites on capped brood (multiplying the average number of mites per pupa by the number of capped brood in the colony). The number of capped brood can be calculated using a grid.

b) Washing the brood over a double sieve system:

1) Cut out the selected sample/samples from the frame/frames

2) Remove the cappings of the brood cells with a knife

3) Wash the brood cells directly into a sieve system with warm water from a hand-held shower

4) Collect the mites in the lower fine sieve (mesh width < 0.5 mm) while the brood is gathered in the upper coarse sieve (mesh width 2–3 mm)

5) Place the contents of the sieve on a bright plate, where the mites can be easily identified and counted

6) The results after can be expressed as the average number of mites per cell.

2. Serological tests

No serological tests are available for routine laboratory diagnosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines available.

- **Acknowledgement**

Illustrations by Karl Weiss, extracted from Bienen-Pathologie, 1984. Reproduced with the kind permission of the author and Ehrenwirth-Verlag, Munich (Germany). Photographs by Dr Denis Anderson, Dr Rob Manning, Dr Nazzi, Dr Le Conte, Dr Rosenkranz, Dr Mondet, Dr Schäfer and Dr San Martin.

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* *

NB: There are WOAH Reference Laboratories for infestation of honey bees with *Varroa* spp. (varroosis) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for bee diseases

NB: FIRST ADOPTED IN 1989 AS VARROASIS. MOST RECENT UPDATES ADOPTED IN 2021.

SECTION 3.3.

AVES

CHAPTER 3.3.1.

AVIAN CHLAMYDIOSIS

SUMMARY

Description and importance of the disease: *Avian chlamydiosis* (*AC*) *is caused by a* Chlamydia *species in birds. The taxonomy of the family* Chlamydiaceae *was recently revisited. The genus* Chlamydia *currently includes 11 recognised species, and among them* C. psittaci, C. avium *and* C. gallinacea *have been isolated from birds.*

Outbreaks of AC in psittacine birds and domestic poultry farms cause considerable economic damage. The infection can lead to systemic and occasionally fatal disease in birds. The clinical signs are generally nonspecific and vary greatly in severity, depending on the species and age of the bird and the virulence of the Chlamydia *strain, but respiratory distress is mostly involved. Many birds, especially older psittacine birds and poultry, may show no clinical signs; nevertheless, they may often shed the agent for extended periods.*

Special laboratory handling as determined by biological risk analysis (*see Chapter 1.1.4* Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities) *is recommended and even obligatory in many countries because avian chlamydial strains can cause serious illness* (*pneumonia*) *and death in humans when left untreated.*

Identification of the agent: *The preferred method for the identification of AC is no longer isolation of the organism. Considering the time involved, the need for high-quality samples, the fact that some strains will never grow* in vitro *and the hazard to laboratory personnel, nucleic acid amplification tests* (*NAATs*) *are currently recommended for quick, sensitive and specific diagnosis. These methods include conventional and real-time polymerase chain reaction* (*PCR*)*, DNA microarray-based detection and DNA sequencing. Isolation, cytological staining of smears of exudate or faeces, and of impression smears of tissues, immunohistochemical staining of cytological and histological preparations and antigen-capture enzyme-linked immunosorbent assays* (*ELISA*) *can be used if NAATs are not available.*

Serological tests: *Serology alone is not particularly useful in diagnosing a current chlamydial infection in birds because of the high prevalence of this infection in birds and the long-term* (*up to several months*) *persistence of antichlamydial antibodies. In most bird species, there is a high background rate of antichlamydial antibodies. Thus, to determine if a single bird is infected, serology should always be used in conjunction with gene or antigen detection, or paired sera should be examined. A positive test is evidence that the bird was infected by the bacterium but does not necessarily indicate an active infection. False negative results can occur in birds with acute infections that are sampled before seroconversion. Treatment with antibiotics may also delay or diminish the antibody response.*

The main serological methods that are used for detecting chlamydial antibodies are: (*1*) *various methods of elementary body agglutination* (*EBA*)*,* (*2*) *the complement fixation test and* (*3*) *ELISA. ELISA is highly sensitive and specific when using recombinant proteins/peptides as antigen targets and it detects IgM, IgG and IgA.*

Requirements for vaccines: *There are no commercial vaccines available for chlamydiosis control in poultry.*

A. INTRODUCTION

Avian chlamydiosis (AC) is caused by infection with a *Chlamydia* species in birds. In 2015, the taxonomy of the family *Chlamydiaceae* was revisited by Sachse *et al*. (Sachse *et al*., 2015). The genus *Chlamydia* currently includes 11 recognised species, namely *C. abortus* (sheep, goats, cattle), *C. caviae* (guinea-pigs), *C. felis* (cats), *C. muridarum* (mouse, hamster), *C. psittaci* (birds and others), *C. pecorum* (sheep, cattle), *C. pneumonia* (human and others), *C. suis* (swine), *C. trachomatis* (human) and two recently established species isolated from birds, *C. avium* and *C. gallinacea* (Sachse *et al.,* 2014). While most of these organisms are highly host specific, *C. pneumonia* and *C. psittaci* have a broader host range. The latter has been reported to occur not only in birds and humans, but also in cattle, sheep, swine, horses and other animals.

Until very recently, *C. psittaci* was considered to be the sole causative agent of the disease in birds. Originally called psittacosis, the term ornithosis was introduced later to differentiate the disease in domestic and wild fowl from the disease in psittacine birds. The two syndromes are currently considered to be the same (Andersen & Vanrompay, 2008). Their earlier separation was based on the assumption that in humans, ornithosis was a milder disease than psittacosis. However, it should be noted that the disease in humans contracted from turkeys and ducks is often as severe as that contracted from psittacine birds.

Infection of birds with *C. psittaci* is common all over the world and has been found in about 465 avian species (Kaleta & Taday, 2003). Outbreaks of AC in psittacine birds and domestic poultry farms cause considerable economic damage. The infection can lead to systemic and occasionally fatal disease in birds. The clinical signs are generally nonspecific and vary greatly in severity, depending on the species and age of the bird and the chlamydia strain. AC can produce lethargy, hyperthermia, abnormal excretions, nasal and eye discharges, and reduced egg production. Mortality rates will vary greatly. In pet birds, the most frequent clinical signs are conjunctivitis, anorexia and weight loss, diarrhoea, yellowish droppings, sinusitis, biliverdinuria, nasal discharge, sneezing, lachrymation and respiratory distress. Many birds, especially older psittacine birds and poultry, may show no clinical signs; nevertheless, they may often shed the agent for extended periods. Necropsy of affected birds will often reveal multifocal hepatic necrosis, spleen and liver enlargement, fibrinous airsacculitis, pericarditis and peritonitis (Andersen & Vanrompay, 2008; Vanrompay, 2013). Histological lesions are suggestive of infection but are non-pathognomonic unless there are identifiable chlamydiae present.

Until recently, nine different genotypes based on the *omp*A gene coding for the major outer membrane protein (MOMP) were distinguished within *C. psittaci* strains. Seven of these genotypes are thought to occur predominantly in a particular order or class of Aves and two in non-avian hosts, i.e. genotype A in psittacine birds, B in pigeons, C in ducks and geese, D in turkeys, E in pigeons, ducks and others, E/B in ducks, turkeys and pigeons, F in parakeets, WC in cattle, and M56 in rodents. Most of the avian genotypes have also been identified sporadically in isolates from cases of zoonotic transmission to humans, particularly A, B and E/B (Heddema *et al.,* 2006; Vanrompay *et al.,* 2007). Meanwhile, subgroups for three of the more heterogeneous genotypes have been introduced, i.e. A-VS1, A-6BC, A-8455, EB-E30, EB-859, EB-KKCP, D-NJ1, D-9N, and provisional genotypes to cover the strains that were previously non-typable have been suggested (Sachse *et al.,* 2008).

Antibiotics are the only current means of control. *Chlamydia psittaci* is susceptible to a number of antibiotics: the drug of choice varies from country to country. Chlortetracycline, doxycycline, and other tetracyclines are the most commonly used. Treatment needs to be maintained for extended periods of time. For pet birds, 45 days is often recommended (Vanrompay, 2013).

Evidence suggests that other chlamydial species, such as *C. abortus*, *C. pecorum*, *C. trachomatis*, *C. suis* and *C. muridarum* can also be harboured by birds (Guo *et al*., 2016; Pantchev *et al*., 2009), as well as by the avian species *C. avium* and *C. gallinacea* described by Sachse *et al*., in 2014. Their epidemiological importance is still unclear, however *C. avium* and *C. gallinacea* appear to be quite widespread in pigeons and psittacines or poultry birds, respectively. The pathogenicity of these two newly introduced species has yet to be systematically investigated. In surveys reported to date, no clinical signs have been observed in chickens carrying *C. gallinacea* (Guo *et al*., 2016; Laroucau *et al*., 2009), nor in most of the *C. avium* carriers among pigeons. However, it seems likely from currently available data that *C. avium* is able to cause respiratory disease in parrots and pigeons (Sachse *et al*., 2014). It is now recommended to do a differential diagnosis and use diagnostic methods that are capable of differentiating between

C. psittaci and the other species that can be hosted by birds. To date only molecular methods can make this distinction.

1. Zoonotic risk and biosafety requirements

The strains of avian chlamydiae can infect humans and should be handled with appropriate biosafety and containment procedures (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Risk assessment and management are essential when performing diagnosis of AC. Adequate information, communication, and health surveillance by an occupational physician are recommended.

Most infections occur through inhalation of infectious aerosols. While the disease from psittacine birds is best known, the infection in poultry is of particular concern as transmission to humans is common during handling and slaughter of the birds (Dickx *et al.,* 2010; Lagae *et al*., 2014). Post-mortem examinations of infected birds and handling of cultures should be done in certified Class II laminar flow hoods whenever possible or with proper protective equipment. Appropriate zoonotic agent decontamination procedures should be followed because human infection can result from transient exposures. The incubation period is usually 5–14 days; however, longer incubation periods are known. Human infections vary from inapparent to severe systemic disease with interstitial pneumonia and encephalitis. The disease is rarely fatal in properly treated patients; therefore, awareness of the danger and early diagnosis are important. Infected humans typically develop headache, chills, malaise and myalgia, with or without signs of respiratory involvement. Pulmonary involvement is common. Auscultatory findings, however, may appear to be normal or to underestimate the extent of involvement. Diagnosis can be difficult and in the past was usually established through testing paired sera for antibodies to chlamydia by the complement fixation test (CFT). However, some patients remain seronegative though hospitalised with psittacosis; serology is therefore being replaced by nucleic acid amplification tests (NAATs), which also allow the bird source to be traced. In humans, tetracycline, doxycycline, or azithromycin are usually the drugs of choice unless contraindicated. The length of treatment will vary with the drug, but should be continued for at least 14 days for tetracycline.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian chlamydiosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Conventional PCR	–	–	–	++	+	–
Real-time PCR	–	–	–	+++	++	–
DNA microarray	–	–	–	++	+	–
Cytological staining	–	–	–	+	–	–
Isolation in cell culture or embryonated eggs	–	–	–	++	+	–
IHC on fixed tissue	–	–	–	++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
CFT	+	+	–	+	+	–
ELISA	++	+	–	+	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; IHC = immunohistochemistry; CFT = complement fixation test;
ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

The preferred method for the identification of AC is no longer isolation of the organism. Considering the time involved, the need for high-quality samples, the fact that some strains will never grow *in vitro* and the hazard to laboratory personnel, NAATs are currently recommended for quick, sensitive and specific diagnosis. These include conventional and real-time polymerase chain reaction (PCR), DNA microarray-based detection and DNA sequencing. Isolation, cytological staining of smears of exudate or faeces, and of impression smears of tissues, immunohistochemical staining of cytological and histological preparations and antigen-capture enzyme-linked immunosorbent assays (ELISA) can be used if NAATs are not available.

The samples to be collected will depend on the disease signs in evidence. Specimens from acute cases should include inflammatory or fibrinous exudate in or around organs that display lesions, ocular and nasal exudates, impression smears of liver, whole blood and tissue samples from kidney, lung, pericardium, spleen, and liver. In cases with diarrhoea, colon contents or excrement should be used. In live birds, the preferred samples are pharyngeal and nasal swabs. Intestinal excrement, cloacal swabs, conjunctival scrapings, and peritoneal exudate can also be taken.

1.1. Molecular methods – detection of nucleic acids

Chlamydia psittaci can be identified and sub-typed using: (1) species-specific conventional PCR; (2) real-time PCR (reviewed in Sachse *et al.,* 2009); (3) *omp*A-sequencing (Sachse *et al*., 2008); (4) multi-locus sequence typing (MLST) (Pannekoek *et al*., 2010); and (5) DNA microarray (Sachse *et al.,* 2005, 2008).

As mentioned above, *C. psittaci* is not the only chlamydial agent encountered in birds. The new chlamydial agents described by Sachse *et al.* in 2014 have to be taken into consideration when a given avian sample is positive in a general chlamydial test, e.g. *Chlamydiaceae*-specific PCR or immunohistochemistry, but negative in a species-specific test for *C. psittaci*. In such a case, partial or complete sequencing of the *omp*A gene and the rRNA operon or alternative species-specific PCR assays will reveal the identity of the strain. The occurrence of *Chlamydia* strains that are phylogenetically in between *C. psittaci and C. abortus* has also been described (Van Loock *et al*., 2003; Pannekoek *et al*., 2010), and should likewise be considered as a possible differential diagnosis.

Reagents designed to stabilise the DNA should be considered when a delay in processing the sample is anticipated (DeGraves *et al.,* 2003). DNA samples can be prepared using inexpensive reagents or commercially available kits.

1.1.1. Conventional polymerase chain reaction

PCR techniques have been replacing isolation for the detection of chlamydiae from animal tissue. Infection risks to laboratory staff are avoided by inactivation of the sample prior to testing. The sensitivity of conventional PCR assays will usually exceed that of isolation. Current conventional PCR tests for detection of *C. psittaci* target the 16S–23S rRNA or the *omp*A gene (reviewed in Sachse *et al.,* 2009). Sensitivity and specificity vary on sample preparation and PCR test, but are considered inferior when compared with quantitative real-time PCR assays. Sensitivity is

increased by targeting a relatively short DNA segment or using a nested procedure. However, there is the risk of contamination if extreme care is not taken when manipulating the reactions (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

1.1.2. Real-time PCR

Real-time PCR has become the preferred method in diagnostic laboratories for its rapidity, high throughput, potential for quantification and ease of standardisation (Sachse *et al.,* 2009). This technology requires a fluorescent-labelled probe and special equipment, which increases costs. Its sensitivity can be equivalent to that of the nested system, but contamination problems and labour are reduced as it is based on one reaction in a closed system.

A hierarchical approach is recommended for the detection and identification of *C. psittaci* DNA. Such an approach includes a *Chlamydiaceae*-specific screening PCR based on the sequences of 23S-rRNA in positive cases (DeGraves *et al.,* 2003; Ehricht *et al.,* 2006; Everett *et al.,* 1999), followed by a *C. psittaci*-specific PCR assay based on sequences of the outer membrane protein (ompA) (Pantchev *et al.,* 2009) or of the incA gene (Ménard *et al.,* 2006). Minor groove binding (MGB) probes are used to rule out cross-reactions with *C. abortus*.

The protocol of the *omp*A-based *C. psittaci*-specific assay (Pantchev *et al*., 2009) is given in more detail below. The assay is conducted as a duplex amplification that includes an internal amplification control (IAC). A detection limit of 2 inclusion-forming units per reaction mix was determined.

i) The *C. psittaci*-specific oligonucleotides are primers CppsOMP1-F (5'-CAC-TAT-GTG-GGA-AGG-TGC-TTC-A-3') and CppsOMP1-R (5'-CTG-CGC-GGA-TGC-TAA-TGG-3'), as well as MGB® probe CppsOMP1-S (FAM-CGC-TAC-TTG-GTG-TGA-C-TAMRA). The IAC system includes primers EGFP1-F (GAC-CAC-TAC-CAG-CAG-AAC-AC) and EGFP10-R (CTT-GTA-CAG-CTC-GTC-CAT-GC), as well as TaqMan probe EGFP-HEX (HEX-AGC-ACC-CAG-TCC-GCC-CTG-AGC-A-BHQ1). Plasmid IC2 (available commercially) serves as the IAC template.

ii) The amplification is conducted in 96-well microtitre plates on an Mx3000P thermocycler or comparable equipment. Each 25-µl reaction contains 12.5 µl of 2 × universal real-time PCR Master Mix. The final concentration is 0.8 µM for each *C. psittaci* primer, 0.4 µM for each IAC primer, and 0.2 µM for each probe.

iii) IAC template DNA (500 copies) is added to each reaction before the final volume is made up with water.

iv) The following cycling parameters are used: initial heating cycle at 95°C for 10 minutes (single denaturation step), 45 cycles of 95°C for 15 seconds and 60°C for 1 minute (annealing and extension).

v) The cycle threshold value (Ct = Cq quantification cycle) automatically calculated by the software should be used. Cq values of 35 or lower are considered as positive. Cq values higher than 35 should be treated with caution as they may represent cross-reaction with related microorganisms. In such cases, the samples should be re-examined, preferentially by alternative tests using different genomic targets (Ménard *et al*., 2006; Opota *et al.,* 2015). Samples can also be re-tested in the real-time PCR. In this case, only repeatedly positive samples are judged as true positive.

Other *omp*A-based real-time PCR protocols were developed to differentiate between genotypes of *C. psittaci* (Geens *et al.* 2005, Heddema *et al.*, 2015). The latter PCR is also validated for use on human samples in case of a zoonotic infection and thus helpful to trace chains of zoonotic transmission.

Real-time PCR protocols are available for the specific detection of *C. avium* (Zocevic *et al*., 2013) and *C. gallinacea* (Laroucau *et al*., 2015) species.

1.1.3. DNA microarray

DNA microarray technology was shown to be a powerful tool in the diagnosis of chlamydial infections (Sachse *et al.,* 2005). The assay for detection and identification of *Chlamydiaceae* spp. is based on PCR amplification of the 23S rRNA gene and subsequent identification of *C. psittaci* and the other avian agents *C. avium* and *C. gallinacea* by hybridisation with species-specific probes. It has been validated and proved suitable for routine diagnosis (Borel *et al.,* 2008). This methodological approach enables detection of mixed chlamydial infections and identification of unexpected chlamydial species directly from clinical samples. An extended version of the DNA microarray allows for *omp*A-based genotyping of *C. psittaci* strains and clinical samples (Sachse *et al*., 2008).

1.2. Direct visualisation – cytological staining

Chlamydiae can be detected in smears of cloacal or conjunctival swabs and in impression smears of tissues (lung, liver, spleen, kidney and airsacs if enough material is available) by cytological staining such as Giemsa, Giménez, modified Giménez, Ziehl–Neelsen and Macchiavello's stains (Campbell *et al*., 2015). The modified Giménez technique is most often used (Andersen & Vanrompay, 2008). However, none of the stains specifically detects chlamydia. They are all less sensitive than antibody-based antigen detection methods or specific NAATs. Therefore, use of a cytological staining is losing popularity.

1.2.1. Modified Giménez staining

i) Reagents

a) Solution 1

Distilled H_2O (450.0 ml) and phenol (5.0 ml) added to basic fuchsin (2.5 g) and 95% ethanol (50.0 ml). Incubate at 37°C for 48 hours. Filter and store in the dark at room temperature.

b) Solution 2

Na_2HPO_4 (11.65 g); $Na_2HPO_4.H_2O$ (2.47 g); distilled H_2O, pH 7.5 (to 1.0 litre).

c) Solution 3

Solution 1 (20.0 ml); and solution 2 (25.0 ml). Let stand for 10 minutes, filter and use.

d) Solution 4

0.5% citric acid.

e) Solution 5

Fast green (0.2 g); distilled H_2O (100.0 ml); and glacial acetic acid (0.2 ml).

f) Solution 6

Solution 5 (20.0 ml); and distilled H_2O (50.0 ml).

ii) Procedure for smears

a) Fix in methanol for 5 minutes.

b) Stain in solution 3 for 10 minutes and rinse in tap water.

c) Counterstain in solution 6 for 2 minutes.

d) Rinse in tap water and air-dry.

iii) Procedure for paraffin sections

a) Deparaffinise and hydrate with distilled H_2O.

b) Stain in solution 3 for 10 minutes and rinse in tap water.

c) Dip in solution 4 until no more red runs out of the section. Rinse in tap water.

d) Counterstain in Solution 6 for 20 dips.

e) Dip in two changes of 95% alcohol, for five dips each. Dehydrate, clear, and mount.

Note: a shorter procedure with "ready to use" carbol fuchsin (1/10 in distilled water), acetic acid (0.1%,) and malachite green counterstain (0.8%) is also available (Vanrompay *et al.,* 1992). Chlamydiae will appear red against a green background.

1.3. Isolation in cell culture

1.3.1. Treatment of samples for isolation

Proper handling of clinical samples is necessary to prevent loss of infectivity of chlamydiae during shipping and storage. A special medium consisting of sucrose/phosphate/glutamate (SPG) was developed for rickettsiae and has proven to be satisfactory for transport of chlamydial field samples. The medium as recommended for chlamydiae consists of SPG buffer: sucrose (74.6 g/litre); KH_2PO_4 (0.52 g/litre); K_2HPO_4 (1.25 g/litre); L-glutamic acid (0.92 g/litre), and bovine serum albumin – fraction V (1 g/litre), which can be sterilised by filtering. Added to this are streptomycin, vancomycin, (25-100 μg/ml), amphotericin B and gentamicin (50 μg/ml each). The addition of antibiotics reduces the effect of contamination, even when samples are shipped at ambient temperatures. The organism remains viable for several days even in the absence of refrigerative storage. This medium can also be used as a laboratory diluent and for freezing of chlamydiae.

Contaminated samples must be pre-treated before being used to inoculate cell cultures. There are three basic methods: treatment with antibiotics, treatment with antibiotics together with low-speed centrifugation (Andersen & Vanrompay, 2008), and treatment with antibiotics with filtration (Andersen & Vanrompay, 2008). A number of antibiotics that do not inhibit chlamydia can be used. Samples are homogenised in phosphate buffered saline (PBS), pH 7.2, containing a maximum of the following: streptomycin, vancomycin (100 μg/ml each), and gentamicin (50–200 μg/ml). Amphotericin B or nystatin (50 μg/ml each) can be added to control yeast and fungal growth. Penicillin, tetracycline and chloramphenicol should be avoided as these inhibit the growth of chlamydiae. In some cases, for example porcine faecal samples, treatment with penicillin G (500 IE/ml) can been useful.

When contamination is light, samples should be homogenised in the antibiotic solution prior to inoculation into tissue cultures. Samples are often left to stand in the antibiotic solution for 24 hours at 5°C before inoculation. Heavily contaminated samples, such as faecal samples, should be homogenised in antibiotics and then centrifuged at 500 ***g*** for 20 minutes. The surface layer and the bottom layer are discarded. The supernatant fluid is collected and recentrifuged. The final supernatant fluid is used for inoculation. Samples should be passed through a filter of 450–800 μm average pore size if contamination persists.

Cell cultures are the most convenient method for the isolation of *C. psittaci*. The most common cell lines are buffalo green monkey (BGM), McCoy, HeLa, African green monkey kidney (Vero), and L cells (Vanrompay *et al.,* 1992). The cells are grown as monolayers using standard tissue culture media containing 5–10% fetal calf serum and antibiotics that are not inhibitory to chlamydia (as described previously).

When selecting cell culture equipment, it is important to remember that:

i) Chlamydiae can be identified by direct or indirect immunofluorescence or some other appropriate staining technique;

ii) The inoculum is usually centrifuged on to the monolayer to enhance its infectivity;

iii) The sample may need to be blind passaged at 4–5 days to increase sensitivity of isolation;

iv) The sample will need to be examined from two to three times during any one passage; and

v) Chlamydiae can be infectious to humans.

Small flat-bottomed vials, such as 1-dram (3.7 ml, 15 × 45 mm) shell vials or bottles containing cover-slips that are 12 mm in diameter, will meet these requirements. A number of vials, often four to six, are inoculated with each sample to permit fixing and staining at various intervals, and to permit repassaging of apparently negative samples 6 days after inoculation. When testing multiple samples, 24-well multiwell dishes can also be used as they have a labour-saving advantage. However, it should be noted that cross-contamination between samples can be a problem.

Chlamydiae can be isolated from cells that are replicating normally, but the use of non-replicating cells is preferable as these may provide increased nutrients for the growth of chlamydiae. Suppressed cells can also be observed for longer periods. Host cell division can be suppressed by cytotoxic chemicals, such as cycloheximide, which can be added to the medium at the rate of 0.5–2.0 μg/ml at the time of inoculation of the monolayer (Andersen & Vanrompay, 2008). A similar cytostatic effect that will enhance the growth of most chlamydial strains is reached by the use of serum-free tissue culture medium.

Attachment of chlamydia to cells is increased by centrifuging the inoculum on to the monolayer at 2000–3500 ***g*** for 30–90 minutes at 37°C. After a 2-hour incubation period at 37°C and 5% CO_2, the inoculum is removed and replaced with serum-free or cycloheximide-containing tissue culture medium, and cultures are incubated at 37–39°C. Cultures must be examined for chlamydiae at regular intervals using an appropriate staining method. This is usually done on day 2 or 3, as well as on day 4 or 5. Cultures that appear to be negative at the fifth day are harvested and repassaged. When repassaging chlamydiae, cells and culture media should be passaged without using freeze–thawing to disrupt cells, as this will destroy the chlamydiae.

Before staining the cultures, the medium is first removed, the cultures are washed with PBS and fixed with acetone or methanol for 2–10 minutes. The fixation time will depend on the tissue culture vessel used. As acetone will soften most plastics, the use of a mixture of 50% acetone and 50% methyl alcohol may be preferable. A number of staining methods can be employed to demonstrate chlamydial inclusions. The preferred method is direct immunofluorescence (Andersen & Vanrompay, 2008). A chlamydial fluorescein-conjugated antiserum is applied to the infected cells and incubated in a humid chamber for 30 minutes at 37°C. The cover-slips are then washed three times with PBS, mounted immediately, and examined. Chlamydial inclusions fluoresce in green. Commercial conjugate preparations using monoclonal antibodies (MAbs) are available and are highly specific. Conjugates may also be prepared from polyclonal sera, but it is important to obtain specific, high-titred antisera. Polyclonal antisera can be prepared in rabbits, guinea-pigs, sheep or goats. Conjugates are then prepared using standard techniques (Andersen & Vanrompay, 2008). Chlamydial inclusions can also be demonstrated by indirect fluorescent antibody and immunoperoxidase techniques (Andersen & Vanrompay, 2008). Direct staining can be done with Gimenez (see Section B.1.2.1), Giemsa, Ziehl–Neelsen, or Macchiavello's stains. Except for immunofluorescence, all these stains have the advantage that standard light microscopes can be used.

1.4. Isolation in embryonated eggs

Chicken embryos are still used for the primary isolation of chlamydiae. Samples should be handled and pre-treated with antibiotics as described in Section B.1.3. The standard inoculation procedure is to inject up to 0.5 ml of inoculum into the yolk sac of a specific pathogen free 6–7-day-old embryo (Andersen & Vanrompay, 2008). The eggs are then incubated in a humid atmosphere at 39°C, rather than at 37°C, as multiplication of chlamydiae is greatly increased at the higher temperature. Replication of the organism usually causes the death of the embryo within 3–10 days. If no deaths occur, two additional blind passages are usually made before designating any sample as negative. Chlamydial infections will give rise to a typical vascular congestion of the yolk sac membranes. These are harvested and homogenised as a 20% (w/v) suspension in SPG buffer, and can be frozen to preserve the strain, or inoculated into eggs or on to cell cultures.

The organism can be identified by preparing an antigen from an infected yolk sac and testing it by direct staining of smears using appropriate stains or by using the antigen in a serological test. Cell culture monolayers can be inoculated with the yolk sac suspension and examined by direct immunofluorescence 48–72 hours later for the presence of chlamydial inclusions. Typical inclusions are intracytoplasmic round or hat-

shaped bodies. With some virulent strains, the inclusions rapidly break up and the chlamydial antigen is dispersed throughout the cytoplasm.

1.5. Antigen detection

1.5.1. Immunohistochemical staining

Immunohistochemical staining can be used to detect chlamydiae in cytological and histological preparations and is an indispensable tool to show the association of chlamydial agents and pathological lesions in tissues. The technique is more sensitive and specific than histochemical staining. Antigen detection can be performed using commercially available anti-*Chlamydia* antibodies directed against LPS or MOMP (major outer membrane protein).

The selection of the primary antibody is very important. Both polyclonal and monoclonal antibodies have been used. Because formalin affects chlamydial antigens, it is recommended that polyclonal antibodies be made to purified formalin-inactivated chlamydiae. The chlamydial strain used is not important, as the antibodies will be reactive mainly to the group-specific antigens. MAbs should also be selected for reactions to formalin-fixed chlamydiae. A pool of group-reactive MAbs can be used.

1.5.2. Enzyme-linked immunosorbent assays

The ELISA has been extensively promoted in kit format for use in the diagnosis of human chlamydiosis. These test kits detect the lipopolysaccharide (LPS) antigen (group reactive) and will detect all species of *Chlamydiaceae*. A number of these kits have been tested for use in detecting chlamydiae in birds (Vanrompay *et al.,* 1994), but none of the kits has been licensed for detection of *C. psittaci*. One problem with some of these tests is that the chlamydial LPS shares some epitopes with other Gram-negative bacteria, and these epitopes can cross-react, resulting in a high number of false-positive results. This problem has been reduced or eliminated in more recently developed kits by careful selection of the MAbs used. These kits, however, still lack sensitivity because a few hundred organisms are still needed to give a positive reaction. Most diagnosticians believe that a diagnosis of AC can be made when a strong positive ELISA reaction is obtained from birds with signs of psittacosis. Because of the number of false-positive results, a positive in an individual bird without signs of disease is not considered to be significant, but indicates the need for further testing using different methods.

2. Serological tests

Serology alone is not particularly useful in diagnosing a current chlamydial infection in birds because of the high prevalence of this infection in birds and the long-term (up to several months) persistence of antichlamydial antibodies. In most bird species, there is a high background rate of antichlamydial antibodies in birds. Thus, to determine if a single bird is infected, serology should always be used in conjunction with antigen or gene detection, or paired sera should be examined (Vanrompay, 2013). However, obligatory examination of paired sera removes serology from immediate clinical relevance. A positive test is evidence that the bird was infected by the bacterium but does not necessarily indicate an active infection. False negative results can occur in birds with acute infections that are sampled before seroconversion. Treatment with antibiotics also may delay or diminish the antibody response. The main serological methods that are being used for detecting chlamydial antibodies are: (1) various methods of elementary body agglutination (EBA), (2) the CFT and (3) ELISA.

EBA detects primarily IgM antibodies and thus can detect early infections. A negative result does not guarantee that a bird is free of infection because the sensitivity of the test is rather low. The direct CFT detects avian IgG but not IgM, so recent infections can be 'missed'. Its disadvantages are that: (1) the test antigens are not commercially available, (2) the test cannot be used for testing sera from avian species whose immunoglobulins do not fix complement, (3) it is only relatively sensitive, (4) it cannot be used to differentiate between IgG and IgM antibodies, and (5) it is fairly laborious when there is a large number of samples to be tested. The modified CFT is more sensitive but has the same disadvantages as the CFT.

The CFT is more and more being replaced by highly sensitive and specific ELISAs based on the use of recombinant proteins (Verminnen *et al*., 2006) or peptide antigens (Sachse *et al*., 2009). ELISAs can detect avian IgM, IgG and IgA as long as the correct isotype-specific conjugate is used.

2.1. Modified direct complement fixation test for *Chlamydia*

For AC, a modified direct CFT method is used. This method differs from the direct CFT in that normal, unheated chicken serum from chickens without chlamydial antibody is added to the complement dilution. The normal serum increases the sensitivity of the CFT procedure so that it can be used to test sera from avian species whose antibodies do not normally fix guinea-pig complement.

2.1.1. Test procedure

i) Serum to test is heat-inactivated at 60°C for 30 minutes prior to use.

ii) Serum is then diluted 1/5 in veronal (barbiturate) buffer saline (VBS)

iii) Twofold dilutions of the diluted serum are prepared in 96-well round bottom microtitre plates.

iv) Guinea-pig complement is diluted in VBS prior to the addition of the antigen and 2 complement haemolytic units are used.

v) Sera complemented with 5 % of fresh chicken serum, complement and antigen are reacted in the plates and incubated for 1 hour at 37°C (and alternate acceptable procedure is overnight incubation at 4°C).

vi) A 2–4% suspension of sensitised washed red blood cells is added.

vii) Plates are incubated for 30 minutes at 37°C, and the centrifuged for 5 minutes at 600 ***g***.

When using commercially available CFT antigens and ready-to-use CFT reagents, the manufacturers' instructions should be applied.

Recommended controls to verify test conditions:

i) Positive control: a control serum that gives a positive reaction;

ii) Negative control serum: a control serum that gives a negative reaction;

iii) Anti-complementary control (serum control): diluent + inactivated test serum + complement + haemolytic system;

iv) Antigen control: diluent + antigen + complement + haemolytic system;

v) Haemolytic system control: diluent + haemolytic system;

vi) Complement control: diluent + complement titration + antigen + haemolytic system.

vii) The absence of anti-complementary activity must be checked for each serum; anticomplementary sera must be excluded from analyses.

A sample that produces 100%haemolysis at the 1/5 dilution is negative and a sample that produces 25–100% haemolysis is positive.

2.1.2. CFT antigen preparation

The simplest methods start with the growth of chlamydiae in cell culture. The two methods described below produce antigens that can be used in the micro-CFT. The procedures are quite similar: both include the growth of chlamydiae in cell culture, the inactivation of the chlamydiae, partial purification of the antigen, mechanical disruption, and dilution into the appropriate buffer. The method selected will depend on the equipment available.

The first procedure (Grimes, 1985) starts with the chlamydiae and cell culture debris harvested when cytopathic effects are noted. The culture is inactivated by the addition of phenol to a final concentration of 1.0%, incubated for 24 hours at 37°C, and concentrated by centrifugation at 10,000 ***g*** for 1 hour. The sediment is reconstituted to 10% of the original volume using VBS, pH 7.2, containing 1.0% phenol and 1.0% glycerol.

The sediment is then homogenised in an omnimixer at top speed for three 1-minute periods while cooled in ice water. The homogenate is centrifuged for 15 minutes at 100 ***g*** to remove debris.

Some procedures suggest heating the antigen for 30 minutes in a boiling water bath at this time. The supernatant is saved and diluted to the desired concentration.

In the second procedure for the production of antigen for the CFT (Bracewell & Bevan, 1986), antigen is prepared from L cells infected with a psittacine strain. The cell culture medium is discarded, and the cells are heated for 40 minutes at 56°C. The cells are lysed in distilled water, the chlamydiae are disrupted by ultrasonication, and then made isotonic in VBS. The antigen is tested against a standard sheep convalescent serum and used at 2 units in the micro- CFT.

There are a number of procedures for preparing the antigen from infected yolk sacs, some of which are quite elaborate. However, with the following procedure it is relatively easy to prepare a crude infected yolk sac antigen that works well in the modified direct CFT. An egg-adapted strain of *Chlamydia* is used to inoculate 6–7-day-old embryonated chicken eggs via the yolk sac. The yolk sacs are harvested from embryos that die between 3 and 7 days post-inoculation. The yolk-sac harvest is diluted 1/3 in PBS, Tris buffer, or cell culture medium, and then autoclaved for 20 minutes. The suspension is cooled and then homogenised thoroughly. The use of a high-speed tissue homogeniser for 3–5 minutes is recommended. After homogenisation, phenol is added to make a final concentration of 0.5% phenol (prepare a 5% phenol stock solution and add 1 ml for every 9 ml of antigen). The antigen preparation is prepared, held for 3 days, and then the supernatant is used after centrifugation for 20 minutes at 1000 ***g***. The antigen can be stored for long periods of time at 4°C.

2.2. Recombinant major outer membrane protein ELISA

The recombinant major outer membrane protein (MOMP) ELISA (Verminnen *et al*., 2006) can be performed on chicken and turkey sera pretreated with kaolin to remove background activity. MOMP-specific antibody titres are determined using a standard ELISA protocol and micro-well plates coated with recombinant MOMP. Serum, diluted 1/100, is added to the coated wells. Recombinant MOMP is produced in pcDNA4::MOMPHis transfected COS7 cells (Vanrompay *et al*., 1999). Briefly, COS7 cells are cultured in Dulbecco's modified Eagle's medium supplemented with 3.7 g of sodium bicarbonate/litre, 1 mM L-glutamine, and 10% fetal calf serum. Transfection with plasmid DNA is performed by the diethylaminoethyl dextran method. Forty-eight hours post-transfection recombinant MOMP production is monitored by an indirect immunofluorescence staining using serovar and genus-specific MAbs. His-tag labelled recombinant MOMP is purified by affinity chromatography and the protein concentration is determined by the bicinchoninic acid protein assay. For the determination of antibody titres, 1/2000 and 1/4000 dilutions of biotinylated anti-chicken/turkey IgG (H+L) antibody and peroxidase-conjugated streptavidin are used, respectively. The results are positive if the absorbance exceeds the cut-off value of the mean of the negative control sera plus three times the standard deviation.

2.3. Other tests

Other tests include the agar gel immunodiffusion test, the latex agglutination (LA) test, the EBA test (Grimes & Arizmendi, 1996) and the micro-immunofluorescence test (MIFT). Immunodiffusion is less sensitive than the CFT. The LA test will detect antibodies to *C. psittaci*, and is easy and rapid to perform (Grimes *et al.,* 1993). Latex beads are coated with purified chlamydial antigen, mixed thoroughly with the test serum on a glass plate, and rotated for 2 minutes to enhance agglutination. The test is read against a dark background. Sera giving positive reactions should be retested with uncoated beads to eliminate possible nonspecific agglutination. The LA and direct CFTs correlate in 72.5% of tests with paired sera. The LA test has a sensitivity of 39.1% and a specificity of 98.8% relative to the direct CFT (Grimes *et al.,* 1993). The test detects both IgM and IgG, but it is best at detecting IgM. It has been suggested for use in detecting recent or active infections. The EBA test detects only IgM, and it is indicative of a current infection. The MIFT is rapid and easy to perform; however, fluorescence-conjugated anti-species sera are not always available.

C. REQUIREMENTS FOR VACCINES

To date, no commercial vaccines against avian chlamydiosis are available, although vaccination with a recombinant DNA plasmid containing the *C. psittaci omp*A gene provided significant (partial) protection in experimentally infected specified pathogen free (SPF) turkeys (Verminnen *et al*., 2010) and budgerigars (Harkinezhad & Schautteet,

2009). DNA vaccination has the advantage that it can be used in the presence of maternal antibodies (Van Loock *et al*., 2004) and the antigen is processed in the same way as during a natural infection, resulting in humoral and cell-mediated immune responses.

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NB: There are WOAH Reference Laboratories for avian chlamydiosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for avian chlamydiosis

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.3.2.

AVIAN INFECTIOUS BRONCHITIS

SUMMARY

Description and importance of the disease: *Avian infectious bronchitis* (*IB*) *is caused by the* Gammacoronavirus *infectious bronchitis virus* (*IBV*). *It has no zoonotic relevance. The virus causes infections mainly in chickens and is a significant pathogen of commercial meat and egg type birds. IB is an acute, contagious disease characterised primarily by respiratory signs in growing chickens. In hens, decreased egg production and quality are often observed. Several strains of the virus are nephropathogenic and may produce interstitial nephritis and mortality. The severity of IBV-induced respiratory disease is enhanced by the presence of other pathogens, including bacteria, leading to chronic complicated airsacculitis. Diagnosis of IB requires detection of the virus by virus isolation, antigen staining techniques or demonstration of viral nucleic acid from diseased flocks. Demonstration of a rising serum antibody response may also be useful. The widespread use of live and inactivated vaccines may complicate both the interpretation of virus detection and serology findings. The occurrence of antigenic variant strains may overcome immunity induced by vaccination.*

Identification of the agent: *For the common respiratory form, IBV is most successfully isolated from tracheal mucosa and lung several days to one week following infection. For other forms of IB, or in a more chronic phase of the infection, kidney, oviduct or the caecal tonsils of the intestinal tract tissues may be better sources of virus depending on the pathogenesis of the disease. Reverse-transcriptase polymerase chain reaction* (*RT-PCR*) *is increasingly being used to identify the spike* (*S*) *glycoprotein genotype of IBV field strains. Genotyping using primers specific for the S1 subunit of the S gene or sequencing of the same gene generally provides similar but not always identical findings to serotyping by haemagglutination inhibition* (*HI*) *or virus neutralisation* (*VN*) *tests. Supplementary tests include electron microscopy, the use of monoclonal antibodies, VN, immuno-histochemical or immunofluorescence tests, and immunisation–challenge trials in chickens. Specific pathogen free chicken embryonated eggs or chicken tracheal organ cultures* (*TOCs*) *from embryos may be used for virus isolation. Following inoculation of the allantoic cavity, IBV produces embryo stunting, curling, clubbing of the down, or urate deposits in the mesonephros of the kidney, often within three serial passages. Alternatively, VN or HI tests using specific antiserum may be used to identify the serotype.*

Serological tests: *Commercial enzyme-linked immunosorbent assays* (*ELISA*) *kits are often used for monitoring serum antibody responses. The antigens used in the kits are broadly cross-reactive among serotypes and allow for general serological monitoring of vaccinal responses and field challenges. The HI test is used for identifying serotype-specific responses to vaccination and field challenges especially in young growing chickens. Because of multiple infections and vaccinations, the sera of breeders and layers contain cross-reactive antibodies and the results of HI and VN test testing cannot be used with a high degree of confidence for serotyping the infection.*

Requirements for vaccines: *Both live attenuated and oil emulsion inactivated vaccines are available. Live vaccines, usually attenuated by serial passage in chicken embryos, confer better local immunity of the respiratory tract than inactivated vaccines. The use of live vaccines carries a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application will generally result in safe application of live vaccines.*

Inactivated vaccines are injected and a single inoculation does not confer significant protection unless preceded by one or more live IBV priming vaccinations.

A. INTRODUCTION

Avian infectious bronchitis (IB) was first described in the United States of America (USA) in the 1930s as an acute respiratory disease mainly of young chickens. A viral aetiology was established, and the agent was termed avian infectious bronchitis virus (IBV). The virus is a member of the genus *Gammacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae*, in the order *Nidovirales*. IBV and other avian coronaviruses of turkeys and pheasants are classified as gammacoronaviruses, with mammalian coronaviruses comprising *Alpha* and *Betacoronaviruses*. Novel related coronaviruses have been discovered in wild birds and pigs and have been designated *Deltacoronaviruses* (Woo *et al*., 2012), interestingly the avian *Deltacoronaviruses* have a different genomic order and show no close relationship to the *gammacoronaviruses*. Coronaviruses have a non-segmented, positive-sense, single-stranded RNA genome.

IB affects chickens of all ages (Britton & Cavanagh, 2007; Cavanagh *et al*., 2002); IBV-like viruses have been isolated from turkeys, pheasants and guinea fowl. The disease is transmitted by the air-borne route, direct chicken-to-chicken contact and indirectly through mechanical spread (contaminated poultry equipment or egg-packing materials, manure used as fertiliser, farm visits, etc.). IB occurs world-wide and assumes a variety of clinical forms, the principal one being respiratory disease that develops after infection of the respiratory tract tissues following inhalation or ingestion. Infection of the oviduct at a very young age can lead to permanent damage and, in hens, can lead to cessation of egg-laying or production of thin-walled and misshapen shells with loss of shell pigmentation. IB can be nephropathogenic causing acute nephritis, urolithiasis and mortality, especially in young birds. After apparent recovery, chronic nephritis can produce death at a later time. Vaccine and field strains of IBV may persist in the caecal tonsils of the intestinal tract and be excreted in faeces for weeks or longer in clinically normal chickens. For an in-depth review of IB, refer to Jackwood & de Wit, (2013). A detailed discussion of IBV antigen, genome and antibody detection assays prepared by de Wit (2000) is also available.

There have been no reports of human infection with IBV.

B. DIAGNOSTIC TECHNIQUES

Confirmation of diagnosis is based on virus detection or seroconversion. Extensive use is made of live and inactivated vaccinations, which may complicate diagnosis by serological methods as antibodies to vaccination and field infections cannot always be distinguished. Persistence of live vaccines may also confuse attempts at recovering or identifying the causative field strain of IBV.

Table 1. Test methods available for the diagnosis of infectious bronchitis virus and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Virus isolation (embryos or TOCs)	+[a]	++[c]	–	+++	+	+[h]
Staining by immunohistochemistry	–	–	–	++	+	+[h]
Gene sequencing (virus identification)	–	–	–	–	++[f]	–
RT-PCR (detection of virus genome)	+[a]	++	++	++	+	+[h]

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Real-time RT-PCR	+[a]	++	++	++	+	+[h]
Haemagglutination test (virus identification)	–	–	–	–	+	–
VN (virus identification)	–	–	–	–	+	–
Detection of immune response						
VN (antibody detection)	–	–[d]	–	+[e]	–	+[e]
HIT (antibody detection)	–	–[d]	+	+[e]	+	++[e]
ELISA (antibody detection)	++[b]	++	++	++[e]	++[g]	++[e]

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
[a]Suitable for ensuring lack of infection during the past 10 days; [b]suitable for ensuring lack of infections dating back to more than 10 days; [c]suitable at the individual level only during excretion periods; [d]limited suitability for this purpose as it may be too specific of the serotype used as an antigen; [e]Suitable provided paired samples collected a few weeks apart can be analysed; [f]especially suitable for surveillance of a given or an emerging genotype; [g]especially suitable when IB surveillance is not focused on a given serotype; [h]sometimes used in the evaluation of vaccines to assess protection against viral excretion, but can be positive even when good clinical protection is achieved. TOC = tracheal organ culture; RT-PCR = reverse-transcriptase polymerase chain reaction; VN = virus neutralisation; HIT = haemagglutination inhibiton test;
ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

1.1. Sampling

Samples appropriate to the form of IB observed must be obtained as soon as signs of clinical disease are evident. For acute respiratory disease, swabs from the upper respiratory tract of live birds or tracheal and lung tissues from diseased birds should be harvested. For birds with nephritis or egg-production problems, samples from the kidneys or oviduct, respectively, should be collected in addition to respiratory specimens. Although virus isolation is a well established method, IBV identification by reverse-transcriptase polymerase chain reaction (RT-PCR) and subsequent sequencing or genotype-specific RT-PCRs will be the most used methods in the field. A positive RT-PCR result is not proof that IBV is present in the kidney cells and causing damage; it could also be caused by viraemia or contamination of the swabs by organs other than the airsac. In situations where IB-induced nephritis is suspected, kidney samples should also be selected from fresh carcases for histochemistry or immunofluorescense to demonstrate local replication. A high rate of virus recovery has been reported from the caecal tonsil or faeces. However, isolates from the intestinal tract may have no relevance to the latest infection or clinical disease. IBV isolation may be facilitated using sentinel specific pathogen free (SPF) chickens placed at one or more times in contact with commercial poultry. Blood samples from acutely affected birds as well as convalescent chickens can also be submitted for serological testing.

1.2. Culture

Samples must be placed in cold transport media containing penicillin (10,000 International Units [IU]/ml) and streptomycin (10 mg/ml) and kept on ice and be frozen as soon as possible. Suspensions of

tissues (10–20% w/v) are prepared in sterile phosphate buffered saline (PBS) or nutrient broth for egg inoculation, or in tissue culture medium for chicken tracheal organ culture (TOC) inoculation (Cook *et al*., 1976). The suspensions are clarified by low-speed centrifugation and filtration through bacteriological filters (0.2 μ) before inoculation of SPF embryonated chicken eggs or TOCs.

SPF embryonated chicken eggs and/or TOCs are used for primary isolation of IBV. Cell cultures are not recommended for primary isolation as it is often necessary to adapt IBV isolates to growth in chicken embryos before cytopathic effect (CPE) is produced in chick embryo kidney cells.

Embryonated eggs used for virus isolation should originate preferably from SPF chickens or from breeder sources that have been neither infected nor vaccinated with IBV. Most commonly, 0.1–0.2 ml of sample supernatant is inoculated into the allantoic cavity of 9–11-day-old embryos. Eggs are candled daily for 7 days with mortality within the first 24 hours being considered nonspecific. The initial inoculation usually has limited macroscopic effects on the embryo unless the strain is derived from a vaccine and is already egg adapted. Normally, the allantoic fluids of all eggs are pooled after harvesting 3–6 days after infection; this pool is diluted 1/5 or 1/10 in antibiotic broth and used to infect another set of eggs for up to a total of three to four passages. Typically, a field strain will induce observable embryonic changes consisting of stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros on the second to fourth passage. Embryo mortality in later passages may occur as the strain becomes more egg adapted. Other viruses, notably adenoviruses that are common to the respiratory tract, also produce embryo lesions indistinguishable from IBV. The IBV-laden allantoic fluid should not agglutinate red blood cells and isolation of IBV must be confirmed by serotyping or genotyping. Infective allantoic fluids are kept at –20°C or below for short-term storage, –60°C for long-term storage or at 4°C after lyophilisation.

TOCs prepared from 19- to 20-day-old embryos can be used to isolate IBV directly from field material (Cook *et al*., 1976). An automatic tissue-chopper is desirable for the large-scale production of suitable transverse sections or rings of the trachea for this technique (Darbyshire *et al*., 1978). The rings are about 0.5–1.0 mm thick, and are maintained in a medium consisting of Eagle's N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) in roller drums (15 rev/hour) at 37°C. Infection of tracheal organ cultures usually produce ciliostasis within 24–48 hours. Ciliostasis may be produced by other viruses and suspect IBV cases must be confirmed by serotyping or genotyping methods.

1.3. Methods for identification and detection

The initial tests performed on IBV isolates are directed at eliminating other viruses from diagnostic consideration. Chorioallantoic membranes from infected eggs are collected, homogenised, and tested for avian adenovirus group 1 by immunodiffusion or PCR. Group 1 avian adenovirus infections of commercial chickens are common, and the virus often produces stunted embryos indistinguishable from IBV-infected embryos. Furthermore, harvested allantoic fluids do not hemagglutinate (HA) chick red blood cells. Genetic-based tests, RT-PCR, and sequence analysis, are used commonly to identify an isolate as IBV. The presence of IBV in infective allantoic fluid or TOCs is usually detected by RT-PCR amplification. Other techniques may be used as well, for example cells present in the chorioallantoic membranes or allantoic fluid of infected eggs or TOCs may be tested for IBV antigen using fluorescent antibody tests, immunohistochemistry or an antigen- enzyme-linked immunosorbent assays (ELISA) using a group-specific monoclonal antibody (MAb) or polyclonal antiserum.

1.4. Serotype identification

Antigenic variation among IBV strains is common (Jackwood & de Wit, 2013), but at present there is no agreed definitive classification system (Valastro *et al.,* 2016). Nevertheless, antigenic relationships and differences among strains are important, as vaccines based on one particular serotype may show little or no protection against viruses of a different antigenic group. As a result of the regular emergence of antigenic variants, the viruses, and hence the disease situation and vaccines used, may be quite different in different geographical locations. Ongoing assessment of the viruses present in the field is necessary to produce vaccines that will be efficacious in the face of antigenic variants that arise. Serotyping of IBV isolates and strains has been done using haemagglutination inhibition (HI) (and virus neutralisation (VN) tests in chick embryos, TOCs and cell cultures. MAbs have been described and used in antigen-ELISAs or immunofluorescence tests for typing of IBV strains, but the number of suitable

MAbs is very limited (de Wit, 2000). Owing to the number of variants and the complexity of serotyping, serotyping is hardly used anymore.

1.5. Genotype identification

Development of improved techniques for nucleotide sequencing, the availability of IBV sequence data, especially relating to the S gene, in computer databases and the demonstration that even relatively short sequence lengths can allow meaningful results in phylogenetic analyses have led to an increase in such studies and have largely replaced HI and VN serotyping for determining the identity of a field strain. The molecular basis of antigenic variation has been investigated, usually by nucleotide sequencing of the gene coding for the spike (S) protein or, more specifically, nucleotide sequencing of the gene coding for the S1 subunit of the S protein where it is believed that the epitopes to which neutralising antibodies bind are found. A high correlation with HI or VN results has not been seen, while different serotypes generally have large differences (20–50%) in the deduced amino acid sequences of the S1 subunit, other viruses that are clearly distinguishable in neutralisation tests show only 2–3% differences in amino acid sequences.

The primary advantages of genotyping methods are a rapid turnaround time, and the ability to detect a variety of genotypes, depending on the primer sets used. Genetic diversity between IBV isolates has been confirmed, but viruses sharing temporal, geographical, antigenic or epidemiological parameters tend to fall into specific lineages and this has proven valuable in assessing both the global epidemiology and local spread of IBV (de Wit *et al.*, 2011; Jackwood, 2012; Valastro *et al.*, 2016). Sequence analysis of the S gene provides the required information for determining the level of genetic homology and the relationship of an IBV S gene to other IBV isolates including vaccine strains.

It has been shown that coronaviruses isolated from turkeys, pheasants and guinea fowl are genetically similar to IBV, having approximately 90% nucleotide identity in the highly conserved region II of the 3' untranslated region (UTR) of the IBV genome (Cavanagh *et al*., 2001; 2002). The potential role of these coronaviruses in IBV infections has not been determined. This is the same for the gammacoronaviruses that have been detected in wild birds (Muradrasoli *et al.*, 2010)

The major uses of RT-PCR tests are virus identification at the genetic level and its application in the understanding of epidemiological investigations during IBV outbreaks. The RT-PCR tests, as they now exist however, do not provide information on viral pathogenicity.

1.5.1. Reverse-transcriptase polymerase chain reaction (RT-PCR)

The wider availability and increased speed of production of results obtained using commercially available kits for RT-PCR and automatic sequencers now means such studies are within the capabilities of many more diagnostic laboratories, providing more accurate and meaningful results. Tracheal or oropharyngeal swabs are often used as the specimens of choice because they are easy to obtain and process. Such samples usually contain little extraneous organic material that can interfere with RNA recovery and amplification by PCR. The system used for RNA extraction will also affect the success of RT-PCR on clinical specimens, and even with commercial kits care should be taken in selecting the most appropriate system validated for the samples to be analysed. The usual target region for IBV characterisation is the S1 subunit of the S glycoprotein gene.

Many one and two-step RT-PCR kits are commercially available from manufacturers claiming superior enzyme sensitivity and fidelity. Reverse transcription is performed according to the manufacturer's instructions. Most diagnostic laboratories determine the genotype of IBV isolates using partial sequence analysis, usually about 700 nt long, of the S1 region of the S gene. Given the diverse nature of the IBV S1 sequence, the actual region sequenced may vary. Such analyses allow rapid epidemiological assessment of the origins and spread of the viruses responsible for IBV outbreaks and the detection of vaccine strains within flocks. It should be noted that for more refined epidemiological studies and for more accurate studies on the evolution of IBV, the phylogenetic studies need to use complete S gene and even whole genome sequences.

1.5.2. Real-time RT-PCR analysis

Rapid confirmation of IBV can be determined using a strategy that avoids post-amplification processing (sequence analysis). The most common method for IBV is real-time RT-PCR. The real-time RT-PCR assay uses specific probes against the target sequence, the use of fluorogenic hydrolysis probes or fluorescent dyes eliminate the requirement of any post-amplification processing step and results can be obtained in less than 3 hours.

A generic IBV real-time PCR that targets a conserved region in the IBV genome (e.g. the 5' UTR) can be used for the detection of IBV in a clinical sample (Callison *et al.*, 2006). Genotype-specific real-time PCRs targeting the S1 gene for genotypes that are known to circulate in the region may be used in conjunction with this generic IBV PCR. Genotype-specific primers and probes have been published for a number of genotypes such as Massachusetts (primer sets XCE3-(CAG-ATT-GCT-TAC-AAC-CAC-C) and MCE1+ (AAT-ACT-ACT-TTT-ACG-TTA-CAC), 793B primer sets XCE3-(CAG-ATT-GCT-TAC-AAC-CAC-C) and BCE1+ (AGT-AGT-TTT-GTG-TAT-AAA-CCA), Arkansas and others (Cavanagh *et al.,* 1999; Roh *et al.,* 2014). However, due to the high variability between and on-going evolution within genotypes (Valastro *et al*., 2016), continual updating of the genotype-specific primers and probes is needed. When the genotype-specific RT-PCRs are negative and the generic RT-PCR is positive, additional sequencing may be needed to identify the strain involved and to adapt the primers and probes used in the genotype specific PCRs accordingly.

2. Serological tests

A number of tests have been described. Those considered here include VN, agar gel immunodiffusion (AGID), HI and ELISA. Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost. In general, ELISAs are most suitable for routine serological testing and can detect antibodies caused by vaccination and field exposure. ELISA can detect antibody responses to all serotypes. AGID also detects IBV antibodies against all types of IBV but lacks sensitivity and is liable to yield inconsistent results as the presence and duration of precipitating antibodies may vary with individual birds. VN and HI tests are more serotype specific, especially in young birds that have not been exposed to different types of IBV (de Wit, 2000). VN tests are too expensive and impractical for use in routine conditions. VN and HI when used on serial sera from young growing chickens such as pullets and broilers can give information on the serotype-specific antibody status of a flock. Regular monitoring of sera from flocks for IB antibody titres may help to indicate the level of vaccine or field challenge responses. Because chicken sera from older birds that have been in contact with vaccines and the field strain of several serotypes contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB at the serotype level cannot be used with a high degree of confidence as the detected antibodies against a certain serotype might be induced by infections of other types of IBV.

2.1. Virus neutralisation

In VN tests, all sera should first be heated to 56°C for 30 minutes. Virus is mixed with serum and incubated for 30–60 minutes at 37°C or room temperature. Chicken embryos are most often employed, but antibodies can be measured using TOC or cell culture systems. Two methods have been used to estimate neutralising antibodies. One employs a constant serum concentration reacted with varying dilutions of virus (the alpha method) and the other employs a constant amount of virus and varying dilutions of serum (the beta method).

In the alpha method, tenfold dilutions of egg-adapted virus are reacted with a fixed dilution (usually 1/5) of antiserum, and the mixtures are inoculated into groups of from five to ten eggs. The virus alone is titrated in parallel. End-points are calculated by the Kärber or the Reed and Muench methods. The results are expressed as a neutralisation index (NI) that represents the $\log_{10}$ difference in the titres of the virus alone and that of the virus/antiserum mixtures. The NI values may reach 4.5–7.0 in the case of homologous virus/serum mixtures; values of <1.5 are not specific, but a heterologous virus will give a value as low as 1.5.

The beta method is the more widely used neutralisation test for antibody assay with chicken embryos or cells. Two- or four-fold dilutions of antiserum are reacted in equal volumes with a dilution of virus, usually fixed at 100 or 200 EID_{50} (median embryo-infective doses) per 0.05 ml and 0.1 ml of each mixture inoculated into the allantoic cavity of each of from five to ten embryonated eggs. A control titration of

the virus is performed simultaneously to confirm that the fixed virus dilution in the virus/serum mixtures was between $10^{1.5}$ and $10^{2.5}$ EID_{50}. End-points of the serum titres are determined by the Kärber or Reed and Muench method as before, but here are expressed as reciprocals of log_2 dilutions. This fixed-virus/varying-serum method is also employed for neutralisation tests in tracheal organ cultures using five tubes per serum dilution, as is conventional with other viruses. The results are calculated according to Reed and Muench, and the virus titres are expressed as median ciliostatic doses per unit volume (log_{10} CD_{50}). Serum titres are again expressed as log_2 dilution reciprocals. This test is more sensitive than others, but technical logistics hamper its more widespread adoption.

2.2. Haemagglutination inhibition

A standard protocol for a HI test for IBV has been described (Alexander *et al*., 1983), and the test procedure detailed below is based on that standard. Most strains and isolates of IBV will agglutinate chicken red blood cells (RBCs) after neuraminidase treatment (Schultze *et al*., 1992). The strain selected to produce antigen may be varied, depending on the requirements of diagnosis. The antigen for the HI test is prepared from IBV-laden allantoic fluids.

For HA and HI tests, procedures are carried out at 4°C or a validated higher temperature, such as 20°C.

2.2.1. Haemagglutination test

i) Dispense 0.025 ml of PBS, pH 7.0–7.4, into each well of a plastic U or V-bottom microtitre plate.

ii) Place 0.025 ml of virus antigen in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/2, 1/3, 1/5, 1/7 and 1/9.

iii) Make twofold dilutions of 0.025 ml volumes of the virus antigen across the plate.

iv) Dispense a further 0.025 ml of PBS into each well.

v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.

vi) Mix by tapping the plate gently and allow the RBCs to settle for 40–60 minutes at 4°C, when control RBCs should be settled to a distinct button.

vii) HA is more easily determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA in which there is no sedimentation or streaming; this is 100% HA and represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

2.2.2. Haemagglutination-inhibition test

The HI test is used in the diagnosis and routine flock monitoring of vaccine responses.

i) Dispense 0.025 ml of PBS into each well of a plastic U or V-bottom microtitre plate.

ii) Place 0.025 ml of serum into the first well of the plate.

iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.

iv) Add 4 HAU of virus antigen in 0.025 ml to each well and leave for 30 minutes.

v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and, after gentle mixing, allow the RBCs to settle for 40–60 minutes when control RBCs should be settled to a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed more exactly by tilting the plates. Only those wells in which the RBCs 'stream' at the same rate as the control wells (containing 0.025 ml RBC and 0.05 ml PBS only) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre $>2^2$, and a positive control serum, for which the titre should be within one dilution of the known titre.

viii) Sera are usually regarded as positive if they have a titre of 2^4 or more. However, it should be noted that even in SPF flocks, a very small proportion of birds may show a nonspecific titre of 2^4, but usually in birds over 1 year of age. Because chicken sera from older birds that have been in contact with several types of IBV contain antibodies that can be highly cross-reactive against antigenically unrelated strains, a cut-off of the HI test of 2^4 will be too low.

2.3. Enzyme-linked immunosorbent assay

Commercial kits for ELISAs are widely available and used. These ELISAs use different cut-offs and mathematical formulas to convert the ELISA result into a titre. This means that every ELISA has its own interpretation and the titre results of different ELISAs on the same serum will differ.

C. REQUIREMENTS FOR VACCINES

1. Background

Strains used in live virus vaccines generally require attenuation. At present, a minority of countries only permits the use of attenuated live vaccines of the Massachusetts type, such as the H120 strain. Most countries also have licensed vaccines against other serotypes depending on the local situation and needs. Commonly used administration routes for live vaccines are spray (course spray or aerosol) or in the drinking water (oral route). Administration by eye-drop, if carefully done, is a very good method but in many cases too expensive.

The grouping of IBV strains that confer cross-protective immunity is the most important typing system from a practical point of view because it provides direct information about the efficacy of a vaccine (de Wit, 2000). The number of cross-protective groups that exists is unknown, but cross-challenge experiments in chicken tend to identify a smaller number of groups compared with serotypes and genotypes, presumably because they are measuring the complete immune response and not just a part of it. In general, there is a higher chance of a good level of cross-protection between strains with a high level of genetic homology in the S-gene than between strains with a low homology. However, the vaccination-challenge experiments have shown that the relationship is not very strong (de Wit *et al*., 2011). Therefore, a cross-immunisation study has to be performed to be able to determine the cross-protective immunity of a strain. Many factors can influence the outcome of such studies and should be accounted for (de Wit & Cook, 2014).

Live vaccines confer better local immunity in the respiratory tract and suitable combinations of vaccines of different serotypes also may protect against a wider antigenic spectrum of field strains (Cook *et al*., 1999; de Wit *et al*, 2013). Live vaccines carry a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper application of vaccine can achieve uniform distribution of the vaccine in the flock and avoid back-passage. Furthermore, the use of vaccines at the manufacturer's recommended dosages will also help avoid back-passage reversion that may be caused by fractional dose application. For long-living birds, vaccination with live vaccines only during the rearing period will often not be sufficient to induce a long-lasting protection against challenge in the laying period. Boosting with inactivated vaccines of a good quality can be very efficient in increasing the level of protection against challenge in the laying period. The efficacy of inactivated vaccines depends heavily on proper priming with a live vaccine(s). Inactivated vaccines must be administered to birds individually, by intramuscular or subcutaneous injection. Variant strains may be used to prepare inactivated autogenous vaccines for controlling IB in layers and breeders, subject to local legislative requirements.

There are prospects for genetically engineered vaccines (Armesto *et al*., 2011; Casais *et al*., 2003), and *in-ovo* vaccination (Tarpey *et al*., 2006; Wakenell *et al*., 1995), but the progress for live vaccines is slow compared with other poultry diseases.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature. National and international standards that apply in the country in which IB vaccines are manufactured must be complied with. The licensing authority should provide information and guidance on requirements. These are now often presented in general terms, as applying to all vaccines – avian and mammalian, live and inactivated, or viral and bacterial vaccines. There may also be specific requirements applying to IB vaccines, live and inactivated. As examples, references are given to the European and USA regulations (European Pharmacopoeia [2017a; 2017b]; USDA, 2017).

For IB vaccines, important differences among countries may arise regarding the challenge virus to be used for potency tests, and its validation. Traditionally, the virulent M41 (Mass 41) strain of the Massachusetts (Mass) type has been used for challenge tests of both live and inactivated vaccines of the Mass serotype. Although this type is still common, it is not the only or the dominant type in any country and many countries allow the use of vaccines from other serotypes of IBV. Establishing criteria for validating the challenge virus may be more difficult for non-Massachusetts types, because of their varying virulence. Inactivated vaccines are often expected to protect against drops in egg production. The traditional M41 challenge should cause a drop of at least 67% in the unvaccinated controls, which was considered by many IB specialists as being excessive and also too dependent on the chicken genetic line and on particular challenge parameters. For other types of IBV, much lower drops in egg production may be regarded as satisfactory, depending on published evidence of the effects of these strains in the field. It therefore seems necessary to relax the criteria for Massachusetts type challenges, and the European Pharmacopoeia now defines a satisfactory drop in egg production for Massachusetts types to be at least 35%, and for non-Massachusetts types to be at least 15% in non-vaccinated birds, provided that the drop is 'commensurate with the documented evidence' (European Pharmacopoeia). However, under laboratory conditions, in many cases even a 35% drop in egg production by M41 is hard to achieve. It can be recommended that an egg drop of at least 15% in non-vaccinated birds should be the minimal drop produced by any IB challenge virus. Appropriate statistical analyses should also be included in any vaccination-challenge study. Inactivated and live vaccines may also claim protection against other clinical signs such as respiratory signs.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production* and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials for veterinary use*.

The seed-lot (master seed) system should be employed for whatever type of vaccine is produced. Each virus must be designated as to strain and origin and must be free from contamination with other strains of IBV and extraneous agents. The master seed virus is the backbone of the vaccine. A few samples of the master seed are used to produce a working seed. This working seed is used for production batches. The master seed should be of such a size that it is sufficient for the life span of a product.

For live virus vaccines, a minority of countries permit only strains of the Massachusetts type. Many countries allow other strains as well, usually on the basis that those strains are already present in their national flocks. The antigenic type incorporated in both live and inactivated vaccines requires justification if there is doubt as to its existence in a country.

2.1.1. Biological characteristics of the master seed

i) Live vaccines

Currently live IBV vaccines are normally attenuated by multiple repeat passage of a virulent virus in specific pathogen free (SPF) embryonated chicken eggs (Cavanagh, 2003). Spontaneous mutations may arise throughout the IBV genome some of which lead to attenuation of the virus, or minor populations present in the inoculum may be selected during passaging, leading to attenuation. However, as a consequence of this method the attenuated viruses produced by this approach have only a few mutations that are responsible for loss of virulence and these will differ between vaccine strains. Two major drawbacks of this method are that once the virus is used to inoculate chickens the mutations within the attenuated vaccine viruses may back-mutate or be re-selected resulting in virulent virus, an undesirable consequence, or that as a consequence of multiple passage the immunogenicity of the attenuated virus will not result in adequate protection. Therefore, it is recommended that the number of passages from the master seed to the working seeds is preferably not more than five. Results from efficacy studies using experimental batches at the highest passage claimed for production should be taken into account when setting the upper limit of passage allowed for the vaccine. To test the worst-case scenario, it is recommended to test the efficacy on the working seed that is used to produce the final product that will be used in the field. The master seed has to comply satisfactorily with the required tests for extraneous agents and safety. The working seeds must be grown in SPF chicken eggs to minimise the risk of introduction of potential pathogens.

ii) Inactivated vaccines

The IBV strains, for inactivated vaccines, are grown in SPF eggs, non-SPF eggs from healthy flocks (where allowed by the regulatory authority) or cell culture and are chemically inactivated usually by an agent that binds to and destroys the genomic RNA. Batches of inactivated virus suspension must be tested for residual infectivity using embryonated eggs.

Every seed lot must be free from bacterial, fungal, mycoplasmal and viral contamination.

For the detection of extraneous viruses, the seed is first treated with a high-titred monospecific antiserum prepared against the strain under examination or against one of identical type. This mixture is cultured in a variety of ways, designed to confirm the absence of any viruses considered from past experience to be potential contaminants. The antiserum must not contain antibodies to adenovirus, avian encephalomyelitis virus, avian rotavirus, chicken anaemia virus, fowlpox virus, infectious laryngotracheitis virus, influenza A virus, Newcastle disease virus, infectious bursal disease virus, leukosis virus, reovirus, Marek's disease virus, turkey herpesvirus, adeno-associated virus, egg-drop syndrome 76 (EDS76) virus, avian nephritis virus, avian pneumovirus or reticuloendotheliosis virus. The inoculum given to each unit of the culture system used should contain a quantity of the neutralised IBV component under test that had an initial infectivity of at least ten times the minimum field dose. These systems include:

1. SPF chicken embryos, incubated for 9–11 days, inoculated via both allantoic sac and chorioallantoic membrane (two passages);
2. Chicken embryo fibroblast cultures or other cells that are genetically susceptible for leukosis virus subgroups A, B, and J but not to endogenous avian leukosis virus. The COFAL test (test for avian leukosis using complement fixation), or double-antibody sandwich ELISA for group-specific leukosis antigen is performed on cell extracts harvested at 14 days. An immunofluorescence test for reticuloendotheliosis virus is done on cover-slip cultures after two passages.
3. SPF chicken kidney cultures that are examined for CPEs, cell inclusions and haemadsorbing agents passaged at intervals of no fewer than 5 days for up to 20 days' total incubation.
4. SPF chickens of minimum vaccination age inoculated intramuscularly with 100 field doses, and on to the conjunctiva with ten field doses; this is repeated 3 weeks later when the chickens are also inoculated both into the foot pad and intranasally with ten field doses. Observations are made for 6 weeks overall, and serum is collected for tests for avian encephalomyelitis, infectious bursal disease, Marek's disease, Newcastle disease and *Salmonella* Pullorum infection.

2.1.2. Validation as a vaccine strain

The vaccine virus shall be shown to be satisfactory with respect to safety and efficacy for the chickens for which it is intended. Tests on vaccine virus should include a test for any potential ability to revert to virulence. Live and inactivated vaccine seed must be tested for safety as in Section C.2.2.4.

Efficacy should be demonstrated using a batch vaccine at the highest passage level intended to be registered.

For live vaccines, a minimum of ten SPF chickens that are not older than the minimum age to be recommended for vaccination are vaccinated by the route intended for field use (e.g. intranasally or by eyedrop) at the recommended dose. Ten unvaccinated control birds from the same age and source are retained separately. All birds of both groups are challenge inoculated either intranasally or by eyedrop 3–4 weeks later or other time interval in line with the desired claim for onset or duration of immunity, with $10^{3.0}$–$10^{5.0}$ EID_{50} of reference challenge virus, the optimal challenge dose may depend on the challenge strain. A swab of the trachea is taken from each bird 4–5 days after challenge and placed in 3 ml of antibiotic broth. Each fluid is tested for IBV by the inoculation (0.2 ml) of five embryonated eggs, 9–11 days of age. An alternative test to that of taking swabs is to kill birds at 4–6 days after challenge and examine microscopically the

tracheal rings for ciliary activity. Failure to resist challenge is indicated by an extensive loss of ciliary motility. The live vaccine is suitable for use if at least 80% of the challenge vaccinated birds show no evidence of IBV in their trachea, while 80% or more of the control birds should have evidence of the presence of the virus.

To assess an inactivated vaccine intended to protect laying birds, 30 or more SPF chickens are vaccinated as recommended at the earliest permitted age. If a primary vaccination with live vaccine is first undertaken, an additional group of birds is given only the primary vaccination. In both cases, these primary vaccinations should be done at no later than 3 weeks of age. The inactivated vaccine is given after the live priming vaccination according to the vaccination schedule to be recommended. A further group of 30 control birds are left unvaccinated. All groups are housed separately until 4 weeks before peak egg production, and then are housed together or in similar conditions. Individual egg production is monitored and once it is regular, all birds are challenged, egg production being recorded for a further 3–4 weeks. The challenge should be sufficient to ensure loss of production during the 3 weeks after challenge. The loss in the non-vaccinated control group should be at least 35% where challenge has been made with a Massachusetts-type strain unless justified. Where it is necessary to carry out a challenge with a strain of another serotype for which there is documented evidence that the strain will not cause a 35% drop in egg production, the challenge must produce a drop in egg production commensurate with the documented evidence and not less than 15% unless justified; the group that received primary live virus vaccine followed by inactivated vaccine should not significantly drop in production compared with the previous level, and the group given only a primary vaccination should show an intermediate drop in production. The vaccine complies with the test if egg production or quality is significantly better in the group having received the inactivated vaccine than in any control group. Sera are collected from all birds at vaccination, 4 weeks later, and at challenge; there should be no response in the control birds.

To assess an inactivated vaccine intended to protect birds against respiratory disease, 20 SPF chickens aged 4 weeks are vaccinated as recommended. An additional 20 control birds of the same age and origin are housed with this first group. Antibody responses are determined 4 weeks later; there should be no response in the control birds. All birds are then challenged with 10^3 CID_{50} (50% chick infective dose) or other dose depending on the strain of virulent virus, killed 4–7 days later, and tracheal sections are examined for ciliary motility, or tracheal swabs evaluated for challenge virus recovery. At least 80% of the unvaccinated controls should display complete ciliostasis, whereas the tracheal cilia of a similar percentage of the vaccinated birds should remain unaffected. Tracheal swabs from at least 90% of the vaccinated birds should be negative for virus isolation, while tracheal swabs from at least 90% of the control birds should be positive for virus isolation.

Both live and inactivated multivalent vaccines containing other fractions such as Newcastle disease, infectious bursal disease, avian metapneumo-, reo- and EDS76 viruses are available. The efficacy of the different fractions of these vaccines should each be established; this should also be evaluated in the combination vaccine to assess possible interference between the different vaccine components.

2.2. Method of manufacture

2.2.1. Procedure

All virus strains destined for live attenuated vaccines are cultured in the allantoic sac of SPF chicken embryos or in suitable cell cultures. For inactivated vaccines, hens' eggs from healthy non-SPF flocks (where permitted by the regulatory authority) or suitable cell cultures may be used. The pooled fluid is clarified and then titrated for infectivity. For live vaccines this fluid is lyophilised or frozen in vials, tablets or other forms, and for inactivated vaccines it is blended with e.g. high-grade mineral oil or other suitable adjuvant to form an emulsion to which a preservative can be added.

2.2.2. Requirements for ingredients

See chapter 1.1.8 with special focus on products of biological origin (POBs) originating from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

2.2.3. In-process controls

The required antigen content is based on initial test batches of vaccine of proven efficacy in laboratory and field trials. Infectivity titrations are done in chicken embryos.

Live vaccine should have a titre not less than the minimum dose, which has been proven to be efficacious in the laboratory studies. For inactivated vaccines, which are produced on hens' eggs from healthy non-SPF flocks, validated methods must be in place to exclude the presence of potential contaminants in the eggs. Otherwise, the inactivating agent and inactivation procedure must be shown under manufacture not only to be effective on IBV, but also on other potential contaminants; with the use of beta-propiolactone or formalin, any live leukosis viruses and Salmonella species must be eliminated; and with other inactivating agents, the complete range of potential contaminants must be rendered ineffective. Before inactivation procedures, it is important to ensure homogeneity of suspensions, and a test of inactivation should be conducted on each batch of both bulk harvest after inactivation and the final product.

2.2.4. Final product batch tests

i) Sterility

Every batch of live vaccine should be tested for the absence of extraneous agents as for the seed virus (see chapter 1.1.9). For vaccines administered via drinking water, spray or skin scarification one non-pathogenic micro-organism per dose is acceptable.

ii) Safety (target animal batch safety test)

a) For live attenuated vaccines

Use no fewer than ten chickens from an SPF flock that are of the minimum age stated on the label for vaccination. Administer by eyedrop to each chicken ten doses of the vaccine reconstituted so as to obtain a concentration suitable for the test. Observe the chickens for 21 days. For vaccines intended for chickens that are 2 weeks old or more, use the chickens inoculated in the 'test for extraneous agents using chickens' (see Section C.2.1.1 point 4). If during the period of observation, more than two chickens die from causes not attributable to the vaccine, repeat the test. The vaccine complies with the test if no chicken shows serious clinical signs, in particular respiratory signs, and no chicken dies from causes attributable to the vaccine.

b) For inactivated vaccines

Inject a double dose of vaccine by the recommended route into each of ten 14- to 28-day-old chickens from an SPF flock. Observe the chickens for 21 days. Ascertain that no abnormal local or systemic reaction occurs.

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

iii) Batch potency

The potency test is developed from the results of efficacy tests on the furthest passage from the master seed virus. Live vaccines are tested for potency by titration of infectivity, and inactivated vaccines by measuring antibody production or alternative methods. An example of a potency test for inactivated vaccine consists of vaccinating 10 SPF chickens, at least 2 weeks of age, and showing that their mean HI titre 4 weeks later is not significantly less than those obtained for a batch that has shown satisfactory efficacy.

iv) Stability

Vaccine must be shown to have the required potency to achieve the claimed duration of immunity at the end of the claimed shelf life.

At least three batches should be tested for stability and must give satisfactory results for 3 months beyond the claimed shelf life. The stability of a live vaccine must be measured by

maintenance of an adequate infectivity titre. The stability of an inactivated vaccine is measured at intervals by batch potency tests. The concentration of preservative and persistence through the shelf life should be assessed. There should be no physical change in the vaccine and it should regain its former emulsion state after one quick shake.

There are maximum level requirements for the use of antibiotics, preservatives and residual inactivating agents.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 and 2) should be submitted to the authorities. Information shall be provided from three consecutive vaccine batches to demonstrate consistency of production.

2.3.2. Safety requirements

Additional testing required for live IB vaccines and precautions:

i) Reversion-to-virulence for attenuated/live vaccines and environmental considerations (dissemination and spread of live vaccines and their potential to cause problems for non-vaccinated animals and non-target animals in case of genetically modified vaccines). The vaccine complies when there is no indication of an increase in virulence of virus recovered from the final bird passage compared with the original vaccine that was used for the first passage. It is recommended to use at least five sequential passages over birds by natural spreading or eye-drop application of a suspension of fresh mucosal tissue. Validated alternative methods may be used as well.

ii) Precautions (hazards)

IBV itself is not known to present any danger to staff employed in vaccine manufacture or testing. Extraneous agents may be harmful, however, and the initial stages of handling a new seed virus should be carried out in a safety cabinet. It is a wise precaution with all vaccine production to take steps to minimise exposure of staff to aerosols of foreign proteins. Persons allergic to egg materials must never be employed in this work. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); Efficacy should be demonstrated using a batch vaccine at the highest passage level intended to be registered. Each batch of live vaccine should contain sufficient live virus per dose per bird to last until the expiry date, indicated as the minimum dose that has been proven to be efficacious in laboratory studies.

Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to challenge. Vaccine efficacy should be established for each serotype of IBV against which protection is claimed.

The challenge models for determining efficacy are as outlined in Section C.2.2.2.

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NB: At the time of publication (2018) there were no WOAH Reference Laboratories for avian infectious bronchitis (adenomatosis) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.3.3.

AVIAN INFECTIOUS LARYNGOTRACHEITIS

SUMMARY

Description of the disease: *Avian infectious laryngotracheitis (ILT) is a respiratory disease caused by* gallid alphaherpesvirus 1*. It is principally a disease of chickens, although it can also affect pheasants, partridges and peafowl. The clinical signs and observed pathology reactions may vary from extremely severe, with some birds dying from asphyxiation, to very mild, indistinguishable from other mild respiratory diseases of chickens. The principal lesion is tracheitis. In infected birds, the virus can become latent and be re-excreted at a later date without clinical signs.*

Laboratory diagnosis depends on isolation of the virus, and demonstration of the presence of the virus, viral antigens or DNA. Histopathological examination of the trachea for characteristic intranuclear inclusions and syncytial cell formations are of diagnostic value. Many of the diagnostic methods described in this chapter have not undergone formal validation to modern standards, but are accepted through widespread use over a prolonged period ("historical validation").

Detection of the agent: *Virus isolation may be done by inoculation of suspected material on to the dropped chorioallantoic membrane of embryonated hens' eggs, or into avian embryonic cell cultures. These methods are time-consuming but remain useful. Rapid methods include immunofluorescence on tracheal exudate or frozen sections, and an enzyme-linked immunosorbent assay (ELISA) to demonstrate viral antigen in mucosal scrapings. Polymerase chain reaction (PCR) methodology has been shown to be more sensitive than virus isolation for the examination of clinical material and is now widely used. Both conventional and real-time PCR are used to detect ILTV DNA. Real-time PCR provides a relative quantification of viral genomes per sample, which can indicate the stage of infection and level of virus transmission in the flock. Currently, histopathology examination of tissues and real-time PCR are the most common pair of rapid assays used for the detection of ILTV infection. Virus characterisation and differentiation of vaccine and wild-type viruses are possible using PCR followed by restriction fragment length polymorphism or sequencing.*

Serological tests: *Antibodies to ILT virus can be detected by virus neutralisation (VN) tests conducted in eggs or in cell cultures, or by AGID reactions, indirect immunofluorescence, or ELISA. The latter is preferred for screening flocks.*

Requirements for vaccines: *Vaccines against ILT are usually prepared from attenuated live virus. Those available at present afford some degree of protection, but are not ideal. A number of recombinant vaccines are also available commercially with various degrees of protective efficacy.*

A. INTRODUCTION

Avian infectious laryngotracheitis (ILT) is a respiratory disease of chickens caused by *gallid alphaherpesvirus 1*. It can also affect pheasants, partridges and peafowl. In the virulent form, the history, clinical signs and very severe tracheal lesions are highly characteristic of the disease, but the mild form may be indistinguishable from other agents causing mild respiratory diseases. Laboratory diagnosis depends on the demonstration of the presence of the virus or viral components or specific antibodies in the serum.

Clinically, the disease may appear in three forms, namely peracute, subacute, and chronic or mild. In the peracute form, onset of disease is sudden with a rapid spread. The morbidity is high and mortality may exceed 50%. Some birds may die in good body condition before the appearance of signs, which are characteristic and comprise difficulty in breathing with extension of the neck and gasping in an attempt to inhale. There is also gurgling, rattling and coughing when birds try to expel obstructions in the trachea. Conjunctivitis may also be observed. Clots of

blood may be coughed up and can be found on the floor and walls of the house. Post-mortem changes are confined to the upper respiratory tract and are also characteristic, consisting of haemorrhagic tracheitis with blood clots, mucoid rhinitis, and blood-stained mucus along the length of the trachea.

In the subacute form, the onset of illness is slower and respiratory signs may extend over some days before deaths are seen. The morbidity is high but the mortality is lower than in the peracute form, between 10% and 30%. Post-mortem findings are less severe and consist of mucoid exudate with or without blood in the trachea. Yellow caseous diphtheritic membranes may be found adherent to the larynx and upper tracheal mucosa.

Chronic or mild ILT may be seen among survivors of either of the above forms of the disease, although some outbreaks themselves may be entirely mild. Incidence of chronic ILT within a flock may be only 1–2%. Signs include coughing, nasal, ocular and oral discharge, and reduced egg production.

Differential diagnosis of ILT from other respiratory diseases, especially Newcastle disease, which may also cause severe tracheitis, is important. Also, diphtheritic oral lesions should be differentiated from the wet form of fowlpox.

Infection is acquired via the upper respiratory tract and transmission occurs most readily from acutely infected birds, but clinically inapparent infection can persist for long periods with intermittent re-excretion of the virus, and these recovered carrier birds are also a potential means of transmission of the disease (Hughes *et al*., 1987). Given that transmission of ILT takes place by close contact, transmission is slower in cage houses than where birds are loose-housed, and the path of infection through a cage house may be apparent. Recent work has confirmed considerable variation among ILT virus (ILTV) strains in their tropism for trachea or conjunctiva and those with affinity for the latter site can severely affect weight gain (Kirkpatrick *et al*., 2006a). There is increasing evidence that highly virulent and transmissible strains can emerge as a result of recombination between two or more different strains (Lee *et al*., 2015).

There is no known risk of human infection with ILTV. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.*

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian infectious laryngotracheitis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Virus isolation	–	–	–	++	–	–
Immuno-fluorescence for antigen	–	+	–	++	–	–
ELISA – antigen detection	–	++	+	+++	+	–
PCR	++	+++	++	+++	+++	–
Histopathology	–	–	–	++	–	–

Detection of immune response						
VN	+	–	+	–	–	+
ELISA – antibody detection	++	+	+++	+	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
ELISA = Enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; VN = virus neutralisation.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

The virus may be isolated in chicken embryo liver, chicken embryo kidney or in chicken kidney cell cultures. Of these, monolayers of chicken embryo liver cells have been found to be the most sensitive (Hughes & Jones, 1988). The virus can also be grown on the dropped chorioallantoic membrane (CAM) of 10- to 12-day-old specific pathogen free (SPF) embryonated chicken eggs (Jordan, 1964).

The causative herpesvirus may be demonstrated directly in tracheal exudate by electron microscopy (Van Kammen & Spadbrow, 1976). Viral antigens may be detected by immunofluorescence (Braune & Gentry, 1985; Wilks & Kogan, 1979), or enzyme-linked immunosorbent assay (ELISA), using tracheal mucosal scrapings (York & Fahey, 1988). Histopathological examination of the trachea for typical herpesvirus intranuclear inclusions and syncytial cell formation may also be helpful (Pirozok *et al*., 1957). Both conventional and real-time polymerase chain reaction (PCR) are used to detect ILTV nucleic acid. An additional advantage of real-time PCR is that it provides a relative quantification of viral genomes per sample, which can indicate the stage of infection and level of virus transmission in the flock. Conventional PCR is also useful in the diagnosis of ILTV because amplification products generated by conventional PCR can be sequenced for further virus genotyping (Menendez *et al.,* 2014).

1.1. Virus isolation

When samples are taken from live birds for virus isolation, tracheal swabs are superior to oropharyngeal or conjunctival swabs. These are placed in transport medium containing antibiotics. When selecting material for virus isolation from chronic outbreaks, it is more productive to cull a bird in the early stages of the infection, rather than to attempt to isolate virus from a bird that has died of asphyxiation after a long illness. The quality of sample is further improved if the bird is killed by barbiturate or other injection rather than by cervical dislocation. The whole head and neck from dead birds may be submitted, or only the trachea and larynx after their removal with minimal contamination. Tracheas should be transported in antibiotic broth for virus isolation, but wrapped in moist tissue paper if destined for electron microscopy. Any prolonged storage of infected tissues should be at –70°C or below to minimise loss of virus titre. Repeated freezing and thawing must be avoided as this reduces virus infectivity.

Exudate and epithelial cells are scraped from the tracheas, diluted approximately 1/5 in nutrient broth containing penicillin and streptomycin, and agitated vigorously. The resulting suspension is centrifuged at low speed to remove debris, and 0.1 ml of the supernatant fluid is inoculated on to the dropped CAM of at least three embryonated chicken eggs of 10–12 days' incubation. The eggs are sealed with paraffin wax and incubated at 37°C for up to 7 days. They are candled daily and the CAMs of dead embryos or of those surviving for 7 days are examined for typical pocks. Alternatively, at least two confluent chick embryo liver or chicken embryo kidney cell monolayers, with their medium removed, are inoculated and allowed to adsorb for 1–2 hours. Cultures are overlaid with fresh medium, incubated for up to 7 days and examined daily under the microscope for evidence of a typical syncytial cell cytopathic effect (CPE).

In each instance, up to three passages of material may be necessary before a specimen is considered to be negative. A virus isolate can be confirmed as ILTV by a neutralisation test in eggs or cell culture using hyperimmune antiserum to ILTV. Alternatively, virus particles may be identified rapidly in cell culture fluid or in pocks on CAMs by electron microscopy, viral antigens by immunofluorescence in acetone-fixed ILT-virus-infected cell cultures or in frozen sections of CAM and viral nucleic acid by PCR.

1.2. Immunofluorescence

In immunofluorescence tests for viral antigens, epithelial cell scrapings from the trachea are smeared on to a glass slide. Alternatively, 5 μm thick cryostat sections of trachea, snap-frozen in liquid nitrogen may be used. The preparations are fixed in acetone at room temperature for 10 minutes. These can be stained directly by applying chicken anti-ILTV immunoglobulin labelled with fluorescein isothiocyanate (FITC) for 1 hour, followed by rinsing for 15 minutes in a bath of phosphate-buffered saline (PBS), pH 7.2, agitated with a magnetic stirrer. Otherwise, they can be stained indirectly by applying an appropriate dilution of chicken anti-ILT serum for 1 hour. The slide is rinsed thoroughly with PBS for 15 minutes as above, and an FITC-labelled anti-chicken immunoglobulin is applied for 30 minutes. After a final rinse, cover-slips are applied over non-fade mountant. The preparations are examined for specific intranuclear fluorescence in the epithelial cells using a microscope with epifluorescent ultraviolet illumination. Suitable controls include the use of known uninfected specimens and, for the indirect method, the application of nonimmune chicken serum. Particular care should be taken in the reading of indirect immunofluorescence preparations, as endogenous chicken IgG in the trachea may cause unwanted attachment of FITC-labelled anti-chicken IgG.

1.3. Enzyme-linked immunosorbent assay

A number of antigen detection ELISA kits are available commercially, and the assay procedure may vary depending on the kit; the manufacturer's instructions should always be followed. When the monoclonal antibody (MAb) ELISA is used for detecting viral antigens (McNulty *et al*., 1985), tracheal exudate is mixed with an equal volume of PBS containing 1% (v/v) of a nonionic, non-denaturing detergent, then vortexed for 30 seconds and centrifuged at 10,000 ***g*** for 1 minute. The supernatant fluid is dropped in 50 μl volumes in wells of microtitre plates previously coated with rabbit IgG against ILTV, diluted 1/200 in 0.05 M carbonate/bicarbonate buffer, pH 9.0, and incubated for 1 hour. Next, 50 μl of MAb against major glycoproteins of ILTV, diluted 1/50 in PBS, is added to each well, followed by 50 μl of a 1/1000 dilution of affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase. The chromogen/substrate, 5-aminosalicylic acid (6.5 mM) with hydrogen peroxide (to a final concentration of 0.0005%), is added to the wells in 100 μl volumes. After 30 minutes, the plates are read at 450 nm on a spectrophotometer and the absorbance reading for each well is corrected by subtracting the reading obtained for wells containing diluting buffer instead of tracheal exudate. The positive/negative cut-off point is taken as the mean absorbance value for several negative (i.e. tracheal material without ILTV) samples plus 3 standard deviations.

1.4. Histopathology

Birds selected for post-mortem examination should be in the acute phase of disease. Euthanasia should be by intravenous injection of barbiturates or exposure halothane, to avoid damaging the trachea. Tracheas for histopathological examination should be placed in 10% neutral buffered formalin or Bouin fixative (preferable for detection of intranuclear inclusion bodies) immediately after removal from the birds and, after fixation, embedded in paraffin wax. Eyelid and lung are sometimes examined. Intranuclear inclusions may be seen in the epithelial cells of the trachea after staining with haematoxylin and eosin. Syncytial cell formations are often present within the exudates and commonly contain intranuclear inclusion bodies. Inclusion bodies are the classical Cowdry type A inclusions of herpesviruses, but they may be present for only 3–5 days after infection. In severe cases where most infected cells have detached from the tracheal lining, inclusions may be seen in intact cells among the cellular debris in the lumen of the trachea. Longitudinal rather than transverse sections of trachea permit examination of the whole length of the organ.

1.5. Molecular methods

Several molecular methods for identifying ILTV DNA in clinical samples have been reported, but the PCR has proved the most useful. Using a nested PCR, Humberd *et al*. (2002) showed that ILTV DNA could be detected in formalin-fixed, paraffin-embedded tissues independently of the presence of syncytial cells, intranuclear inclusions or both.

PCR has been found to be more sensitive than virus isolation for clinical samples, especially when other contaminant viruses such as adenoviruses are present (Williams *et al*., 1994). Alexander & Nagy (1997)

found that during the middle to the end of the infection phase, PCR and virus isolation were similar in sensitivity, but PCR was superior in the recovery phase.

The combination of PCR with restriction fragment length polymorphism (RFLP) analysis of single and multiple viral genes and genome regions has enabled the characterisation of different strains within a country or region (Chang *et al*., 1997). Several reports have shown that while some field strains are closely related to and likely to be derived from vaccine viruses, others are true 'wild types' (Ojkic *et al*., 2006). Genes commonly examined by different authors include ICP4, TK (thymidine kinase), glycoprotein G (gG), glycoprotein E (gE) and UL47. Oldoni & Garcia (2007) used 36 restriction enzymes, while others have used as few as four.

There is no molecular test that is universally recognised for strain identification of ILTVs, but a protocol has been provided below in Section B.1.5.1.iv *Restriction fragment length polymorphism* that has been used in a number of poultry diagnostic laboratories for differentiation of vaccines and field strains and epidemiological investigations. A good history of vaccination in the flock may assist with interpretation of results although it should be noted that field strain infection can occur in vaccinated birds. Vaccine strains can also occasionally be isolated from unvaccinated birds,

1.5.1. Test procedures

i) Conventional PCR

In a typical PCR protocol for ILTV, viral DNA is extracted from clinical samples (swabs, tissues), chorioallantoic membrane plaques, cell culture supernatants or vaccines using DNA extraction kits. Primers used can be obtained from previously published work or designed using ILTV sequences on the Genbank international database.

The following protocol is routinely used in a number of veterinary diagnostic laboratories for detection of ILTV in clinical specimens and has potential for the preliminary typing of the virus by restriction fragment length polymorphism of the PCR amplicons. Full details of this PCR are provided in Kirkpatrick *et al.* (2006b). In brief, an approximately 2.24 kbp region of the ILTV thymidine kinase gene is amplified using a pair of forward (5'-CTG-GGC-TAA-ATC-ATC-CAA-GAC-ATC-A-3') and reverse (5'-GCT-CTC-TCG-AGT-AAG-AAT-GAG-TAC-A-3') primers and the resultant amplicons are separated by electrophoresis through 0.8% agarose gels, stained with an appropriate nucleic acid stain and exposed to UV light for visualisation. The 50 µl amplification reaction contains 200 µM each of dATP, dCTP, dGTP and dTTP, 1 mM $MgCl_2$, 250 µM each of the primers, 1 µl (2.5 units) Taq DNA polymerase, 5 µl of 10 × Taq DNA polymerase buffer and 5 µl extracted DNA as template. The reaction mixture should be incubated at 94°C for 3 minutes, then subjected to 35 cycles of 94°C for 15 seconds, 60°C for 45 seconds and 72°C for 150 seconds, and finally incubated at 72°C for 3 minutes. In each series of PCRs, a control tube containing sterile distilled H_2O, instead of extracted DNA, should be included as a negative control.

Another protocol that has been used in veterinary diagnostic laboratories around the world for the detection and preliminary typing of the virus involves the amplification of two regions of the ICP4 gene. Primers ICP4-1F (5'-ACT-GAT-AGC-TTT-TCG-TAC-AGC-ACG-3') and ICP4-1R (5'-CAT-CGG-GAC-ATT-CTC-CAG-GTA-GCA-3') amplify a 688 bp fragment at position 181 to 869[1]; ICP4-2F (5'-CTT-CAG-ACT-CCA-GCT-CAT-CTG-3') and ICP4-2R (5'-AGT-CAT-GCG-TCT-ATG-GCG-TTG-AC-3') amplify a 635 bp fragment at position 3804 to 4440[2]. Full details of these PCRs are provided by Chacon & Ferreira (2009).

The real-time PCR protocol described by Mahmoudian *et al*. (2011) can also be used as a conventional PCR with the final products can be examined by electrophoresis through 2% agarose gels.

1 Genbank accession number NC 006623
2 Genbank accession number NC 006623

ii) Real-time PCR

Real-time PCR assays have been described for ILTV (Creelan *et al*., 2006; Mahmoudian *et al*., 2011). These have the advantage that they can be conducted in less than 2 hours and therefore provide a very rapid method of ILT diagnosis in comparison with traditional virus isolation, or even the standard PCR followed by gel electrophoresis. The following protocol can be used for rapid detection of ILTV in clinical specimens. Full details of the methodology are provided in Callison *et al.* (2007). In brief, two oligonucleotide primers, ILTVgCU771 (5'-CCT-TGC-GTT-TGA-ATT-TTT-CTG-T-3') and ILTVgCL873 (5'-TTC-GTG-GGT-TAG-AGG-TCT-GT-3'), are used to amplify a 103-bp product from the ILTV gC gene. A fluorescent-labelled probe, ILTV probe817 (5'-FAM-CAG-CTC-GGT-GAC-CCC-ATT-CTA-BHQ1-3'), is also used. The reaction is conducted with appropriate PCR kits, for example, using 25 µl total volume containing 10 µl of the 2× master mix, 0.5 µM of forward and reverse primers, 0.1 µM of the probe, 1 µl of HK-UNG, and 5 µl of DNA template. Incubation and data acquisition are performed using a real-time thermocycler although PCR protocols will need to be optimised for the machine used. Reactions are incubated for 2 minutes at 50°C, 15 minutes at 95°C, then 40 cycles of 15 seconds at 94°C and 1 minute at 60°C. For each assay, the threshold cycle number (CT value) is the PCR cycle number at which the fluorescence of the reaction exceeds 30 units of fluorescence.

iii) Nucleotide sequence analysis

A number of ILTV genes can be targeted for PCR followed by nucleotide sequence analysis of the resultant amplicons for strain identification purposes, for example, ICP4 may be amplified by PCR using the primers described by Chacon & Ferreira, (2009) and the resultant amplicons purified using a disposable mini column method and submitted to bi-directional DNA sequencing using the PCR primers as sequencing primers. Various software programs including clustal W may be used for analysis of the sequences and comparison with the existing sequences in GenBank. It should be noted that sequence analysis of multiple genes may be required for proper identification of the ILTV strains.

Whole genome sequencing has also been reported in several publications as a means of fully characterising the viral strains, but, at least currently, equipment, reagents and expertise are available only in a limited number of laboratories and therefore this technique has not been discussed here.

iv) Restriction fragment length polymorphism (RFLP)

The combination of PCR and RFLP has enabled field strains of ILTV to be distinguished from vaccine strains (Creelan *et al*., 2006; Han & Sim, 2001; Kirkpatrick *et al*., 2006a; Ojkic *et al*., 2006; Oldoni & Garcia 2007). A range of restriction endonucleases (RE) have been described for RFLP analysis of ILTV PCR products and several genes have been targeted for digestion. They include ICP4, TK, UL15, UL47 glycoprotein G and ORF-BTK genes. Detailed description of the methodology can be found in Creelan *et al*. (2006), Han & Sim (2001), Kirkpatrick *et al*. (2006a), Ojkic *et al*., (2006) and Oldoni & Garcia (2007). A brief description of methodology for TK, ICP4, ICP18.5 and ORFB-TK PCR RFLP is provided below from Kirkpatrick *et al*. (2006a) with some modifications.

For amplification of TK, PCR is performed in a 50-µl reaction mixture containing 200 µM each dATP, dCTP, dGTP, and dTTP, 1 mM $MgCl_2$, 250 µM of each primer (TK forward: CTG-GGC-TAA-ATC-ATC-CAA-GAC-ATC-A; and TK reverse: GCT-CTC-TCG-AGT-AAG-AAT-GAG-TAC-A), 1.25 U of Taq DNA polymerase, 5 µl of 10× Taq DNA polymerase buffer, and 2 µl of extracted DNA. The reaction mixture is incubated at 94°C for 3 minutes, then subjected to 35 cycles of 94°C for 15 seconds, 60°C for 45 seconds and 72°C for 60 seconds, and finally incubated at 72°C for 3 minutes.

PCR amplification of ICP4, ICP18.5 and ORFB-TK, is performed in separate tubes using primers ICP4 forward: AAA-CCT-GTA-GAG-ACA-GTA-CCG-TGA-C and ICP4 reverse: ATT-ACT-ACG-TGA-CCT-ACA-TTG-AGC-C; ICP18.5 forward: TCG-CTT-GCA-AGG-TCT-TCT-GAT-GG and ICP18.5 reverse: AGA-AGA-TGT-TAA-TTC-ACA-CGG-ACA-C; and ORFB-TK forward: TCT-GCG-ATC-TTC-GCA-GTG-GTC-AG and ORFB-TK reverse: TGA-CGA-GGA-GAG-CGA-ACT-TTA-ATC-C. A 50-µl reaction mixture contains 200 µM each dATP, dCTP, dGTP, and

dTTP, 2 mM $MgSO_4$, 250 µM each primer, 1 U of Platinum Taq DNA Polymerase high fidelity, 5 µl of 10× Platinum Taq DNA polymerase buffer, and 2 µl of extracted DNA. The reaction mixture is incubated at 94°C for 1 minute, then subjected to 35 cycles of 94°C for 1 minute, 68°C for 7 minutes, and finally incubated at 68°C for 10 minutes.

10-µl volumes of TK, ICP4, ICP18.5 and ORFB-TK PCR products are digested separately with the restriction endonucleases Msp*I*, Hae*III*, Hae*III*, and Fok*I* respectively, at 37°C for 1 hour. After digestion, the resultant DNA fragments are separated in a 15% poly-acrylamide gel and restriction DNA fragments visualised by an appropriate nucleic acid stain and exposed to UV light for visualisation. Pattern differences are recorded for each enzyme and results can be developed into dendrograms.

2. Serological tests

The method of choice for detection of antibodies to ILTV in chicken serum is ELISA. Virus neutralisation (VN) can also be used (Adair *et al*., 1985). AGID and indirect immunofluorescence tests are little used nowadays.

2.1. Enzyme-linked immunosorbent assay

A number of ELISA kits are available commercially, and the assay procedure may vary depending on the kit; the manufacturer's instructions should always be followed. Most commercial ELISAs use whole virus as detecting antigen, but some use recombinant glycoproteins. For an in-house ELISA, the whole virus antigen can be obtained by sonication of heavily infected cell cultures at the time of maximum CPE, which is then centrifuged and the supernatant absorbed on to the wells of microtitre plates. A negative antigen is provided by uninfected cell culture material treated in the same way. The test consists essentially of the addition of 0.1 ml of 1/10 dilutions of test sera to duplicate wells coated with positive or negative antigen. After incubation at 37°C for 2 hours, the plates are washed four times and a rabbit anti-chicken IgG conjugated with peroxidase at a recommended dilution (1/1000 to 1/4000 depending on the manufacturer) is added. After incubation at 37°C for 1 hour, the plates are washed again four times. Finally, a chromagen consisting of 5-aminosalicylic acid is added to each well followed by hydrogen peroxide substrate to a final concentration of 0.0005%, and the absorbance of the fluid in each well is read at 450 nm on a spectrophotometer. The result for each serum is expressed as the difference between the mean absorbance produced with the positive and negative antigens. The positive/negative cut-off point is taken as the mean absorbance value for numerous negative sera plus 3 standard deviations. The test is very sensitive and may be useful for surveillance purposes. Antibody responses as measured by ELISA are detectable 7–10 days after infection and peak at about 2 weeks. The response to ILT vaccines may be variable and testing is often done at 14 days post-vaccination or after.

2.2. Virus neutralisation

VN tests may be conducted on the dropped CAMs of embryonating chicken eggs that have been incubated for 9–11 days, where antibody specifically neutralises pock formation caused by ILTV. Alternatively, the tests can be performed in cell cultures, where antibody specifically neutralises the ILTV thus preventing CPE. Doubling dilutions of serum are added to equal volumes of a constant concentration of virus. This concentration may either be 100 median egg infectious doses (EID_{50}) for egg inoculations, or 100 median tissue culture infectious doses ($TCID_{50}$) for the inoculation of cultures. The mixtures are incubated at 37°C for 1 hour to allow any neutralisation to occur.

When the test is performed in eggs, the virus/serum mixtures are inoculated on to the dropped CAMs, using at least five eggs per dilution. Eggs are sealed and incubated at 37°C for 6–7 days. The end-point is recorded as the highest dilution of the serum where no pocks are present on the CAMs. When the tests are done in cell cultures, serum dilutions are prepared in 96-well microculture plates and virus is then added. After the period allowed for neutralisation, freshly prepared chicken embryo liver or kidney cells are added to each well. The plates are incubated at 37°C in an atmosphere of 5% CO_2 and examined daily for CPE; 50% end-points are read after approximately 4 days when the virus control titre indicates that 30–300 $TCID_{50}$ of virus have been used in the test. For the cell culture method of testing, virus neutralisation at 1/8 (initial dilution) or greater is considered positive.

C. REQUIREMENTS FOR VACCINES

1. Background

ILT is usually controlled with live vaccines. Vaccines may be used in response to disease outbreaks, or may be used routinely in endemic areas. Repeated doses may be required to afford good protection. Live attenuated ILT vaccines produced in cell cultures or embryonated hens' eggs are commonly used. Live recombinant (vectored) vaccines are also used. These vaccines use herpesvirus of turkeys or fowlpox virus as vectors to express ILTV proteins. There has also been some work with genetically engineered deletion-mutant vaccines and the results of these initial studies look promising (Coppo *et al.,* 2013). For attenuated ILT vaccines, the live virus seed is a suitably attenuated or naturally avirulent strain of ILTV. Live attenuated ILT vaccines may be administered by eyedrop, spray or in the drinking water. Recombinant vaccines may be delivered by wing-web puncture, subcutaneous injection or *in-ovo* inoculation. There are advantages and disadvantages associated with each of the different types of vaccine and the different methods of delivery (Coppo *et al*., 2013; Garcia, 2017). For example, vectored vaccines may only be partially protective and live attenuated ILT vaccines may have residual virulence that can cause clinical disease, especially if administered by spray and a small droplet size is produced and inhaled. Live attenuated ILT vaccines may also revert to higher levels of virulence following bird-to-bird passage and persist in the field (Coppo *et al.,* 2013). For this reason, it may be difficult to discontinue vaccination once it has been started. Subclinical mixed infections of vaccine and field virus, in vaccinated birds, can cause severe disease in unvaccinated in-contacts. Natural recombination between attenuated vaccine strains of ILTV to produce virulent viruses has been reported and is also a risk for other live vaccines. The use of multiple different ILTV vaccines in the same populations should therefore be avoided (Coppo *et al*., 2013).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements (e.g. Code of Federal Regulations of the United States of America [USA], 2000; European Pharmacopoeia, 2010).

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

The virus seed for attenuated live ILT vaccines is a suitably attenuated or naturally avirulent strain of ILTV. Attenuation is achieved by serial passage of virus in embryonated eggs or tissue cultures. Recombinant vectored vaccines are created using recombinant DNA methodologies. The master seed virus (MSV) can be propagated in SPF chicken embryos or tissue cultures derived from such embryos. Initial tests are performed to demonstrate the safety and efficacy of the chosen master seed and the master seed must be tested for purity, virus identity and the presence of extraneous pathogens. The MSV is stored in aliquots at –70°C. The viruses used in manufacture should be no more than five passages from the MSV.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Details on the origin of the virus seed, including passage history and any genetic manipulations should be recorded. The MSV is tested in chicken embryos or chickens for sterility and freedom from extraneous agents (see chapter 1.1.9). Virus content is determined by virus titration in chicken embryos or cell culture.

2.1.3. Validation as a vaccine strain

ILT vaccines cross-protect against different strains of ILTV. Infection of non-target species with vaccine strains of ILTV is not a major concern because of the narrow host range of ILTV.

2.2. Method of manufacture

2.2.1. Procedure

In large-scale vaccine production, the virus is propagated in SPF chicken embryos or tissue culture derived from such embryos, up to the fifth passage from the MSV. The acceptable passage

level is supported experimentally by the passage level used to prepare the experimental product used in efficacy studies. The vaccine is made by inoculation of the production seed virus into 9- to 11-day-old chicken embryos or tissue culture prepared from chicken embryos derived from SPF flocks. Eggs are inoculated through a hole in the shell, on to the dropped CAM or into the allantoic sac. They are sealed and incubated at 37°C for 4–6 days. All eggs are candled before harvest and only those with living embryos are used. To harvest the virus, the eggs are chilled, then cleansed and opened aseptically. The CAMs or allantoic fluids are pooled in sterile, cooled containers. The CAMs should show the thick grey plaques typical of ILTV growth. Tissue culture-derived product would be prepared from virus-bearing cell culture fluids, which would also be subsequently pooled and tested.

2.2.2. Requirements for ingredients

Bovine serum used in vaccine production should be sourced from a country with negligible risk for transmissible spongiform encephalopathies [TSEs]. The origin of animal pancreatic trypsin used for cell culture should be considered. See chapter 1.1.8 with a special focus on products of biological origin originating from a country with negligible risk for TSEs.

2.2.3. In-process controls

The infected tissue or tissue culture homogenate may be tested for purity, potency, virus content and safety.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

The presence of ILTV may be confirmed by mixing the vaccine with ILTV antiserum and demonstrating that it is no longer able to infect embryonated hens' eggs from an SPF flock, or susceptible cell lines into which it is inoculated.

iii) Safety

Each batch of vaccine is delivered by eyedrop (10 doses per bird) or by intratracheal injection to SPF chickens, or other target species. The birds are observed for 14–21 days for adverse effects attributable to the vaccine.

iv) Batch potency

Once the *in-vivo* efficacy of the vaccine has been established, the batch potency may be determined by measuring the virus content. This is done by virus titration using serial dilutions of vaccine inoculated onto the dropped CAM of embryonated hens' eggs, or inoculation of suitable cell cultures. The virus content (tested at any time within the expiration period) should be at or above the minimum titre stated on the label. The release and expiration titres are based on the minimum protective dose of the vaccine. Although efficacy testing of each vaccine batch may not be required, efficacy testing of a representative batch (using a vaccinating dose containing not more than the minimum virus titre stated on the label) may be needed.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

In the USA, 25 susceptible chickens, 3- to 4-weeks old, are injected intratracheally and observed for 14 days. Deaths are counted as failures. Four or fewer failures are allowed for satisfactory serials. In the European Union (EU), initial safety tests use the vaccine virus at the least attenuated passage level between the MSV and a batch of the vaccine. Twenty SPF chickens are inoculated with 10 times the vaccine dose and observed for 21 days. Each route and method of administration that is recommended for vaccination should be tested in chickens at the youngest age recommended for vaccination. No notable clinical signs or deaths attributable to ILT are allowed. Batch tests for safety in the EU require eye-drop inoculation of 10 SPF chickens, at the youngest age recommended for vaccination, with 10 times the vaccine dose. Chickens are observed for 21 days. No notable clinical signs or deaths attributable to ILT are allowed.

i) Reversion to virulence

The EU requires five *in-vivo* sequential passages of vaccine virus. Each passage is performed in groups of five SPF chickens. Sequential passage may be achieved by natural spreading, or by collecting and pooling infectious material from the respiratory tract of one group of birds and using this material to infect the subsequent group of birds via eye-drop inoculation. Passaged virus recovered after five sequential passages (or earlier if passage cannot be maintained) is compared with the unpassaged virus using an index of respiratory virulence test. The maximally passaged virus must show no increase in virulence. The index of respiratory virulence tests involves intratracheal inoculation of 0.2 ml of vaccine at three different doses, starting with stock that has titre of 10^5 EID_{50} or 10^5 $CCID_{50}$ per 0.2 ml (or maximal obtainable titre) and then two serial tenfold dilutions of that stock. At least 20 SPF chickens are inoculated per dose. Chickens are observed for 10 days and deaths are recorded. The index of respiratory virulence is the total number of deaths observed in the three groups divided by the total number of chickens

ii) Precautions (hazards)

Care should be taken over diluting and administering the vaccine, and over the proper disposal of unused vaccine.

2.3.3. Efficacy requirements

A test must be carried out to establish the efficacy of the vaccine in groups of birds of the minimum age for which the product is destined and also for each avian species. Each route and method of administration recommended for vaccination must be tested. Twenty (USA) or 30 (EU) chickens are vaccinated with the most attenuated passage level of vaccine virus. Ten additional chickens are held as controls. After 10–14 days (USA) or 21 days (EU) vaccinated chickens and controls are challenged with virulent ILTV intratracheally or in the orbital sinus. At least 80% (USA) or 90% (EU) of unvaccinated controls must die or show severe clinical signs of ILT, or show notable macroscopic lesions. To be satisfactory, only 5% or 10% of the vaccinated birds should die or show severe clinical signs of ILT, or show notable macroscopic lesions.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

The advent of recombinant (vectored) ILT vaccines has brought the potential to differentiate serologically between infected and vaccinated birds and the potential to use DIVA control strategies. Many of the gene-deleted ILT vaccines that are currently under development offer the same potential. A number of studies have reported the use of serological screening tools to detect antibodies against specific ILTV glycoproteins (Coppo *et al.,* 2013).

2.3.5. Duration of immunity

The results of vaccination will depend on many factors, including dose schedule and route of administration. Some degree of protection should be given, over a period of several months.

2.3.6. Stability

Stability is tested by taking samples of correctly stored vaccine at intervals and measuring virus content. Tests should be carried out on at least six batches of the vaccine or until a statistically

valid number of serials have been evaluated and be continued for 3 months after the claimed shelf-life.

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NB: At the time of publication (2021) there were no WOAH Reference Laboratories for avian infectious laryngotracheitis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.3.4.

AVIAN INFLUENZA (INCLUDING INFECTION WITH HIGH PATHOGENICITY AVIAN INFLUENZA VIRUSES)

SUMMARY

Influenza A is caused by specified viruses that are members of the family Orthomyxoviridae *and placed in the genus* Alphainfluenzavirus (Influenzavirus *A or influenza A virus*). *There are seven influenza genera but only influenza A viruses are known to infect birds. Diagnosis is by isolation of the virus or by detection and characterisation of fragments of its genome. This is because infections in birds can give rise to a wide variety of clinical signs that may vary according to the host, strain of virus, the host's immune status, presence of any secondary exacerbating organisms and environmental conditions.*

Detection of the agent: *Suspensions in antibiotic solution of oropharyngeal and cloacal swabs* (*or faeces*) *taken from live birds, or of faeces and pooled samples of organs from dead birds, are inoculated into the allantoic cavity of 9- to 11-day-old embryonated chicken eggs. The eggs are incubated at 37°C* (*range 35–39°C*) *for 2–7 days. The allantoic fluid of any eggs containing dead or dying embryos during the incubation and all eggs at the end of the incubation period are tested for the presence of haemagglutinating activity. The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleoprotein and/or matrix antigens, both of which are common to all influenza A viruses, or by real-time reverse-transcription polymerase chain reaction* (*real-time RT-PCR*) *on the allantoic fluids. Isolation in embryos has largely been replaced for initial diagnosis by direct detection in samples, of one or more segments of the influenza A genome using real-time RT-PCR or other validated molecular techniques.*

For serological subtyping of the virus, a reference laboratory should conduct haemagglutination and neuraminidase inhibition tests against a battery of polyclonal or monospecific antisera to each of the 16 haemagglutinin (*H1–16*) *and 9 neuraminidase* (*N1–9*) *subtypes of influenza A virus. Alternatively, the genome of specific H and N subtypes is identified using RNA detection technologies with subtype specific primers and probes* (*e.g. real-time RT-PCR*) *or sequencing and phylogenetic analysis.*

As the general term 'highly pathogenic avian influenza' and the historical term 'fowl plague' refer to infection with high pathogenicity strains of influenza A virus, it is necessary to assess the pathogenicity of Influenza A virus isolates for domestic poultry. All naturally occurring high pathogenicity avian influenza (*HPAI*) *strains isolated to date have been either of the H5 or H7 subtype, with a subset of H5 or H7 isolates being of low pathogenicity. The methods used for the determination of strain virulence for birds have evolved over recent years with a greater understanding of the molecular basis of pathogenicity. Regardless of their pathogenicity for chickens, H5 or H7 viruses with a HA0 cleavage site amino acid sequence similar to any of those that have been observed in high pathogenicity viruses are considered to be influenza A viruses with high pathogenicity. H5 and H7 isolates that are not highly pathogenic for chickens and do not have an HA0 cleavage site amino acid sequence similar to any of those that have been observed in highly pathogenic viruses are considered to have low pathogenicity. However in some circumstances it is necessary to verify high or low pathogenicity of a virus isolate using the intravenous inoculation of a minimum of eight susceptible 4- to 8-week-old chickens with infectious virus; strains are considered to be of high pathogenicity if they cause more than 75% mortality within 10 days, or inoculation of 10 susceptible 4- to 8-week-old chickens resulting in an intravenous pathogenicity index* (*IVPI*) *of greater than 1.2. Characterisation of suspected highly pathogenic strains of the virus should be conducted in a virus-secure biocontainment laboratory. Low pathogenicity avian influenza* (*LPAI*) *in poultry may be accompanied*

by a sudden and unexpected increase in virulence (emerging disease) or have proven natural transmission to humans associated with severe consequences. In these disease scenarios there should be formal monitoring in relevant poultry populations by national authorities. The occurrence of H5 and H7 low pathogenicity avian influenza viruses should be monitored as some have the potential to mutate into high pathogenicity avian influenza viruses.

Serological tests: *As all influenza A viruses have antigenically similar nucleoprotein and matrix antigens, these are preferred targets of influenza A group serological methods. Enzyme-linked immunosorbent assays (ELISA) are widely used to detect antibodies to these antigens in either host species-dependent (indirect) or species-independent (competitive) test formats. Haemagglutination inhibition tests have also been employed in routine diagnostic serology, but it is possible that this technique may miss some particular infections because the haemagglutinin is subtype specific.*

Requirements for vaccines: *The first use of vaccination in an avian influenza eradication programme was against LPAI. The programmes used inactivated oil-emulsion vaccines with the same haemagglutinin and neuraminidase subtypes as the circulating field virus, and infected flocks were identified by detection of virus or antibodies against the virus in non-vaccinated sentinel birds. During the 1990s the prophylactic use of inactivated oil-emulsion vaccines was employed in Mexico and Pakistan to control widespread outbreaks of HPAI and H5/H7 LPAI. During the 1999–2001 outbreak of H7 LPAI in Italy, an inactivated vaccine was used with the same (i.e. homologous) haemagglutinin subtype to the field virus, but with a different (i.e. heterologous) neuraminidase. This allowed the serological differentiation of non-infected vaccinated birds from vaccinated birds infected with the field virus and ultimately resulted in eradication of the field virus. Prophylactic use of H5 and H7 vaccines has been practised in parts of Italy, aimed at preventing H5/H7 LPAI infections, and several countries in Asia, Africa and the Middle East as an aid in controlling HPAI, in China (People's Rep. of) for H7N9, and in Mexico for H7N3 HPAI virus infections. HPAI viruses should not be used as the seed virus for production of vaccine.*

If LPAI and HPAI viruses are used in challenge studies, an appropriate level of containment should be used as determined by risk assessment.

A. INTRODUCTION

Influenza in birds is caused by infection with viruses of the family *Orthomyxoviridae* placed in the genus *Alphainfluenzavirus* (*influenzavirus A* or influenza A virus) (International Committee on Taxonomy of Viruses (ICTV), 2019). Influenza A viruses are the only orthomyxoviruses known to naturally affect birds (Swayne & Sims, 2020). Many species of birds have been shown to be susceptible to infection with influenza A viruses; aquatic birds form a major reservoir of these viruses, and the overwhelming majority of isolates have been of low pathogenicity (low virulence) for chickens and turkeys. Influenza A viruses have antigenically related nucleoprotein and matrix proteins, but are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (World Health Organization Expert Committee, 1980). At present, 16 H subtypes (H1–H16) and 9 N subtypes (N1–N9) are recognised with proposed new subtypes (H17, H18) for influenza A viruses from bats in Guatemala (ICTV 2019; Swayne *et al.,* 2020; Tong *et al.,* 2013). To date, naturally occurring high pathogenicity influenza A viruses that produce acute clinical disease in chickens, turkeys and other birds of economic importance have been associated only with the H5 and H7 subtypes. Low pathogenicity H5 and H7 occur widely in poultry and aquatic wild birds, although intercontinental spread of HPAI has received greater attention in recent years. There is the risk of a H5 or H7 virus of low pathogenicity (H5/H7 low pathogenicity avian influenza [LPAI]) becoming highly pathogenic by mutation. Some avian influenza virus strains have caused sporadic zoonotic infections principally of H5, H7 and H9 subtypes and these three subtypes have been highlighted as potential pandemic risks should additional mutations occur that support sustained human-to-human transmission (Cox *et al.,* 2017).

Throughout this chapter of the *Terrestrial Manual,* the following terms will be used: 1) HPAI as an infection by an avian influenza virus that meets the definition of high pathogenicity, 2) LPAI as an infection with any H1–H16 avian influenza virus that is not of high pathogenicity, and 3) influenza A as an infection with any HPAI or LPAI virus.

Depending on the species, age and type of bird, specific characteristics of the viral strain involved, and on environmental factors, the highly pathogenic disease, in fully susceptible birds, may vary from one of sudden death with no overt clinical signs, to a more characteristic disease with variable clinical presentations including respiratory signs, such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head,

apathy, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination and nervous signs and diarrhoea (Swayne *et al.,* 2020). In laying birds, additional clinical features include a marked drop in egg production, usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic. In certain host species such as Pekin ducks (*Anas platyrhynchos domesticus*) some HPAI viruses do not necessarily produce significant clinical disease. In addition, LPAI viruses which normally cause only a mild or no clinical disease, may in certain circumstances produce a spectrum of clinical signs, the severity of which may approach that of HPAI, particularly if exacerbating infections and/or adverse environmental conditions are present. Confirmatory diagnosis of the disease, therefore, depends on the isolation or detection of the causal virus and the demonstration that it fulfils one of the defined criteria described in Section B.1.1.1. Testing sera from suspect birds using antibody detection methods may supplement diagnosis, but these methods are not suitable for a definitive identification. Diagnosis for official control purposes is established on the basis of agreed official criteria for pathogenicity according to *in-vivo* tests or to molecular determinants (i.e. the presence of a cleavage site of the haemagglutinin precursor protein HA0 consistent with HPAI virus) and haemagglutinin subtyping. These definitions evolve as scientific knowledge of the disease increases.

HPAI should be subject to official control by national authorities. In addition LPAI, particularly H5 and H7 subtypes, may be subject to national or state/provincial control. The viruses that cause influenza A have the potential to spread from the laboratory if adequate levels of biosecurity and biosafety are not in place. Avian influenza viruses should be handled with appropriate measures as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4. The measures required may vary among the subtypes and pathotypes of influenza A viruses, with higher level containment being indicated for some LPAI and HPAI viruses, and may require additional procedural, equipment and facility enhancements under specific conditions such as high virus concentrations, housing infected animals or conducting procedures with aerosol generating activities. Countries lacking access to such a specialised national or regional laboratory should send specimens to a WOAH Reference Laboratory.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian influenza and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Virus isolation	+	+++	+	+++	+	–
Antigen detection	+	+	+	+	+	–
Real-time RT-PCR	++	+++	++	+++	++	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
AGID	+ (Influenza A)	+ (Influenza A)	++ (Influenza A)	+ (convalescent)	++ (Influenza A)	++ (Influenza A)
HI	+++ (H5 or H7)	++ (H5 or H7)	+++ (H5 or H7)	++ (convalescent)	+++ (H5 or H7)	+++ (H5 or H7)
ELISA	+	+	++	+ (convalescent)	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; AGID = agar gel immunodiffusion;
HI = haemagglutination inhibition test; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

Identification of influenza A viruses as the cause of infections and disease in poultry and other birds requires a thorough diagnostic investigation to differentiate from similar diseases caused by other viral agents especially avian paramyxovirus type 1 (APMV-1). Individual influenza A and APMV-1 virus isolates vary greatly in virulence, causing various syndromes evident as subclinical infections, drops in egg production, respiratory disease, and severe and high mortality disease. The latter clinical syndrome can be caused by either HPAI or Newcastle disease viruses. Therefore, it is judicious to have a single sampling procedure and simultaneously conduct specific differentiating diagnostic tests for both influenza A and APMV-1 viruses on field samples to obtain an accurate aetiological diagnosis of a single agent or, on occasion, confirmation of dual infection.

1.1. Samples for virus isolation

Virus isolation is the reference method but is laborious and time intensive, used primarily for diagnosis of a first clinical case in an outbreak and to obtain virus isolates for further laboratory analysis.

For investigations of severe disease and high mortality in poultry flocks, it is usual to attempt virus isolation from recently dead birds or moribund birds that have been killed humanely. Samples taken from dead birds should include intestinal contents (faeces) or cloacal swabs and oropharyngeal or tracheal swabs. Samples from trachea, lungs, air sacs, intestine, spleen, caecal tonsils, kidney, brain, liver and heart should also be collected and processed either separately or as a pool. When pooling samples the brain should be collected and processed first (to avoid cross contamination with other tissue types) and kept separate as presence of virus in the brain may be an indicator of HPAI or NDV. Further pools should be made consistent with known virus tropisms between HPAI and LPAI, i.e. grouped at the level of respiratory, systemic and gastrointestinal.

Samples from live birds should include both oropharyngeal or tracheal and cloacal swabs, the latter should be visibly coated with faecal material. To avoid harming them, swabbing of small delicate birds should be done with the use of especially small swabs that are usually commercially available and intended for use in human paediatrics or the collection of fresh faeces may serve as an adequate alternative (caution that some influenza A viruses and type 1 avian paramyxoviruses in birds can have a strong respiratory tropism). Similar swab samples can be pooled from the same anatomical site (i.e. cloacal swabs with cloacal swabs, oropharyngeal swabs with oropharyngeal swabs), and most commonly pooling of 5 or occasionally more, if appropriately validated not to reduce sensitivity of detection, but specific swab types should be used (Spackman *et al*., 2013). Further the type of swabs used may affect test sensitivity or validity with thin wire or plastic shafted swabs preferred.

The samples should be placed in isotonic phosphate-buffered saline (PBS), pH 7.0–7.4 with antibiotics or a solution containing protein and antibiotics. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 μg/ml) and mycostatin (1000 units/ml) for tissues and oropharyngeal or tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to re-adjust the pH of the solution to pH 7.0–7.4 following the addition of the antibiotics. It is recommended that a solution for transport of the swabs should contain protein to stabilise the virus (e.g. brain–heart infusion, up to 5% [v/v] cattle serum, 0.5% [w/v] bovine albumen or similar commercially available transport media). If control of *Chlamydophila* is desired, 0.05–0.1 mg/ml oxytetracycline should be included. Faeces and finely minced tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impractical, samples may be stored at 4°C for up to 4 days. For prolonged storage, diagnostic samples and isolates should be kept at −80°C but for transport on dry ice (≤−50°C) is widely used. Repeated freezing and thawing should be avoided.

1.2. Virus isolation

The preferred method of growing influenza A viruses is by the inoculation of specific pathogen free (SPF) embryonated chicken eggs, or specific antibody negative (SAN) eggs. The supernatant fluids of faeces, swabs or tissue suspensions obtained through clarification by centrifugation at 1000 ***g*** for about 10 minutes at a temperature not exceeding 25°C. Clarified preparations can be inoculated using a number of routes including the amniotic sac, chorioallantoic sac or membrane (one of which is recommended for primary isolation) and in all cases allantoic sacs of three to five embryonated SPF or SAN chicken eggs of 9–11 days' incubation. The eggs are incubated at 37°C (range 35–39°C) for 2–7 days. Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C for 4 hours or overnight. After checking that the embryos have died, the amnio-allantoic fluids should be recovered and tested with a screening test (such as haemagglutination [HA] test), influenza A type-specific test (such as agar gel immunodiffusion test [AGID] or solid-phase antigen-capture enzyme-linked immunosorbent assays [ELISA]) or influenza A subtype-specific test (such as haemagglutination inhibition [HI] and neuraminidase [N] inhibition [NI] tests) or a molecular test to detect influenza A specific nucleic acid signatures (such as real-time reverse transcription polymerase chain reaction [RT-PCR]) as described later (see Section B.1.2.2). Detection of HA activity, in bacteria-free amnio-allantoic fluids verified by microbiological assay, indicates a high probability of the presence of an influenza A virus or of an avian orthoavulavirus (formerly avian paramyxovirus). Fluids that give a negative reaction should be passaged into at least one further batch of eggs, and up to three passages.

Routine checks for bacterial contamination should be conducted by streaking samples in Luria Broth agar plates and reading these at 24 and 48 hours of incubation against a light source. BHI agar and blood agar plates may also be used. For larger numbers of sample initial culture could be in tryptose phosphate broth. Contaminated samples can be treated by incubation with increased antibiotic concentrations for 2–4 hours (gentamicin, penicillin g, and amphotericin b solutions at final concentrations to a maximum of 1 mg/ml, 10,000 U/ml, and 20 μg/ml, respectively). Samples heavily contaminated by bacteria that cannot be removed by centrifugation or controlled by antibiotics can be filtrated through 0.45 and 0.2 micron sterile filters. Filtration should be used only when other methods fail because aggregation may significantly reduce virus titre.

1.3. Virus identification

The presence of influenza A virus can be confirmed in AGID tests by demonstrating the presence of the nucleoprotein or matrix antigens, both of which are common to all influenza A viruses (see Section B.2.2). The antigens may be prepared by concentrating the virus from infective allantoic fluid or extracting the infected chorioallantoic membranes; these are tested against known positive antisera. Virus may be concentrated from infective allantoic fluid by ultracentrifugation, or by precipitation under acid conditions. The latter method consists of the addition of 1.0 M HCl to infective allantoic fluid until it is approximately pH 4.0. The mixture is placed in an ice bath for 1 hour and then clarified by centrifugation at 1000 ***g*** at 4°C. The supernatant fluid is discarded. The virus concentrates are resuspended in glycin/sarcosyl buffer: this consists of 1% (w/v) sodium lauroyl sarcosinate buffered to pH 9.0 with 0.5 M glycine. These concentrates contain both nucleoprotein and matrix polypeptides.

Preparations of nucleoprotein-rich antigen can also be obtained from chorioallantoic membranes for use in the AGID test (Beard, 1970). This method involves removal of the chorioallantoic membranes from infected eggs that have allantoic fluids with HA activity. The membranes are then homogenised or ground to a paste. This is subjected to three freeze–thaw cycles, followed by centrifugation at 1000 ***g*** for 10 minutes. The pellet is discarded and the supernatant is used as an antigen following treatment with 0.1% formalin or 1% betapropiolactone.

Use of the AGID test to demonstrate nucleoprotein or matrix antigens is a satisfactory way to indicate the presence of influenza A virus in amnioallantoic fluid, but lacks sensitivity compared to other methods including molecular (see Section 1.2.2) but various experimental and commercial rapid, solid-phase antigen-capture ELISAs (AC-ELISAs) are an effective alternative (Swayne *et al.,* 2020). Most AC-ELISAs have been approved and marketed to detect human influenza A virus in clinical specimens. Some have demonstrated effectiveness for detection of influenza A, but many of these commercial tests have had low sensitivity (Slomka *et al.,* 2012). Those validated for veterinary use are preferred.

Any HA activity of sterile fluids harvested from the inoculated eggs is most likely to be caused by an influenza A virus or an avian paramyxovirus, but a few strains of avian reovirus, as well as nonsterile fluid containing HA of bacterial origin can cause the agglutination of RBCs. There are currently 21 recognised serotypes of avian paramyxoviruses (ICTV, 2019). Most laboratories will have antiserum specific to Newcastle disease virus (avian paramyxovirus type 1, APMV1), and in view of its widespread occurrence and almost universal use as a live vaccine in poultry, it is best to evaluate its presence by haemagglutination inhibition (HI) tests (see Chapter 3.3.14 *Newcastle disease*).

Alternatively, the presence of influenza virus can be confirmed by the use of conventional RT-PCR or real-time RT-PCR using nucleoprotein-specific or matrix-specific conserved primers (Nagy *et al.,* 2020; Spackman *et al*., 2002). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers (Slomka *et al*., 2007; Spackman *et al*., 2002).

Antigenic subtyping can be accomplished by monospecific antisera prepared against purified or recombinant H and N subtype-specific proteins, used in HI and NI tests, or polyclonal antisera raised against a range of intact influenza viruses and used in HI and NI tests. For laboratories conducting the HI test to H subtype it is strongly recommended that two sera for each H subtype is used but with a heterologous N and should ideally use antisera to contemporary viruses relevant to the region in which the virus is detected. Subtyping can also be accomplished using H and N subtype specific primers in RT-PCR and real-time RT-PCR tests; or using sequence analysis of H and N genes. Subtype identification by these techniques is becoming increasingly common but is beyond the scope of many diagnostic laboratories not specialising in influenza viruses. Assistance is available from the WOAH Reference Laboratories and Collaborating Centres (see WOAH website for up-to-date list).

1.4. Assessment of pathogenicity

The term HPAI relates to the assessment of pathogenicity in chickens and implies the involvement of high pathogenicity strains of virus. It is used to describe a disease of fully susceptible chickens with clinical signs that may include one or more of the following: ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, listlessness, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination, nervous signs and diarrhoea. In laying birds, additional clinical features include a marked drop in egg production usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic and high mortality may occur in their absence. In addition, LPAI viruses that normally cause only mild or no clinical disease, may cause a much more severe disease if exacerbating infections or adverse environmental factors are present and, in certain circumstances, the spectrum of clinical signs may mimic HPAI.

The historical term ‘fowl plague’ has been abandoned in favour of the more accurate term HPAI. Because all naturally occurring HPAI viruses to date have been H5 and H7 subtypes and genomic studies have determined HPAI viruses arise by mutation of H5/H7 LPAI viruses, all H5/H7 LPAI viruses may potentially become HPAI but predicting which LPAI strains will mutate to HPAI is not possible. Pathogenicity shifts have been associated with changes to the proteolytic cleavage site of the haemagglutinin including: 1) substitutions of non-basic with basic amino acids (arginine or lysine); 2) insertions of multiple basic

amino acids from codons duplicated from the haemagglutinin cleavage site; 3) short inserts of basic and non-basic amino acids from unknown source; 4) recombination with inserts from other influenza A virus gene segments or avian host cellular genome (e.g. 28S rRNA) that lengthen the proteolytic cleavage site; and 5) loss of the shielding glycosylation site at residue 13 in combination with multiple basic amino acids at the cleavage site1. Amino acid sequencing of the cleavage sites of H5 and H7 subtype influenza A isolates of low pathogenicity for birds may identify viruses that have the capacity, following simple mutation, to have high pathogenicity for poultry.

The following criteria have been adopted by the WOAH for determining pathogenicity of an influenza A virus:

a) One of the two following methods to determine pathogenicity in chickens is used. A high pathogenicity influenza A virus is:

i) any influenza A virus that is lethal[2] for six, seven or eight of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid

or

ii) any influenza A virus that has an intravenous pathogenicity index (IVPI) greater than 1.2. The following is the IVPI procedure:

- Fresh infective allantoic fluid, confirmed free from APMV-1 and other extraneous agents, with a HA titre >1/16 (>24 or >log2 4 when expressed as the reciprocal) is diluted 1/10 in sterile isotonic saline.
- 0.1 ml of the diluted virus is injected intravenously into each of ten 4- to 8-week-old SAN susceptible chickens; if possible, SPF chickens should be used.
- Birds are examined at 24-hour intervals for 10 days. At each observation, each bird is scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead. (The judgement of sick and severely sick birds is a subjective clinical assessment. Normally, 'sick' birds would show one of the following signs and 'severely sick' more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Dead individuals must be scored as 3 at each of the remaining daily observations after death[3].)
- The IVPI is the mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 hours, and an index of 0.00 means that no bird showed any clinical sign during the 10-day observation period.

b) For all H5 and H7 viruses of low pathogenicity in chickens, the amino acid sequence of the connecting peptide of the haemagglutinin molecule (HA0) (i.e. the cleavage site) must be determined. The presence of several basic amino acids, inserts of cellular or viral nucleic acids or loss of specific glycosylation sites in the HA0 cleavage site is the genotypic standard for HPAI strains; therefore, if the isolate being tested has an HA0 cleavage site motif identical to previous HPAI viruses, it should be designated as HPAI irrespective of a low or high pathogenicity determined by pathotyping in chickens (see the table that lists all the reported haemagglutinin proteolytic cleavage sites of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence, which can be found on the OFFLU site (see footnote 2). Furthermore any isolate with a new motif must be tested *in vivo* by IVPI. In case of difficulties in the interpretation of the cleavage site motif, WOAH and/or FAO reference laboratories should be consulted.

The WOAH classification system to identify influenza A viruses for which disease notification and control measures should be taken is defined in the *Terrestrial Code*.

A variety of strategies and techniques have been used successfully to sequence the nucleotides at that portion of the HA gene coding for the cleavage site region of the haemagglutinin of H5 and

1 http://www.offlu.org/wp-content/uploads/2021/01/Influenza_A_Cleavage_Sites.pdf
2 When birds are too sick to eat or drink, they should be killed humanely.
3 When birds are too sick to eat or drink, they should be killed humanely and scored as dead at the next observation.

H7 subtypes of avian influenza virus, enabling the amino acids there to be deduced. This can be done by RNA extraction from the sample and direct sequencing of the haemagglutinin proteolytic cleavage site. Various stages in the procedure can be facilitated using commercially available kits and automated sequencers.

Determination of the cleavage site by sequencing or other methods has become the method of choice for initial assessment of the pathogenicity of these viruses and has been incorporated into agreed definitions. This has reduced the number of *in-vivo* tests, although the initial Sanger sequencing result of a HA cleavage site for an H5 or H7 LPAI virus should be confirmed by either inoculation of birds or deep sequencing using high throughput sequencing with a minimum of 1000 reads to to exclude the presence of any HPAI virus.

Although all the truly HPAI viruses isolated to date have been of H5 or H7 subtypes, at least three isolates, all of H10 subtype (H10N1, H10N4 and H10N5), have been reported that would have fulfilled both the WOAH and EU *in-vivo* definitions for HPAI viruses (Bonfante *et al.,* 2014; Wood *et al*., 1996) as they killed 6/10, 7/10 and 8/10 chickens with IVPI values >1.2 when the birds were inoculated intravenously. However, these viruses did not induce death or signs of disease when inoculated intranasally and did not have a haemagglutinin cleavage site sequence compatible with HPAI virus. Similarly, other intravenously inoculated influenza A viruses are nephrotropic and birds that die have high titres of virus in their kidneys indicating a renal pathogenic mechanism (Slemons & Swayne, 1990), but such laboratory-induced pathobiology is not comparable to multi-organ infection and systemic disease caused by HPAI viruses. An H4N2 virus isolated from quail had a multibasic cleavage site sequence (PEKRRTR/GLF) but with an IVPI value of 0.0 (Wong et al., 2014) suggesting the multibasic cleavage site in viruses other than H5 and H7 alone may not be sufficient for declaration of HPAI virus and the in-vivo test should be carried out. Conversely, four viruses (A/chicken/Pennsylvania/1/83 [H5N2] and A/goose/Guangdong/2/96 [H5N1], A/turkey/England/87-92BFC/91 [H5N1] or A/chicken/Texas/298313/04 [H5N2]) have been described that have HA0 cleavage sites containing multiple basic amino acids, but which show low pathogenicity (IVPI <1.2) when inoculated intravenously into 6-week-old chickens (Londt *et al*., 2007).No single explanation including the presence of a glycosylation site masking the HA0 cleavage site was reported emphasising both intra-haemagglutinin and multigenic influences in rare circumstances upon phenotypic expression of high pathogenicity. The presence of high pathogenicity haemagglutinin cleavage site in H5 and H7 influenza A viruses necessitates declaration of high pathogenicity to facilitate immediate control of the disease, otherwise a delay to complete *in-vivo* testing may result in continued onward transmission and spread between premises with severe consequence for future eradication once confirmed as a HPAI virus.

A table is available on the OFFLU website that lists all the reported haemagglutinin proteolytic cleavage site of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence. This table will be updated as new viruses are characterised; it can be found on the OFFLU site (see footnote 2).

1.5. Antigen capture and molecular techniques

At present, conventional virus isolation and characterisation techniques for the diagnosis of influenza A viruses remain a key method, for initial diagnosis of influenza A infection in a primary disease event and to provide virus for more detailed analyses including *in-vivo* testing and gene sequencing. Further they may be invaluable in confirming or disproving the presence of infectious virus when other test results including conventional and real-time RT-PCR are all weakly positive. However, conventional methods tend to be costly, labour intensive and slow. There have been enormous developments and improvements in molecular and other diagnostic techniques, many of which are now routinely applied as a first choice for the diagnosis of influenza A infections.

1.5.1. Antigen detection

There are several commercially available AC-ELISA kits that can detect the presence of influenza A viruses in poultry (Swayne *et al.,* 2020). Most of the kits are enzyme immunoassays or are based on immunochromatography (lateral flow devices) and use a monoclonal antibody against the nucleoprotein; they should be able to detect any influenza A virus. The main advantage of these tests is that they can demonstrate the presence of influenza A within 15 minutes. The disadvantages are that they may lack sensitivity, they may not have been validated for different

species of birds, H and N subtype identification is not achieved and the kits are expensive. The tests should only be interpreted on a flock basis and not as an individual bird test. Oropharyngeal or tracheal samples from clinically affected or dead birds provide the best sensitivity. Nevertheless, the lack of sensitivity is a major drawback to the use of available antigen detection tests. Test sensitivities may vary between cloacal and tracheal swabs, whilst the tests can perform less well with samples from waterfowl or wild birds compared with chickens. Improved but moderate sensitivity in so named lateral flow devices was reported when using samples of feather follicles from birds infected with HPAI (Slomka *et al.,* 2012). Because of low sensitivity, antigen detection is mainly used for field screening of high mortality clinical cases for suspected influenza A virus infections followed by confirmation of results using a more sensitive laboratory-based test.

1.5.2. Direct RNA detection

As demonstrated by the current definitions of HPAI, molecular techniques are used preferentially for diagnosis for some time now. Furthermore, there have recently been developments towards their application to the detection and characterisation of influenza A viruses directly from clinical specimens of infected birds. It is imperative that when using highly sensitive molecular detection methods that allow rapid direct detection of viral RNA for confirmatory laboratory diagnosis of influenza A infections, stringent protocols are in place to prevent the risk of cross-contamination between clinical samples. In addition, RNA detection test methodologies should be validated to the WOAH standard (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*) using clinical material to demonstrate the tests as being 'fit for purpose' for application in a field diagnostic setting, which may include the use of internal test standards. The control reactions enable greater confidence in the integrity of the molecular reactions, clinical samples and results.

Furthermore, these evaluations enable the appropriate setting of test thresholds for interpretation between positive and negative samples. The increased sensitivity of real-time RT-PCR leads to the detection of viral RNA in samples in the absence of infectious virus and care should be taken when interpreting outputs with small detection limits that may not be indicative of active infection. This problem can be overcome, through the testing of multiple samples from the same cohort of infected birds, especially relevant when testing samples from domestic poultry for disease investigation.

In settings with more limited facilities, RT-PCR techniques on clinical samples can, with the correctly defined primers, result in rapid detection and subtype identification (at least of H5, H7 and H9 subtypes, and more recently developed assays are also available for other subtypes), including a cDNA product that can be used for nucleotide sequencing However, these approaches have now been largely replaced by the preferred molecular detection tests for influenza A virus by real-time RT-PCR, a modification to the RT-PCR that reduces the time for both identification of virus subtype and sequencing. For example, Spackman *et al*. (2002) used a single-step real-time RT-PCR primer/fluorogenic hydrolysis probe system to allow detection of influenza A viruses and determination of subtype H5 or H7. The test performed well relative to virus isolation and offered a cheaper and much more rapid alternative, with diagnosis on clinical samples in less than 3 hours. In additional studies, the real-time RT-PCR was shown to have sensitivity and specificity equivalent to virus isolation in numerous settings but updates to primer/probe design can be beneficial over time to accommodate genetic evolution in gene regions targeted by assays (Laconi *et al.,* 2020). These tests provide high sensitivity and specificity similar to those of virus isolation when used on tracheal and oropharyngeal swabs of chickens and turkeys, but may lack sensitivity for detection of influenza A virus in faecal swabs, faeces and tissues in some bird species, because of the presence of PCR inhibitors resulting in false-negative results (Das *et al*., 2006). Incorporation of a positive internal control into the test will verify a proper test run. In addition, improvements in RNA extraction methods have been developed to eliminate most PCR inhibitors from test samples.

Real-time RT-PCR, usually based around the hydrolysis probe method for generation of the target-specific fluorescence signal, has become the method of choice in many laboratories for at least partial diagnosis directly from clinical specimens. The method offers rapid results, with sensitivity and specificity comparable to virus isolation. These are ideal qualities for influenza A outbreak management, where the period of time in which an unequivocal diagnosis can be obtained is crucial for decision making by the relevant Veterinary Authority. In addition, real-time

RT-PCR systems can be designed to operate in a 96-well format and combined with high-throughput robotic RNA extraction from specimens (Aguero *et al*., 2007).

The approach to diagnosis using real-time RT-PCR adopted in most laboratories has been based on initial generic detection of influenza A virus in clinical samples, primarily by initially targeting the matrix (M) gene, which is highly conserved for all influenza A viruses, followed by specific real-time RT-PCR testing for H5 and H7 subtype viruses. Numerous assays have been reported for highly sensitive detection of M (or NP) gene fulfilling the criteria for a suitable screening test. For subtype identification, primers used in real-time RT-PCR are targeted at the HA2 region, as this is relatively well conserved within the haemagglutinin genes of the H5 and H7 subtypes (Spackman *et al*., 2008; Spackman & Suarez, 2008). It has therefore served as the target region for these subtypes. Spackman *et al.* (2002) demonstrated specific detection of these subtypes, but cautioned that their H5 and H7 primer/probe sequences had been designed for the detection of North American H5 and H7 isolates and might not be suitable for all H5 and H7 isolates. This proved to be the case. Slomka *et al.* (2007) described modification of the H5 oligonucleotide sequences used by Spackman *et al.* (2002) to enable the detection of the Eurasian 'Goose/Guangdong lineage' (Gs/GD) H5N1 subtype and other Eurasian H5 subtypes that have been isolated within the past 15 years in both poultry and wild birds. As the group of 'Gs/GD' viruses diversified and spread across several continents it has become important that diagnostics in all settings have proven fit for purpose detection of this H5 lineage of viruses divided into multiple clades (World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization & H5N1 Evolution Working Group, 2014). Newer rapid methods have been developed that enable simultaneous detection and subtyping speeding the time to achieve rapid identification of an influenza A virus using arrays (Hoffmann *et al.,* 2016) or microchip (Kwon *et al.,* 2019) technologies. The validated Eurasian real-time RT-PCR have proven valuable in the investigation of many H5Nx HPAI clinical samples and other subtypes submitted to International Reference Laboratories from Europe, Africa, Asia and North America since 2005 (Liu *et al.,* 2018; Slomka *et al*., 2007). Each set of primers and probes needs to be validated against a diverse set of viruses to make the test applicable in a diverse range of avian species, and in viruses from broad geographic areas and time periods. In addition, real-time RT-PCR methods are now widely used for the rapid and accurate determination of the neuraminidase subtype (James *et al.,* 2018)

One of the problems with rapidly emerging new tests is that methods and protocols may be developed and reported without the test being properly validated. This has been addressed for some of the real-time RT-PCR protocols. In the European Union, National Reference Laboratories have collaborated to define and validate protocols that can be recommended for use within Europe (Hoffmann *et al.,* 2016; Nagy *et al.,* 2020; Slomka *et al*. 2007). Importantly this should include routine analysis of detected viruses (coordinated through WOAH Reference Laboratories) in standard assays to ensure reliable specific detection of contemporary viruses affecting poultry and other populations. In addition, given the high variability in the influenza A genome it is imperative that assays used in routine diagnosis and surveillance have ongoing demonstration of their fitness for detection of contemporary viruses validated for use in the region where they are applied. There should be an appropriate match for local strains taking account of significant regional and intercontinental variability amongst particular endemic viruses. Laconi *et al.* (2020) in reviewing five validated well used real-time RT-PCR methods concluded that continuous monitoring of assay performance using both in silico and *in-vitro* methodology was important as the emergence of new strains containing mutations within primer and probe binding areas might significantly affect the positive outcome of a test. Increasingly with improvements in assay design and using novel biochemical approaches screening assays relevant to all influenza A viruses from all hosts (animal and human) have been developed (Nagy *et al.,* 2020) with high relevance to an avian–'other' host interface.

Real-time RT-PCR protocols have been described that amplify regions across the cleavage site of the HA0 gene. This may result in useful tests for specific viruses. For example, Hoffman *et al.* (2007) have described a real-time RT-PCR test specific to the Eurasian HPAI H5N1 Qinghai-like clade 2.2 viruses that represents a rapid means of determining the pathotype for this subgroup of H5N1 HPAI viruses without sequencing. In situations where large numbers of positive samples/cases are detected during disease events, specific targeted real-time RT-PCR assays have been developed for the simultaneous sensitive detection and pathotyping of viruses. This can prove to be very useful, particularly when applying to early warning systems such as

surveillance of wild bird populations for local presence of HPAI (Graaf *et al.,* 2017; Naguib *et al.,* 2017).

Modifications to the straightforward RT-PCR method of detection of viral RNA have been designed to reduce the effect of inhibitory substances in the sample taken, the possibility of contaminating nucleic acids and the time taken to produce a result. The loop-mediated isothermal amplification (LAMP) system for H5 and H7 detection appears to show high sensitivity and reliable specificity (Ahn *et al.,* 2019; Bao *et al.,* 2014), but may have limited application because of susceptibility to viral mutations affecting the target regions, reducing virus detection (Postel *et al.,* 2010).

Increasing innovation and technological improvements have made it possible that molecular based and improved antigen detection technologies have developed sufficiently to permit rapid flock side tests for the detection of presence of influenza A virus specific subtypes and pathogenicity markers (Inui *et al.,* 2019). Furthermore, innovations in test design have enabled for example the development of point of care chip based ultrafast PCR approaches (Kwon *et al.,* 2018) with increasing application anticipated in the future.

1.5.3. Gene sequencing

Currently real-time RT-PCR is the preferred method of virus surveillance because the test provides rapid sensitive diagnostics for influenza H5, H7 and H9 and is available in high throughputs. However, greater use of sequencing technologies particularly as unit costs reduce with improvement in technology, offer powerful opportunities to simultaneously detect and sequence from clinical samples in a laboratory or field setting, for example applying nanopore technology (King *et al.,* 2020).

Increasingly gene sequencing is being applied not only to detailed characterisation of viruses for use in molecular epidemiology but also in virus subtyping and defining markers for host range including zoonotic risk. Sanger sequencing methodology has been widely used for decades and enables the rapid determination of typically a single (H) target gene in 24-36 hours to define virus pathogenicity (see Section B.1.1.1) and still has widespread utility. However, as genomic data can be rapidly determined using next generation sequencing technology it enables a broader analysis using a range of bioinformatics tools (Zhang *et al.,* 2017). For example, with the advent of greater access to sequencing methodology either through specialised laboratories or commercial providers it is now possible to determine the genomic sequences of influenza A viruses from birds to provide a level of characterisation important in rapid pathogen identification and outbreak intervention. Conventionally nucleotide sequences have been used in outbreak epidemiology to infer virus origin and precise relationships between different viruses associated within the same event (by phylogeny) to support outbreak management. Virus gene sequences of haemagglutinin and neuraminidase can rapidly be compared to known sequences of all subtypes in gene databases and used to reveal closest match thereby identifying the virus subtype and phylogenetic relationships. This often avoids the need to culture the virus for rapid identification although reliability and quality of data reduces with increasing cycle threshold values in samples from real-time RT-PCR testing.

Increasingly such analyses are now being applied at the whole genome level to reveal virus genotypes and provide greater analytical specificity to the analyses. Such approaches are especially valuable to track since virus evolution which can be more precisely mapped including change through genetic reassortment, a key mechanism associated with virus diversity and fitness for birds. This approach is especially valuable for early or first incursions in a new event as it enables greater precision in determining virus origin and the mechanisms leading to the emergence of virus. This has become increasingly important in characterising the rapid evolution and wide diversity of Gs/GD lineage viruses associated with transcontinental spread. Translation of nucleotide sequences of all genomic segments into amino acid sequences enables data mining for other virus characteristics or traits such as tropism, host range markers including zoonotic and predicted antiviral drug susceptibility which are invaluable for informing outbreak management.

2. Serological tests

2.1. Enzyme-linked immunoassay (ELISA)

Commercial ELISA kits that detect antibodies against the nucleoprotein are available. Kits with an indirect and competitive/blocking format have been developed and validated, and are now being used to detect influenza A virus-specific antibodies. Several avian influenza competitive ELISA (AIV C-ELISA) or blocking ELISA (AIV B-ELISA) have been developed and validated as a more sensitive alternative to the AGID test for the detection of influenza A group reactive antibodies in sera from chickens and other bird species (SCAHLS, 2009). This AIV ELISA platform, as either a "competitive" or "blocking" format, detects antibodies to influenza A viruses by allowing these antibodies to compete for antigen binding sites with a monoclonal antibody against an epitope on the nucleoprotein that is conserved in all influenza A viruses.

The kits should be validated for the specific species of interest and for the specific purpose(s) for which they are to be used. Several different test and antigen preparation methods are used. Such tests have usually been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. Please see the WOAH Register for kits certified by the WOAH[4]. ELISA kits are of moderate cost and are amenable to high throughput screening for influenza A virus infections and have strong utility for application to large-scale serosurveillance programmes and compare favourably to HI (Arnold *et al.,* 2018). However, all positive results must be followed by HI test for subtyping to H5 and H7. Some subtype-specific ELISA kits are available, e.g. for antibodies to H5, H7, H9 and some N subtypes i.e. N1 but generally are of lower sensitivity than influenza A ELISA.

2.2. Agar gel immunodiffusion

All influenza A viruses have antigenically similar nucleoprotein and antigenically similar matrix antigens. Owing to this fact AGID tests are able to detect the presence or absence of antibodies to any influenza A virus. Concentrated virus preparations, as described above, contain both matrix and nucleoprotein antigens; the matrix antigen diffuses more rapidly than the nucleoprotein antigen. AGID tests have been widely and routinely used to detect specific antibodies in chicken and turkey flocks as an indication of infection, but AGID tests are less reliable at detecting antibodies following infection with influenza A viruses in other avian species. These have generally employed nucleoprotein-enriched preparations made from the chorioallantoic membranes of embryonated chicken eggs (Beard, 1970) that have been infected at 10 days of age, homogenised, freeze–thawed three times, and centrifuged at 1000 ***g***. The supernatant fluids are inactivated by the addition of 0.1% formalin or 1% betapropiolactone, recentrifuged and used as antigen. Not all avian species may produce precipitating antibodies following infection with influenza viruses, for example ducks. The AGID is a low-cost serological screening test of reduced sensitivity for detection of generic influenza A infections, but must be followed by HI tests for subtyping influenza A positives as to H5 and H7.

Tests are usually carried out using gels of 1% (w/v) agarose or purified type II agar and 8% (w/v) NaCl in 0.01 M phosphate buffer, pH 7.2, poured to a thickness of 2–3 mm in Petri dishes or on microscope slides, and incubated in a humidified chamber. Using a template and cutter, wells of approximately 5 mm in diameter are cut into the agar at a distance of about 3 mm from each other. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. Each well should have reagent added to fill the well, corresponding to the top of the meniscus with the top of the gel, but do not over fill. Approximately 25–30 µl of each reagent should be required per well, but this depends on thickness of the gel, with thicker gels requiring an additional volume of reagent.

Wells should be examined for precipitin lines at 24 hours, and weak positive samples or samples for which specific lines have not formed should be incubated longer and examined again at 48 hours. The time to formation of visible precipitin line is dependent on the concentrations of the antibody and the antigen. The precipitin lines are best observed against a dark background that is illuminated from behind. A specific, positive result is recorded when the precipitin line between the known positive control wells is

4 https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-5/

continuous with the line between the antigen and the test well. Crossed lines are interpreted to be caused by the test serum lacking identity with the antibodies in the positive control well.

Whilst the AGID is relatively inexpensive and suitable for resource limited settings it is being increasingly replaced by other platforms such as ELISA for flock level serological investigations including pre export/import screening of birds for historical exposure to influenza A.

2.3. Haemagglutination and haemagglutination inhibition tests

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply to the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. U- bottomed plates can be used but care in reading is required as the clarity is less defined. The reagents required for these tests are isotonic PBS (0.01 M), pH 7.0–7.4, and red blood cells (RBCs) taken from a minimum of three SPF or SAN chickens and pooled into an equal volume of Alsever's solution. Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

2.3.1. Haemagglutination test

i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.

ii) Place 0.025 ml of virus suspension (i.e. infective allantoic fluid) in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.

iii) Make twofold dilutions of 0.025 ml volumes of the virus suspension across the plate.

iv) Dispense a further 0.025 ml of PBS to each well.

v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.

vi) Mix by tapping the plate gently and then allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C, if ambient temperatures are high, by which time control RBCs should have formed a distinct button.

vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

2.3.2. Haemagglutination inhibition test

i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.

ii) Place 0.025 ml of serum into the first well of the plate.

iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.

iv) Add 4 HAU of virus/antigen in 0.025 ml to each well and leave for a minimum of 30 minutes at room temperature (i.e. about 20°C) or 60 minutes at 4°C.

v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and mix gently, allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, by which time control RBCs should have formed a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre >1/4 ($>2^2$ or $>\log_2 2$ when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

The HI test is primarily used to determine if antibodies indicating influenza A virus infections are subtyped as H5 and H7 or other H subtypes (H1-4, H6, H8-16). HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2^4 or $\log_2$ 4 when expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (2^3 or $\log_2$ 3) or more. The meaning of a minimum positive titre should not be misinterpreted; it does not imply, for example, that immunised birds with that titre will be protected against challenge or that birds with lower titres will be susceptible to challenge. Appropriate virus/antigen control, positive control serum and RBC control well should be included with each batch of HI tests.

Chicken sera rarely give nonspecific positive agglutination reactions in this test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken RBCs resulting in nonspecific agglutination. Therefore, each serum should first be tested for this idiosyncrasy and, if present, it should be inhibited by adsorption of the serum with chicken RBCs. This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, mixing gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 ***g*** for 2–5 minutes and the adsorbed sera are decanted. Alternatively, RBCs of the avian species under investigation could be used. Nonspecific inhibition of agglutination can be caused by steric inhibition when the tested serum contains antibodies against the same N subtype as the H antigen used in the HI test. The steric inhibition reaction can result in RBC buttoning in the bottom of the plate or streaming at the same rate as the control. If using whole virus antigen in HI test for subtyping, it is important to ensure that two antigens for each haemagglutinin subtype are used with heterologous neuraminidase i.e. H5N1 and H5N6 to eliminate the possibility of interference in the assay with anti N antibodies that can lead to false typing results. Alternatively the H antigen used can be recombinant or purified H protein that lacks N protein. The HI test is based on antigenic binding between the H antigen and antisera and thus other factors may cause nonspecific binding of the H antigen and sera leading to a nonspecific inhibition reaction. At this time there are no documented cross reactions or nonspecific inhibition reactions between the different haemagglutinin subtypes of influenza A.

2.4. Neuraminidase inhibition test

The neuraminidase-inhibition test has been used to identify the influenza A neuraminidase type of isolates as well as to characterise the antibody in infected birds. The procedure requires specialised expertise and reagents; consequently, this testing is usually done in a WOAH Reference Laboratory. The DIVA (differentiating infected from vaccinated animals) strategy used previously in Italy also relies on a serological test to detect specific anti-N antibodies; the test procedure has been described (Capua *et al*., 2003).

C. REQUIREMENTS FOR VACCINES

1. Background

Vaccination alone is not the solution to the control of HPAI if eradication is the desired result. Without the application of monitoring systems, strict biosecurity and depopulation in the face of infection, HPAI will become endemic in vaccinated poultry populations. Long-term circulation of the virus in a vaccinated population may result in both antigenic and genetic changes as has occurred with H5Nx (Gs/GD lineage), H7N3, H7N9 and H9N2 influenza A viruses in Mexico, and various Middle Eastern and Asian countries (Swayne & Sims, 2020). Currently used vaccines and the use of vaccination have been reviewed (FAO, 2016; Swayne & Sims, 2020). The haemagglutinin is the primary influenza A viral protein that elicits a protective immune response used in officially approved poultry vaccines and such immunity is haemagglutinin subtype specific.

To date, the majority of influenza A vaccines used in poultry have been inactivated whole virus vaccines prepared from infective allantoic fluid of embryonated chicken eggs, inactivated by beta-propiolactone or formalin and emulsified with mineral oil adjuvants. Because of the potential for reassortment leading to increased virulence, live conventional influenza vaccines against any subtype are not recommended. However, biotechnology holds great potential to generate live avian influenza virus vaccines with altered gene segments which reduce the risk of reassortment, limit replication and abrogate negative aspects of live influenza A virus vaccines (Song *et al.,* 2007). The existence of a large number of haemagglutinin subtypes (i.e. H1–16), together with the known variation of different strains within a subtype, pose serious problems when selecting strains to produce inactivated influenza A

vaccines. In addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly pre-concentration. While some vaccination strategies use autogenous vaccines, i.e. vaccines prepared from isolates specifically involved in an epizootic, others rely on vaccines prepared from biologically characterised, fully approved seed strain viruses possessing the same haemagglutinin subtype as the field virus and capable of yielding high concentrations of antigen. Historically, inactivated vaccines for LPAI and HPAI control have used LPAI viruses with a matching haemagglutinin subtype from outbreaks as seed strains, but with development of resistance in the field associated with prolonged vaccine use, the majority of inactivated vaccine seed strains are now reverse genetic derived virus with antigenically close matching haemagglutinin, and sometimes neuraminidase, to circulating field viruses. Use of HPAI viruses as inactivated vaccine seed strains is strongly discouraged because of biosafety concerns.

Since the 1970s in the USA, inactivated influenza A vaccines have been used primarily in turkeys against LPAI viruses under emergency vaccination programs, but since the 2000s, most vaccines have been against H1 and H3 swine influenza A viruses used under a routine preventative vaccination program in breeder turkeys (Swayne *et al.,* 2020). Since the early 1990s, vaccination against H9N2 LPAI virus has been used extensively in Asia and the Middle East using billions of inactivated vaccine doses (Swayne & Sims, 2020). Vaccination against HPAI was first used in Mexico during the H5N2 outbreaks of 1994–1995 (Villarreal, 2007), and in Pakistan (Naeem, 1998) during the H7N3 outbreaks of 1995. Beginning with H5N1 goose/Guangdong (Gs/GD) lineage HPAI outbreaks in Hong Kong in 2002 (Sims, 2003), a vaccination policy was adopted using H5N2 LPAI vaccine seed strains and subsequently replaced with H5Nx reverse genetic vaccine seed strains, as the field virus spread throughout and outside of China Between 2002 and 2010, 113 billion doses of vaccine was used to control HPAI with 95% being inactivated and 5% recombinant vaccines, and a similar usage rate continues (Swayne *et al.,* 2011; Swayne & Sims, 2020). As the H5Nx Gs/GD lineage HPAI spread across the global, additional countries have implemented emergency and/or preventative vaccination programs for HPAI control. Similarly, preventive vaccination against H5N1 HPAI has been permitted for outdoor poultry and zoo birds in several European Union countries in in the 2000s.

Live recombinant virus-vectored vaccines with H5 influenza A virus haemagglutinin gene inserts have been approved and used in a few countries since 1997, mostly in chickens, and include recombinant fowl poxvirus (rFPV), recombinant Newcastle disease virus (rNDV) and recombinant herpesvirus turkey vaccines (rHVT). Since 2015, non-replicating, haemagglutinin based H5 RNA particle, H5 expressed baculovirus and H5 DNA vaccines have been approved for poultry but have had limited use (Swayne & Sims, 2020).

1.1. Rationale and intended use of the product

Experimental work has shown, for HPAI and LPAI, that potent and properly administered vaccines increase resistance to, or prevent infection, protect against clinical signs and mortality, prevent drops in egg production, reduce virus shedding from respiratory and intestinal tracts, protect from diverse field viruses within the same haemagglutinin subtype, protect from low and high challenge exposure, and reduce excretion and thus prevent contact transmission of challenge virus (Capua *et al*., 2004; Swayne & Sims, 2020). Although, in experimental vaccination studies, a challenge virus is still able to infect and replicate in clinically healthy vaccinated SPF birds when exposed to high doses, the quantities shed may be insufficient for onward transmission of the virus (Van der Goot et al., 2005). Most of the work evaluating vaccines has been done in chickens and turkeys and some care must be taken in extrapolating the results obtained to other species. Most national HPAI and LPAI control regulations reserve the right to use vaccines in emergencies.

2. Outline of production and minimum requirements for conventional vaccines

The information below is based primarily on the experiences in the USA and the guidance and policy for regulatory approval of influenza A vaccines in that country (United States Department of Agriculture, 1995 [updated 2006]). The basic principles for producing vaccines, particularly inactivated vaccines, are common to several viruses e.g. Newcastle disease (chapter 3.3.14).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If HPAI virus is to be used in challenge studies, the facility used for such studies should meet the competent veterinary authority within the country minimum requirements for Containment Group 3 pathogens as outlined in chapter 1.1.4.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

For any subtype, only well characterised influenza A virus of proven low pathogenicity, preferably obtained from an international or national repository, should be used to establish a master seed for inactivated vaccines. HPAI viruses should not be used as seed virus for vaccine. For HPAI, reverse genetic produced vaccine seed strains based on haemagglutinin gene of the HPAI virus are preferred, but should have the cleavage site sequence altered to that of a H5/H7 LPAI virus.

A master seed is established from which a working seed is obtained. The master seed and working seed are produced in SPF or SAN embryonated eggs. The establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised aliquots (0.5 ml).

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The established master seed should be controlled/examined for sterility, safety, potency and absence of specified extraneous agents.

2.2. Method of manufacture

2.2.1. Procedure

For vaccine production, a working seed, from which batches of vaccine are produced, is first established in SPF or SAN embryonated eggs by expansion of an aliquot of master seed to a sufficient volume to allow vaccine production for 12–18 months. It is best to store the working seed in liquid form at below –60°C as lyophilised virus does not always multiply to high titre on subsequent first passage.

The routine procedure is to dilute the working seed in sterile isotonic buffer (e.g. PBS, pH 7.2), so that about 10^3–10^4 EID_{50} in 0.1 ml are inoculated into each allantoic cavity of 9- to 11-day-old embryonated SPF or SAN chicken eggs. These are then incubated at 37°C. Eggs containing embryos that die within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure maximum yield with the minimum number of embryo deaths.

The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the allantoic fluids collected by suction. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination.

In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000, i.e. 0.1% formalin) or beta-propiolactone (BPL) (a typical final concentration is 1/1000–1/4000, i.e. 0.1–0.025% of 99% pure BPL). The time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are formulated with non-concentrated inactivated allantoic fluid (active ingredient). However, active ingredients may be concentrated for easier storage of antigen. The active ingredient is usually emulsified with mineral or vegetable oil and surfactants. The exact formulations are generally commercial secrets.

2.2.2. Requirements for substrates and media

The inactivated influenza A vaccines prepared from conventional virus are produced in 9- to 11-day-old embryonated SPF or SAN chicken eggs. The method of production is basically the same as for propagating the virus aseptically; all procedures are performed under sterile conditions.

2.2.3. In-process controls

For inactivated vaccines, completion of the inactivation process should be tested in embryonated eggs, taking at least 10 aliquots of 0.2 ml from each batch and passaging each aliquot at least twice through SPF or SAN embryos. Viral infectivity must not remain.

2.2.4. Final product batch tests

Most countries have published specifications for the control of production and testing of vaccines, which include the definition of the obligatory tests on vaccines during and after manufacture.

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

For inactivated vaccines, a double dose is administered by the recommended route to ten 3-week-old birds, and these are observed for 2 weeks for absence of clinical signs of disease or local lesions.

iii) Batch potency

Potency of influenza A vaccine is generally evaluated by testing the ability of the vaccine to induce a significant HI titre in SPF or SAN birds. Conventional potency testing involving the use of three diluted doses and challenge with HPAI virus (e.g. chapter 3.3.14) may also be used for vaccines prepared to give protection against LPAI subtypes. For inactivated vaccines against HPAI or LPAI virus, potency tests may rely on the measurement of immune response or challenge and assessment of morbidity, mortality (HPAI only) and quantitative reduction in challenge virus replication in respiratory (oropharyngeal or tracheal) and intestinal (cloaca) tracts. Assessment of haemagglutinin antigen content could allow for *in-vitro* extrapolation to potency for subsequent vaccine batches.

iv) Preservatives

A preservative may be used for vaccine in multidose containers.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Most inactivated influenza A vaccines are approved for use in chickens and turkeys. Field trials in the target species should be conducted to determine tolerance and safety of the vaccine at full dose. Recently the use of inactivated influenza A vaccines has been expanded to ducks, geese, other poultry and zoo birds. Any extra-label use of the vaccines should be done cautiously and under the supervision of a veterinarian experienced in disease control through vaccination in the test species. Care must be taken to avoid self-injection with oil emulsion vaccines.

ii) Reversion-to-virulence for attenuated/live vaccines

Only inactivated influenza A virus vaccines are recommended. Live conventional influenza vaccines against any subtype are not recommended because of the risk for reassortment of gene segments of vaccine virus with field virus, potentially creating more pathogenic field viruses.

iii) Environmental consideration

None

2.3.2. Efficacy requirements

i) For animal production

For regulatory purposes, influenza A vaccines should pass an efficacy challenge test using a statistically relevant number of SPF or SAN chickens per group. The challenge should occur at a minimum of three weeks post-vaccination, using a challenge HPAI virus dose that

causes 90% or greater mortality in the sham population. A standardised challenge dose of 10^6 mean chicken embryo infectious doses is most widely used. Protection from mortality in the vaccine group should be a minimum of 80%. For LPAI, mortality is not a feature of challenge models, therefore a statistically significant reduction in virus shedding titre and/or the number of birds shedding virus from oropharynx or cloaca should be observed between sham and test vaccine groups. Other metrics of protection can be used to determine efficacy such as prevention of drops in egg production.

In establishing minimum antigen requirements, 50 PD_{50} or 3 µg of haemagglutinin per dose have been recommended (Swayne & Sims, 2020). Minimum HI serological titres in field birds should be 1/32 to protect from mortality or greater than 1/128 to provide reduction in challenge virus replication and shedding for antigenically close related vaccine and challenge viruses.

ii) For control and eradication

Efficacy should be the same as for animal production.

2.3.3. Stability

When stored under the recommended conditions, the final vaccine product should maintain its potency for at least 1 year. Inactivated vaccines must not be frozen.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Recombinant live vaccines for influenza A viruses have been produced by inserting the gene coding for the influenza A virus haemagglutinin into a non-influenza live virus vector and using this recombinant virus to immunise poultry against influenza A (Swayne & Sims, 2020). Recombinant live vector vaccines have several advantages over inactivated influenza A vaccines: 1) they induce mucosal, humoral and cellular immunity; 2) they can be mass administered *in ovo* or to 1-day-old birds in the biosecure hatchery to induce early protection; and 3) they enable easy serological differentiation of infected from non-infected vaccinated birds because they do not induce the production of antibodies against the nucleoprotein or matrix antigens that are common to all influenza A viruses; i.e. differentiation of infected from vaccinated (DIVA) animals. Therefore, only field-infected birds will exhibit antibodies in the AGID test or ELISAs directed towards the detection of influenza group A (nucleoprotein and/or matrix) antibodies. However, recombinant live vaccines have limitations in that they may have reduced replication and thus induce no or only partial protective immunity in birds that have had field exposure to or vaccine induced immunity against the vector virus or the H gene insert (Bertran *et al.,* 2018; Swayne & Sims, 2020). If used in day-old or young birds, the effect of maternal antibodies to the vector virus on vaccine efficacy may vary with the vector type; i.e. most severe inhibition in decreasing order for Newcastle disease virus, fowl poxvirus and HVT vectors. In addition, because the vectors are live viruses that may have a restricted host range, the use of such vaccines must be restricted to species in which efficacy has been demonstrated.

A rFPV-H5 vaccine, with H gene insert for A/turkey/Ireland/1378/1983 (H5N8), was developed in the early 1980s and authorised beginning in 1998 for use against H5N2 LPAI of Mexico (Swayne & Sims, 2020). This vaccine has principally been used in Mexico with expansion into several other countries within Central America and Vietnam with over 9 billion doses used between 1998 and 2016. This rFPV-H5 has had the H gene insert updated to A/chicken/Mexico/P-14/2016 (H5N2) (Bertran *et al.,* 2020). An rFPV-H7 with haemagglutinin insert from A/chicken/Guanajuato/07437-15/2015 (H7N3) has been developed and approved with deployment to Mexico in 2018 against H7N3 HPAI, and a rFPV-H5 with H and N gene inserts from A/goose/Guangdong/1996 (H5N1, clade 0) was used in China against the H5N1 HPAI during 2005 (Chen & Bu, 2009; Criado *et al.,* 2019; Swayne & Sims, 2020). rFPV can be effective when given to 1-day-old chicks with varying levels of maternal immunity (Arriola *et al.,* 1999). However, when very high levels of inhibitory immunity is anticipated because of previous infection or vaccination, the efficacy of the recombinant live vaccine in such day-old chicks should be confirmed and may require a prime-boost application of recombinant vaccine followed at a minimum 10 days later by inactivated influenza A vaccine boost to give optimal immunity (Richard-Mazet *et al.,* 2014; Swayne & Sims, 2020).

Newcastle disease virus can also be used as a vector for expressing influenza haemagglutinin genes. A recombinant Newcastle disease vaccine virus (rNDV) expressing a H5 HA gene (rNDV-H5) was shown to protect SPF chickens against challenge with both virulent Newcastle disease virus and a HPAI H5N2 virus (Veits *et al.,* 2006). A similar recombinant virus based on Newcastle disease virus vaccine strain La Sota and expressing H gene of A/goose/Guangdong/1996 (clade 0)(H5N1) was produced in China (the People's Rep. of) (Ge *et al.,* 2007) and reported to be efficacious in protection studies with either virus. This rNDV-H5 (clade 0) vaccine has been used widely with subsequent updating of HA insert twice with clade 2.3.4 and 2.3.2 clade haemagglutinin inserts (Swayne & Sims, 2020). An rNDV-H5 with H gene insert from A/chicken/Mexico/435/2005 (H5N2) has been developed, approved and deployed in Mexico against H5N2 LPAI (Swayne & Sims, 2020). An rNDV-H5 vaccine with H gene insert from A/chicken/Iowa/04-20/2015 (H5N2) (Gs/GD lineage, clade 2.3.4.4) insert was effective in protecting chickens against challenge with homologous H5N2 HPAI virus in chickens lacking immunity to the Newcastle disease virus vector or the H gene insert, but rNDV-H5 vaccine was ineffective as a primary or booster vaccine in poultry with maternal immunity or well-immunised against Newcastle disease or the H5 haemagglutinin protein (Bertran *et al.,* 2018). rNDV-H5 vaccines are effective as a primary vaccine if used in Newcastle disease or H5 antibody negative chickens, or as a priming vaccine followed by a boost with an inactivated influenza A vaccine in Newcastle disease or H5 antibody positive chickens. The major advantage of rNDV-H5 is the ability for low cost mass application by spray in the hatchery or field (Swayne & Sims, 2020).

Since 2010, a rHVT-H5 with haemagglutinin insert of A/swan/Hungary/4999/2006 (Gs/GD lineage, clade 2.2) has been approved and used in Egypt and Bangladesh against H5Nx Gs/GD lineage HPAI and in Mexico against H5N2 LPAI (Rauw *et al.,* 2011; Swayne & Sims, 2020). This rHVT-H5 vaccine has produced broad protection across diverse H5 HPAI viruses (Rauw *et al.,* 2011). Furthermore, maternally derived antibodies to rHVT vector or H5 haemagglutinin protein have had minimal negative impact on the effectiveness of the vaccine in broiler chickens after a single vaccination at 1 day of age (Bertran *et al.,* 2018). The rHVT-H5 is limited to application only *in ovo* or at 1 day of age to chickens in the hatchery, as application later on the farm is not feasible because of the ubiquitous infection by Marek's disease viruses or use of Marek's disease vaccines.

Because of the induction of broader immunity across mucosal, humoral and cellular areas, recombinant live vectored vaccines have had a longer use life in the field before appearance of field viruses that are resistant to the vaccine strains as compared to inactivated whole virus vaccines which produce primarily a strong humoral immunity. A recombinant duck enteritis virus in domestic ducks has been developed and shown efficacy but is pending regulatory approval and deployment in China (People's Rep. of) (Liu *et al.,* 2011).

Non-replicating haemagglutinin-based RNA particle and DNA vaccines with H gene from A/Gyrfalcon/Washington/40188-6/2014 (H5N8) (Gs/GD lineage, clade 2.3.4.4) have been approved for poultry use in the USA (Swayne & Sims, 2020). The H5 RNA particle vaccine is part of the USA emergency vaccine bank, along with rHVT-H5 and an inactivated H5N2 vaccines. The H5 RNA particle vaccine has been demonstrated to be an effective booster vaccine to replace rg inactivated H5Nx vaccine (Bertran *et al.,* 2017). A baculovirus with H gene insert from A/duck/China/E319-2/2003 (Gs/GD lineage, clade 2.3.3) has been approved for poultry use in Bangladesh, Egypt and Mexico (Swayne & Sims, 2020). Since this category of vaccine only contain the specific influenza A haemagglutinin protein, they are easily amenable to serological DIVA testing using assays designed for identifying antibodies to the nucleoprotein/matrix protein. However, field reports of protection with vectored and conventional influenza A vaccines suggest that protection by single dose of the vectored vaccines for long lived poultry is not feasible, with long-term field protection requiring a booster with inactivated influenza A vaccine or non-replicating, haemagglutinin-based vaccine (Swayne & Sims, 2020).

In addition to these approved vaccines, various experimental haemagglutinin-based H5 and H7 influenza A vaccines have been described using *in-vivo* or *in-vitro* expression systems including recombinant adenoviruses, salmonella, vaccinia, avian leucosis virus, various eukaryotic systems (plants or cell cultures) and infectious laryngotracheitis virus (Swayne & Sims, 2020).

3.2. Special requirements for biotechnological vaccines, if any

Live recombinant vectored vaccines with influenza A haemagglutinin gene inserts should have an environmental impact assessment completed to determine the risk of the vaccine to be virulent in non-target avian species and will not increase in virulence in the target avian species.

4. Surveillance methods for detecting infection in vaccinated flocks and vaccinated birds

A strategy that allows differentiation of infected from vaccinated animals (DIVA), has been put forward as a possible solution to the eventual eradication of HPAI and H5/H7 LPAI without involving mass culling of birds and the resulting economic damage, especially in developing countries (FAO, 2004). This strategy has the benefits of vaccination (less virus in the environment), but the ability to identify infected flocks would still allow the implementation of additional control measures, including stamping out of infected flocks. DIVA strategies use one of two broad detection schemes within the vaccinated population: 1) detection of influenza A virus ('virus DIVA'), or 2) detection of antibodies against influenza A field virus infection ('serological DIVA'). At the flock level, a simple method consists of regularly monitoring sentinel birds left unvaccinated in each vaccinated flock, but this approach does have some management problems, particularly with regards to identifying the sentinels in large flocks. As an alternative or adjunct system, testing for field exposure may be performed on the vaccinated birds either by detection of field virus or antibodies against the virus. To detect the field virus, oropharyngeal or cloacal swabs from baseline daily mortality or sick birds can be tested, individually or as pools, by molecular methods, such as real-time RT-PCR or AC-ELISA of the vaccinated populations (Swayne & Kapczynski, 2008).

To use serological DIVA schemes, vaccination systems that enable the detection of field exposure in vaccinated populations should be used. Several systems have been used. First, use of a vaccine containing a virus of the same haemagglutinin subtype but a different neuraminidase (N) from the field virus. Antibodies to the N of the field virus act as natural markers of infection. This system was used in Italy following the re-emergence of a H7N1 LPAI virus in 2000, and used an H7N3 inactivated vaccine with the detection of N3 antibodies indicating a vaccinated flock, N1 antibodies indicating infection, and both N1 and N3 antibodies indicating an infected, vaccinated flock (Capua *et al*., 2003). Problems with this system would arise if a field virus emerges that has a different N antigen to the existing field virus or if subtypes with different N antigens are already circulating in the field as is present in many low and middle income countries with H5Nx (Gs/GD lineage), H9N2 and other NA subtypes in live poultry markets (Swayne & Sims, 2020). A second serological DIVA option is the use of vaccines that contain only HA, e.g. replicating or non-replicating recombinant vaccines, which allows validated, classical AGID and nucleoprotein (NP)- or matrix protein-based ELISAs to be used to detect antibodies indicative of infection in vaccinated birds. Finally, for inactivated vaccines, a test that detects antibodies to the nonstructural viral or M2e proteins have been described (Avellaneda *et al*., 2010; Lambrecht *et al.,* 2007). These systems are yet to be validated in the field.

5. Continued evaluation and updating of vaccine seed strains to protect against emergent variant field virus strains

Historically, H5 LPAI inactivated vaccine seed strains and recombinant fowl poxviruses with H5 gene inserts have shown broad cross protection in chickens against challenge by diverse H5 HPAI viruses from Eurasia and North America (Swayne & Kapczynski, 2008). In 1995, Mexico implemented influenza A vaccine use for poultry as one tool in the HPAI control strategy, with eradication of HPAI strain by June 1995, but as H5N2 LPAI viruses continued to circulate, H5N2 vaccination was maintained (Villarreal, 2007). Within a few years, multiple lineages of antigenically variant H5N2 LPAI field viruses emerged that escaped from immunity induced by the original 1994 inactivated vaccine seed strain (Lee *et al.,* 2004). Similarly, emergent H5Nx HPAI Gs/GD lineage field viruses have arisen in China (the People's Rep. of), Indonesia, Egypt and various other Asian and Middle Eastern countries since 2005 that escaped from immunity induced by classical H5 inactivated LPAI vaccine seed strains and even rg generated H5 vaccine seed strains used in commercial vaccines (Grund *et al*., 2011; Liu *et al.,* 2020; Swayne & Sims, 2020). Similarly, H9N2 LPAI field viruses resistant to inactivated vaccine seed strains have arisen in multiple countries in Asian and Middle East after prolonged usage of a single inactivated vaccine seed strain. It is not clear whether the emergence of these antigenic variants is related to use of vaccines or improper use of vaccines, but the emergence of resistance necessitated the change in vaccine seed strains to antigenically match the circulating field strains (Cattoli *et al.,* 2011; Lee *et al.,* 2016). China as the largest user of avian influenza vaccines has updated it's inactivated H5Nx (Gs/GD lineage) and H7N9 seed strains eight times and once, respectively, with the life span of a seed strain ranging from 3 to 7 years (Liu *et al.,* 2020; Swayne & Sims, 2020). Mexico has updated its H5N2 inactivated seed strains twice and its rFPV-H5 once over a 20-year period of H5 vaccine use (Swayne & Sims, 2020). Initially H9N2 inactivated vaccine usage in South Korea, was associated with decreased field virus diversity, as vaccinal immunity completely inhibited antigenically closely related field virus replication (Lee et al., 2016). However, over time, field

virus diversity increases as antigenic variants arise in the field and expand their populations. The live recombinant vectored vaccines have been updated less frequently, suggesting a broader immunity, requiring less frequent insert updates as compared inactivated vaccine seed strains.

All influenza A vaccination programmes should have an epidemiologically relevant surveillance programme that includes all relevant geographical regions and production sectors. The resulting isolates, along with viruses obtained from outbreaks, should be assessed for genetic and antigenic variation as part of an ongoing program for assessing vaccine effectiveness in the field. Initially, the viruses should be sequenced and analysed for critical amino acid changes within the five major antigenic epitopes of the HA. A representative subset of antigenic variants should be tested for cross-reactivity in a HI test using a panel of standard antisera produced against diverse influenza A viruses from the same HA subtype and the data analysed for quantitative changes by antigenic cartography (Fouchier & Smith, 2010). Based on this cartographic data, a few of the predominant circulating influenza A viruses and selected antigenic variants should be used in challenge efficacy studies (Swayne *et al.,* 2015). Vaccines that are not protective should be discontinued and replaced with vaccines containing updated inactivated vaccine seed strains or HA inserts within other vaccine platforms. Based on the timeline for emergence of antigenic variants for H5N1 viruses in China (People's Rep. of), vaccines should be assessed at a minimum every 2–3 years for efficacy against predominant circulating field viruses of the country or region. Alternatively, vaccine seed strains should be updated when a vaccine-escape mutant accounts for more than 30% of the relevant AIV subtype (Liu *et al.,* 2020). Based on this scientific information, the competent veterinary authority within the country should establish, in consultation with leading veterinary vaccine scientists and international organisations, naturally isolated or reverse genetics LPAI vaccine seed strains for conventional inactivated vaccines, and H5 and H7 haemagglutinin gene insert cassettes for recombinant vaccines. In some situations, more than one seed strain may be necessary to cover all production sectors within a country. Only high quality and potent vaccines should be approved for use in control programmes. Proper administration of high quality, potent vaccines is critical in inducing protective immunity in poultry populations.

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NB: There are WOAH Reference Laboratories for avian influenza (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for avian influenza

NB: FIRST ADOPTED IN 1989 AS AVIAN INFLUENZA (FOWL PLAGUE). MOST RECENT UPDATES ADOPTED IN 2021.

APPENDIX 3.3.4.1.

BIOSAFETY GUIDELINES FOR HANDLING HIGH PATHOGENICITY AVIAN INFLUENZA VIRUSES IN VETERINARY DIAGNOSTIC LABORATORIES

INTRODUCTION

The spread of high pathogenicity H5Nx avian influenza throughout Asia, Africa and Europe has led to an increase in the number of laboratories performing diagnostics for this pathogen. High pathogenicity avian influenza (HPAI) viruses, in general, are a serious threat to birds and mortality is often 100% in susceptible chickens. In addition, the agents can also pose a serious zoonotic threat, with approximately 60% mortality reported in humans infected with H5N1 HPAI virus. In recognition of the need for guidance on how to handle these viruses safely, the WOAH has established the following biocontainment guidelines for handling specimens that may contain HPAI virus. They are based on Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities,* the World Health Organization[5], and Centers for Disease Control and Prevention[6].

BIOCONTAINMENT LEVELS

Samples for diagnostic testing for HPAI virus using the following techniques do not require high-level containment but should be carried out at an appropriate biosafety and containment level determined by risk analysis (see chapter 1.1.4.):

- Conventional and real-time reverse transcriptase polymerase chain reaction (RT-PCR)
- Antigen-capture assays
- Serology

Virus isolation and identification procedures for handling specimens that may contain high-titred replication-competent HPAI virus should as a minimum, include the following:

- Personnel protective equipment should be worn, including solid-front laboratory coats, gloves, safety glasses and respirators with greater than or equal to 95% efficiency.
- Specimens from potentially infected birds or animals should only be processed in type II or type III biological safety cabinets (BSC).
- Necropsies of birds should be performed in a Type II BSC while wearing respiratory protection, such as a N95 respirator, or in a Type III biological safety cabinet, or other primary containment devices with 95% efficient air filtration.
- Centrifugation should be performed in sealed centrifuge cups.
- Centrifugation rotors should be opened and unloaded in a BSC.

5 WHO laboratory biosafety guidelines for handling specimens suspected of containing avian influenza A virus, 12 January 2005.

6 Biosafety in Microbiological and Biomedical Laboratories, 5th edition. HHS Publication No. (CDC) 21-1112. https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF 1 December 2009.

- Work surfaces and equipment should be decontaminated after specimen processing.
- Contaminated materials should be decontaminated by autoclaving or disinfection before disposal or should be incinerated.

If chickens or other birds or mammals are inoculated with HPAI viruses, inoculation should be done in appropriate containment including:

- Inoculated chickens should be held in animal isolation cabinets or other primary containment devices, or non-isolation cages/floor pens in specially designed containment rooms
- Animal isolation cabinets should be in a separate facility that is equipped to handle the appropriate biocontainment for HPAI.
- The room should be under negative pressure to the outside and the animal isolation cabinets should be under negative pressure to the room.
- Animal isolation cabinets should have HEPA-filtered inlet and exhaust air.
- Biosafety cabinet or other primary containment devices should be available in the animal facility to perform post-mortem examinations and to collect specimens.

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CHAPTER 3.3.5.

AVIAN MYCOPLASMOSIS (*MYCOPLASMA GALLISEPTICUM, M. SYNOVIAE*)

SUMMARY

Description of the disease: *Avian mycoplasmosis is caused by several pathogenic mycoplasmas among which* Mycoplasma gallisepticum (*MG*) *and* M. synoviae (*MS*) *are considered the most important. MG causes chronic respiratory disease of domestic poultry, especially when flocks are stressed and/or other respiratory pathogens are present. The disease is characterised by coryza, conjunctivitis, sneezing, and sinusitis, particularly in turkeys and game birds. It can result in significant production losses and downgrading of meat-type birds, and loss of egg production. MS may cause respiratory disease, synovitis, eggshell alteration, loss of egg production and carcass downgrading or it may result in a silent infection. MG and MS strains vary in infectivity and virulence, and infections may sometimes be inapparent.*

Detection of the agent: *MG and MS can be identified by immunological methods after isolation in mycoplasma media or by detection of their DNA in field samples or cultures.*

Samples for isolation can be swabs of organs or tissues, exudates, diluted tissue homogenates, aspirates from the infraorbital sinuses or joint cavities, material from egg yolk or embryos. Clinical signs and lesions will influence the sample selection. Broth and agar combined with basic biochemical tests are used for isolation and first recognition of the Mycoplasma*, but identification of the genus and species is made through immunological tests (e.g. fluorescent antibody or immunoperoxidase tests) and/or biomolecular tests.*

DNA detection methods based on the polymerase chain reaction are commonly used in several specialised laboratories.

Serological tests: *Several serological tests are used to detect MG or MS antibodies, but due to variations in test specificity and sensitivity, they are recommended only for flock screening rather than for testing individuals.*

The most commonly used are the rapid serum agglutination (RSA) test, the enzyme-linked immunosorbent assay (ELISA) and the haemagglutination inhibition (HI) test. In the RSA test, sera are mixed with commercially produced stained antigen and sera that react within 2 minutes are heated at 56°C for 30 minutes and retested. Sera that still react, especially when diluted, are considered positive and are tested by either ELISA or HI for confirmation. Several commercial MG and MS antibody ELISA kits are available.

Requirements for vaccines: *Although the preferred method of control is maintenance of MG- and MS-free flocks, both live and inactivated vaccines are used in chickens. Vaccination should be considered only in specific cases on the basis of the epidemiological situation in the area or in farms where infection is inevitable. The normal use is to prevent egg-production losses in commercial layers, although vaccines may also be used to reduce egg transmission in breeding stock or to aid MG eradication on multi-age sites. It is important to vaccinate before field challenge occurs.*

Available live vaccines for MG for chickens are produced from the F strain and strains ts-11 and 6/85, which are apathogenic strains with improved safety characteristics. Administration of the F strain by the intranasal or eyedrop route is preferred, but aerosol or drinking water administration may be used. The eyedrop method is recommended for ts-11, and a fine spray for 6/85. Pullets are generally vaccinated between 9 and 16 weeks of age. One dose is sufficient and vaccinated birds remain permanent carriers. Long-term use of the F strain on multi-age sites can result in displacement of field strains. The ts-11 strain has been successfully used to eradicate F strain in multi-age commercial layers. In the past few years, two live MS vaccines produced from the MS-H strain and MS1 have been licensed in several countries. Eyedrop administration is advised for the MS-H strain whereas fine aerosol is recommended for MS1.

The birds should be vaccinated by 5 weeks of age. New MG vaccine candidates are under study, some of them are attenuated strains and others are based on vectored-virus technology.

Inactivated vaccines consisting of concentrated suspension of MG or MS organisms in an oil emulsion are licensed in several countries. They should be administered parenterally to pullets at 12–16 weeks of age. Two doses are desirable. MG bacterins are effective in preventing egg-production losses and respiratory disease, but do not prevent infection with wild-type MG. MS inactivated vaccine are not in common use.

A. INTRODUCTION

1. Description and impact of the disease

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) belong to the class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae*. It should be noted, however, that *M. meleagridis* and *M. iowae* can also cause disease in poultry, but MG and MS are considered to be the most important avian pathogenic mycoplasmas, and both occur world-wide.

MG infection is particularly important in chickens and turkeys as a cause of respiratory disease and decreased meat and egg production (Raviv & Ley, 2013). It can also cause upper respiratory disease in game birds. Some strains of MG have been recognised in North America in house finches as a cause of conjunctivitis with important impacts in wild bird populations such as inability to find food, starvation or death, but they do not appear to be pathogenic for poultry. In poultry the infection is spread vertically through infected eggs and horizontally by close contact; the MG nucleic acid has been identified in environmental samples. Recently it was shown that MG and MS may survive up to 9 days in synthetic fibres though less in human hair (Abolnik *et al.,* 2014) showing a predisposition of these mycoplasmas for attachment to surfaces. Other methods of spread are less well documented.

The clinical signs of MG in infected poultry can vary from subclinical to obvious respiratory signs including coryza, conjunctivitis, coughing and sneezing. Nasal exudate, rales and breathing through the partially open beak may occur. Unilateral or bilateral sinusitis may also be a feature, particularly in turkeys and game birds and the infraorbital sinuses may become so swollen that the eyelids are closed. Conjunctivitis, with frothy ocular exudate is also seen in turkeys, game birds and, sometimes, in chickens. In turkeys there is often soiling of the wing feathers as the result of attempts to remove exudate from the eyes. Infected finches may reveal ocular and nasal discharge and swollen eyelids in addition to the conjunctivitis.

MG may be associated with acute respiratory disease in chickens and turkeys, especially in young birds, with the turkey being more susceptible. The severity of the disease is greatly influenced by the degree of secondary infection with viruses such as Newcastle disease and infectious bronchitis, and/or bacteria such as *Escherichia coli*. In turkeys there is synergism with avian metapneumovirus infection. A more chronic form of the disease may occur resulting in reduced egg production in breeders and layers.

Lesions of the respiratory tract initially present as excess mucous exudate followed by catarrhal and caseous exudate, which may form amorphous masses in the air sacs. In turkeys and game birds the swollen infraorbital sinuses contain mucoid to caseous exudate.

MS may be associated in chickens with infectious synovitis; the birds may exhibit pale combs, lameness and retarded growth. Swellings may occur around joints. Greenish droppings containing large amounts of urates are commonly seen. Joints may contain a viscous, creamy to grey exudate in the joint and along tendon sheaths, along with hepatosplenomegaly and mottled, swollen kidneys (Ferguson-Noel & Noormohammadi, 2013). Respiratory signs and lesions are similar to those observed with MG, except that they are generally milder, and, as with MG, there is a synergistic effect with other respiratory agents. MS strains exhibit significant variability with respect to their virulence and tissue tropism (Catania *et al.,* 2016a; Landman, 2014; Landman & Feberwee, 2004). Recently a new clinical form resulting in high shell breakage and low egg production in chicken layers was reported initially in Europe (Catania *et al.,* 2010; Feberwee *et al.*, 2009a; 2009b) and then reported worldwide. The lesions appear to be confined to the apex of the shell and consist of rough dark areas of 2 cm in diameter with clear edges; in addition, a decrease in egg production is reported (Catania *et al.,* 2010; 2016a).

2. Zoonotic potential and biosafety and biosecurity requirements

There have been no reports of MG or MS infection in humans. Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

3. Differential diagnosis

MG or MS disease in chickens may superficially resemble respiratory disease caused by other pathogens such as mild strains of Newcastle disease virus (Chapter 3.3.14) and avian infectious bronchitis virus (Chapter 3.3.2). These may be also present in mixed infections with MG or MS. Infections with *Avibacterium paragallinarum* and *Pasteurella multocida* should also be ruled out. In broilers, the co-infection with avian metapneumovirus and *E. coli* could show similar features. MG in turkeys may be confused with avian metapneumovirus infections and the presence of sinusitis may also suggest infection with *Bordetella avium, Pasteurella multocida*, *Chlamydia* (Chapter 3.3.1) or MS. Infectious synovitis caused by MS should be differentiated from *Staphylococcus aureus, Riemerella anatipestifer, Ornithobacterium rhinotracheale* and *Enterococcus* joint infections and, in chicken, from infectious tenosynovitis caused by avian orthoreoviruses.

B. DIAGNOSTIC TECHNIQUES

The presence of MG or MS can be confirmed by isolating the organism in a cell-free medium or by detecting its DNA directly in infected tissues or swab samples. Serological tests are also widely used for diagnosis. When results are equivocal the birds are usually resampled.

Table 1. Test methods available for diagnosis of avian mycoplasmosis* (Mycoplasma gallisepticum, M. synoviae) *and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Isolation on culture media	+[b]	–	+	+	–	–
Conventional PCR	++[b]	++[b]	++	+++	++	–
Real-time PCR	+++[b]	+++[b]	+++	+++	+++	–
PCR-DGGE[c]	+	–	+	+	–	–
Detection of immune response						
HI	++[d]	–	+	++[f]	++	+
RSA	+[e]	–	+	+[f]	+	+
ELISA	++[d]	–	++	++[f]	++	++[g]

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]Not suitable for day old birds; [c]applied in culture medium, isolated colonies;
[d]suitable for ensuring lack of infections dating back more than 2–3 weeks;
[e]suitable for ensuring lack of infections dating back more than 5–8 days;
[f]suitable provided paired samples collected a few weeks apart can be analysed;
[g]suitable only for the group vaccinated with killed vaccine, F strain and by temperature sensitive vaccines.
PCR: polymerase chain reaction; DGGE = denaturing gradient gel electrophoresis; HI = hemagglutination inhibition;
RSA = rapid serum agglutination; ELISA = enzyme-linked immunosorbent assay.

1. Detection of the agent

1.1. *In-vitro* culture

Samples are taken from live birds, fresh carcasses or the carcasses of birds that have been frozen quickly after death. The tracheal swab is considered to be the best sample in live animals for most mycoplasma species. In addition, for isolation purposes, it is possible to collect swabs from the choanal cleft. When dead birds are available, mycoplasma isolation can be performed with swabs from different tissues or organs such as the upper and/or mid-trachea, lungs, air sacs, oviduct and joints.

Swabs of the yolk sac should be collected during the last third of the egg incubation period where decreased hatchability of embryonated eggs has occurred (i.e. after the 15th and the 20th day of incubation in chickens and in turkeys, respectively).

Samples can be taken from the inner surface of the vitelline membrane, and from the oropharynx and air sacs of the embryo.

All samples should be tested as soon as possible after collection. If transportation is necessary, collected swabs should be vigorously agitated in 1–2 ml of mycoplasma broth and then discarded; tissues or organs should be frozen. An ice pack or some other means of chilling should be included as MG and MS die rapidly at room temperature. Serial dilutions of samples should be made in mycoplasma broth because the presence of specific antibodies, antibiotics or inhibitory substances in tissues may inhibit mycoplasma growth.

Several suitable culture media have been formulated to support the growth of avian mycoplasma; in addition, several commercial media are also available. mycoplasma media generally contain a digested protein and a meat-infusion base supplemented with serum or a serum fraction, yeast factors, glucose and bacterial inhibitors. It is important that each new batch of medium be tested with recently isolated MG cultures of low *in-vitro* passage because some components, especially the yeast extract and the serum may vary in their ability to support growth.

The medium developed by Frey *et al.* is widely used in the United States of America (USA) and other countries for isolation of MG and MS (Frey *et al*., 1968). Nicotinamide adenine dinucleotide (NAD) is a growth requirement for the primary isolation of MS, but it may be omitted in the medium for the cultivation of MG.

The following broth and agar media are also satisfactory:

i) Part A: Pleuropneumonia-like organism (PPLO) broth base without crystal violet (14.7 g); distilled or deionised water (700 ml).

ii) Part B: Pig serum (heated at 56°C for 1 hour) (150 ml): 25% (w/v) fresh yeast extract (100 ml); 10% (w/v) glucose solution (10 ml); 5% (w/v) thallium acetate (10 ml); 200,000 International Units (IU)/ml penicillin G (5 ml); and 0.1% (w/v) phenol red solution (20 ml). Thallium acetate can be toxic to humans and the precautions for its use should be followed. The pH is adjusted to 7.8. Pig serum may be replaced by horse serum, but it is important to ascertain that it supports the growth of MG. For primary isolation of MS in this medium, a mix of 10% (v/v) NAD solution (1 ml) and 10% (v/v) cysteine solution (1 ml) is also added.

Part A is autoclaved at 121°C, at 1 atmospheric pressure for 15 minutes and, after cooling, is added to Part B, which has previously been sterilised by filtration.

For the corresponding solid medium, 10 g of purified agar, known to support the growth of mycoplasma, is added to part A above. The mixture is autoclaved as before and kept in a water bath at 56°C. The constituents of part B, omitting the phenol red, are mixed separately and then incubated at 56°C. Parts A and B are mixed carefully to avoid the production of air bubbles, and are dispensed into 50 mm dishes using 7–9 ml/dish. Excess surface moisture can be removed by a short incubation at 37°C. Plates are stored in an airtight container at approximately 4°C for up to 4 weeks.

Fresh yeast extract is available commercially, although it is preferable to prepare it 'in-house' by taking active dry baker's yeast (250 g) and suspending it in distilled water (1 litre). This is heated to boiling point, cooled and then centrifuged for 20 minutes at 3000 ***g***. The supernatant fluid is decanted and adjusted to pH 8.0 with 0.1 M NaOH. This is clarified by centrifugation or by filtration, and then sterilised by filtration. The extract is stored at –20°C. Reagent grade glucose (10 g) is dissolved in distilled or deionised water (100 ml) and adjusted to pH 7.8–8.0 with 0.1 M NaOH. It is sterilised by filtration and stored at 4°C. Reagent grade thallium acetate is dissolved (5 g) in distilled or deionised water (100 ml), filter-sterilised and stored at –20°C. Penicillin solution (10^6 IU benzyl penicillin in 5 ml distilled water) is stored at 4°C, and has a shelf life of 1 week. For isolation from heavily contaminated samples, penicillin concentration can be increased to 2000 units/ml or ampicillin, 0.5–1.0 mg/ml, maybe used instead. Phenol red (0.1 g) is ground in 0.1 M NaOH (2.8 ml), and then made up to 100 ml in sterile distilled water and autoclaved at 115°C at 1 atmosphere for 30 minutes. It is stored at 4°C. (Note: thallium acetate is highly toxic and care should be taken, especially when preparing the stock solution.)

Specimens are inoculated on to both mycoplasma agar and into broth. Solid medium may help detection of slow-growing mycoplasmas, which can be overgrown by saprophytes in broth. It may be necessary to make serial dilutions up to 10^{-3} for successful isolation. Inoculated plates are incubated at 37°C in sealed containers. Increased humidity and CO_2 tension in the atmosphere have been reported to enhance growth; these conditions may be obtained by: the inclusion of damp paper or cotton wool; flushing the container with 5–10% CO_2 in nitrogen, placing a lighted candle in the container; or by using a CO_2 incubator or suitable gas-generating system.

The caps of liquid medium containers should be tightly sealed before incubation at 37°C to avoid spurious changes in pH. For the first few days, the plates are examined daily for colonies with a stereoscopic microscope; after that they are examined less frequently. Cultures from field material should be kept for at least 20 days before discarding.

Broth cultures should be examined daily for changes in colour and/or in turbidity. Most mycoplasmas, including MG and MS, metabolise sugar-producing acid causing a change in the pH of the medium from red/orange to yellow. Other mycoplasmas hydrolyse arginine creating alkaline conditions causing a change in pH and consequentially of broth colour from red/orange to strong red or fuchsia. Any observable growth in broth is subcultured onto solid medium immediately. If there is no colour change after 7–14 days, the broth should be subcultured onto solid medium. This should be done because the presence of an arginine-hydrolysing (alkali-producing) mycoplasma species may mask the acid colour change produced by MG or MS, or because there might be mycoplasma strains with less active metabolism.

Mycoplasma colonies on solid medium can usually be recognised, although they may not have the typical 'fried egg' appearance. Bacterial colonies may appear on the first passage, but they are often more pigmented and fail to passage on mycoplasma media.

Biochemical reactions (e.g. fermentation of glucose and failure to hydrolyse arginine) can assist in identification, but they are not specific for MG or MS and necessitate purification of the culture by cloning.

Immunological and DNA detection methods can be used to identify mycoplasma isolates. They include the growth inhibition (GI); and metabolism inhibition (MI) indirect fluorescent antibody (IFA) and immunoperoxidase (IP) tests, the latter two can be considered simple, sensitive and specific. Purified cultures (produced by one colony) are required for the GI and MI tests, but not for the IFA or IP test. IFA and IP can detect the presence of more than one species of mycoplasma, as only the colonies specific for the antiserum will react. However, *M. imitans*, a mycoplasma species that is serologically and biochemically similar to MG has been isolated from ducks, geese and sometimes from other nondomestic bird species in some countries. It may be distinguished from MG by use of specific biomolecular methods. Alternatively, colonies of the isolate can be examined by immunofluorescence using serial dilutions of antisera to MG and *M. imitans* in parallel. The homologous antiserum should have a considerably higher titre.

DNA detection methods for identifying MG or MS directly in tissues or for identifying laboratory isolates are discussed below and are usually based on the polymerase chain reaction (PCR).

In certain circumstances where results of the above methods are not conclusive, inoculation of chick embryos or bioassays in live chicks may be appropriate. However, these techniques are time-consuming and costly, and have largely been replaced by PCR technology, although they remain a useful research tool. The specimens required for inoculation of chicken embryos are the same as those used for the inoculation of artificial media. They are prepared in broth from which thallium acetate is omitted, incubated for 30–60 minutes at 37°C, and then a 0.05–0.1 ml aliquot is inoculated into the yolk sac of several 6- to 8-day-old chicken embryos derived from mycoplasma-free flocks. The eggs are candled daily and embryos that die within 24 hours of inoculation are discarded. Any further dead embryos are kept refrigerated until cultured and those surviving after 5 days are placed at 4°C for 4 hours to kill them and to reduce haemorrhages on opening. The yolk is subcultured into broth and on to agar. Yolk lipid tends to obscure colonies, so it is essential to streak the yolk thinly or, preferably, to dilute it first in mycoplasma broth.

1.2. Antigen detection

Immunofluorescence and IP procedures for diagnosis are generally applied to suspect isolates rather than directly to infected exudates or tissues. This is because the organisms are too small to recognise conclusively under the light microscope and because corresponding negative and positive control samples are unlikely to be readily available.

1.2.1. Indirect fluorescent antibody test

The recommended technique for the IFA test (Rosendal & Black, 1972) requires an agar culture of the unknown isolate, consisting of numerous small discrete colonies, a known MG or MS culture as a positive control, and a culture of another mycoplasma species, such as *M. gallinaceum* or *M. gallinarum* as a negative control. Also required are polyclonal rabbit anti-MG or MS serum, a normal rabbit serum and an anti-rabbit immunoglobulin fluorochrome-conjugated serum. Sera may be prepared in species other than rabbits, but monoclonal antibodies (MAbs) should not be used because MG or MS demonstrate variable expression of their surface epitopes and an MAb may fail to recognise the target microorganism. Suitable working dilutions in sterile phosphate buffered saline (PBS; 0.01 M, pH 7.2) of the anti-MG or MS serum and the conjugate are first determined by cross-titration, and are selected for use at two-to-four-fold dilutions less than the actual end-points. These are applied to the unknown mycoplasma colonies that have been previously grown on agar plates as indicated below.

1.2.1.1. Test procedure

i) From colony-bearing agar plates, cut blocks of about 1.0 × 0.5 cm and place them on to labelled microscope slides with the colonies uppermost.

ii) To make subsequent orientation possible, cut off the lower right-hand corner of the blocks. One block with the unknown isolate, a block with the known MG culture, a block with the known MS culture and a block with a different but known mycoplasma culture are placed on one slide. A block of the unknown isolate is placed on another slide.

iii) Add a drop of suitably diluted MG (or MS) antiserum to the surface of each block of the first slide and add normal rabbit serum to the single block on the second slide.

iv) Incubate all blocks for 30 minutes at room temperature in a humid atmosphere.

v) Place each block in a labelled tube containing PBS, pH 7.2 and wash gently for 10 minutes on a rotary mixer, then rewash as before, and finally return the blocks to the original microscope slides.

vi) Blot excess moisture from the sides of the blocks. Add one drop of the diluted conjugate to each block, and incubate and wash as before.

vii) Return the blocks to their original slides, and examine the colonies by incident light using fluorescence microscopy.

Interpretation of the results is subjective and requires some expertise; comparisons with the controls must give the correct reactions and are essential.

Some laboratories use fluorescein-conjugated antiserum in a direct fluorescent antibody test (DFA). A technique that is widely used for DFA is one in which the reagents are applied successively within stainless steel cylinders placed on the original mycoplasma agar plate. Although this is quick and easy to perform, the results obtained are less specific than using the indirect method, which is therefore preferred.

1.2.2. Indirect immunoperoxidase/immunobinding test

The indirect IP test follows a similar principle to the IFA test except that the binding of specific antibodies to colonies *in situ* is detected by adding an anti-rabbit immunoglobulin that has been conjugated to the enzyme peroxidase. A positive reaction is then visualised by adding an appropriate substrate which, on oxidation, produces coloured colonies. An immunobinding procedure can also be used in which the test colonies are blotted on to nitrocellulose (Kotani & McGarrity, 1985) and then reacted in a similar manner. As with IFA, polyclonal sera should be used for serotyping isolates by IP. The advantage of the IP test over immunofluorescence is that the IP test does not require an expensive fluorescence microscope.

1.2.3. Growth inhibition test

In the GI test, the growth of mycoplasmas is inhibited by specific antiserum, enabling species to be identified. It is relatively insensitive and sera must be high-titred, monospecific and prepared in mammalian hosts as poultry sera do not always inhibit mycoplasma growth efficiently. The organism under test must be in pure culture (cloned) and several dilutions should be tested; a concentration of 10^4 colony-forming units (CFU/ml) is optimal. The rate of growth of the organism may influence growth inhibition, and it is helpful to retard growth initially by incubating at 27°C for 24 hours, followed by incubation at 37°C thereafter. Details of the test and its interpretation are published elsewhere (Clyde, 1983).

1.3. Molecular methods – detection of nucleic acids

PCR assays are routinely used in many laboratories and are characterised by good sensitivity. These methods represent a good alternative to *in-vitro* culture of mycoplasmas because they are based on the detection of specific DNA sequences of the pathogen directly on clinical samples or isolates grown *in vitro*. MG or MS DNA are amplified by PCR using species-specific primers. The real-time PCR with fluorescent labelled probes is becoming increasingly used, shortening the detection time compared with conventional PCR. Great care has to be taken to avoid contamination of samples with MG or MS DNA from nearby necropsy rooms, mycoplasma culture laboratories or from previous PCR runs (see Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases* and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*). Several commercial kits for PCR and real-time PCR of both MG and MS are available and several in-house procedures have also been published (Dijkman *et al.,* 2017; Raviv & Kleven, 2009).

A conventional PCR and a real-time PCR for MG and MS are described below. Within the PCR-based methods, the denaturing gradient gel electrophoresis (DGGE) technique could be applied for the identification of most avian mycoplasmas, including MG and MS, but this test is validated only on isolates from mycoplasma culture. This method is described in Section B.1.3.2.

Genotyping techniques based on the analysis of the *mgc2, pvpA* and *vlh*A genes are currently widely applied for the classification of MG (Armour *et al.,* 2015; Garcia *et al.,* 2005) and MS isolates, respectively (Hammond *et al.,* 2008). Moreover, a core genome multilocus sequence typing (MLST) scheme for *M. gallisepticum* and two *M. synoviae* MLST schemes have been published (Dijkman *et al.,* 2016; El-Gazzar *et al.,* 2017; Ghanem *et al.,* 2017) and are likely to become globally applied epidemiological tools.

1.3.1. Conventional polymerase chain reaction

The assay described is a validated PCR for MG and MS detection based on a 16S rRNA fragment amplification (Lauerman, 1998). Another widely used method based on *mgc2* gene for MG detection (García *et al.,* 2005) is reported. It must be remembered that unrelated strains may occasionally share DNA sequences and give DNA amplification bands in different laboratory conditions. All new PCRs require validation using criterion in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* and Chapter 2.2.3 *Development and*

optimisation of nucleic acid detection assays. The conventional PCR for MS reported in this chapter can also detect *M. bovirhinis* DNA.

1.3.1.1. DNA extraction

DNA is extracted from swab samples (up to 10 swabs may be pooled) suspended in PCR-grade PBS in a 1.5 ml snap-cap Eppendorf tube. Several commercial extraction kits based on the spin column are available for DNA extraction from swabs, tissues, etc. Automated extraction of *Mycoplasma* DNA is possible with specific commercial kits. The appropriate kit for the type of sample should be selected and the manufacturer's protocol for DNA extraction should be followed.

1.3.1.2. Primers

PCR method	PCR primer sequences	Expected amplicon
16S rRNA for MG	MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3'	183 bp
	MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3	
*mgc*2 for MG	MG-1: 5'-CGC-AAT-TTG-GTC-CTN-ATC-CCC-AAC-A-3	236–302 bp
	MG-2: 5'-TAA-ACC-CRC-CTC-CAG-CTT-TAT-TTC-C-3'	
16S rRNA for MS	MS–F:5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3'	211 bp
	MS–R:5'-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3'	

1.3.1.3. Polymerase chain reaction

i) The reaction mixture should be prepared, according to the manufacturer's instruction, in a separate clean area using a set of dedicated pipettes. For each sample, dispense into a PCR tube 45 µl volume of mix containing 0.4 µM of each primer for 16S rRNA MS and *mgc*2 MG, or 0.2 µM for 16S rRNA MG. An internal amplification control (IAC) could be included as a commercial exogenous kit or using designed primers for endogenous sequence (e.g. 18S rRNA for eukaryotic derived samples) amplifying at same PCR conditions. The reaction mixture should be overlaid with a few drops of light weight mineral oil unless the thermocyler is equipped with a heated lid. The tubes are taken to another clean area where the appropriate extracted DNA sample (5 µl) is added to each tube. Positive and negative control DNA should be run in each assay. The tubes are then placed in a thermal cycler for the following cycles: 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and soak at 4°C.

ii) For amplification of 16s rRNA MS and *mgc*2 MG using a hot start Taq polymerase, run the thermocycler at the following profile:

Polymerase activation	95°C	10 minutes	
40 cycles	95°C	45 seconds	
	54°C	60 seconds	
	72°C	60 seconds	
1 cycle (final extension)	72°C	7 minutes	soak at 4°C

1.3.1.4. Electrophoresis

PCR amplification products are detected by conventional gel electrophoresis, incorporating appropriate size markers. The stained products are visualised under UV light or with silver nitrate under a hazardous chemical hood. Alternatively, the amplified product can be run in a capillary electrophoresis machine loaded with appropriate size markers. The base-pair dimension of the amplified fragments could be statistically different by about 10% than expected using this method. Examination of the PCR products should be carried out in a

laboratory area, well separated from places where other steps in the PCR procedure are performed.

1.3.2. 16s-rDNA-PCR and denaturing gradient gel electrophoresis

The 16s-rDNA-PCR-DGGE technique is a method that can be applied for the identification of mycoplasma isolates (McAuliffe *et al.,* 2005), including avian mycoplasma from cultivated broths or agar colonies. It can also be used on DNA extracts from clinical specimens. The genetic target is the V3 region of the 16s gene, which is amplified by the combination of a mycoplasma-specific primer (reverse primer) and a universal bacterial one (forward primer) containing a GC-clamp (40 repeated GC).

The method is based on the migration of DNA fragments following strand separation caused by chemical denaturants in the gel. It is capable of detecting single-base mutations in DNA.

After migration on the denaturing gel, the pattern of the band(s) produced by the unknown samples is compared with positive avian controls, which are run in parallel.

This technique is capable of detecting single avian *Mycoplasma* spp., infection and co-infections, in a single sample (Catania *et al.,* 2014; 2016b).

i) Primers

The following primers are used:

R543: 5'-ACC-TAT-GTA-TTA-CCG-CG-3
GC 341 F: 5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GCC-TAC-GGG-AGG-CAG-CAG-3'

ii) Polymerase chain reaction

The PCR reaction mixture should be prepared in a separate clean area as follows (final volume: 25 µl):

H_2O Ultra-pure	12.5 µl
5 × PCR buffer	5.00 µl
dNTP (l0 mM)	1.00 µl
F Primer (50 µM)	0.25 µl
R Primer (50 µM)	0.25 µl
Taq (5 U/µl)	0.25 µl
DMSO (1%)	0.25 µl
$MgCl_2$ (25 mM)	4.00 µl

A 23.5 µl master mix is dispensed into the tubes and 1.5 µl of nuclease free water/sample/control DNA is then added.

Tubes are then placed in a thermocycler at the following profile:

35 cycles:	95°C	5 minutes	
	95°C	1 minute	
	58°C	45 seconds	
	72°C	60 seconds	
1 cycle (final extension)	72°C	20 minutes	soak at 4°C

iii) Electrophoresis

20 µl of each PCR product is loaded in 10% polyacrylamide/bis (37.5:1) gels, with denaturing gradients from 30 to 60% (where 100% is 7 M urea and 40% [v/v] deionised formamide) in 1× TAE buffer. The electrophoresis is performed at 100 V, 300 mA at a temperature of 60°C for

18 hours, the run time could change based on the DGGE device and on the gel dimension. Gels are then stained with a suitable DNA stain for 30 minutes (5 µl in 50 ml of 1× TAE buffer) and visualised under UV illumination.

1.3.3. Real-time PCR

Species-specific real-time PCRs were developed to increase the throughput of diagnostic samples (Raviv & Kleven, 2009). This method uses specific fluorescent probes that increase the cost of specific analysis, but avoid potential contamination post-amplification. MG gene amplification is targeted to *mgc2* gene and MS amplification on 16S–23S ISR. The assay is conducted as a duplex amplification that includes an internal amplification control (IAC). A detection limit of 10 copies of MS DNA per reaction and 1 copy of MG DNA is determined.

i) Primers

For	Primers and probes
MG	MGFrt F 5'-TTG-GGT-TTA-GGG-ATT-GGG-ATT-3'
	MGRrtr 5'-CCA-AGG-GAT-TCA-ACC-ATC-3'
	MGPrt 5'-Texas Red-TGA-TGA-TCC-AAG-AAC-GTG-AAG-AAC-ACC-BHQ1-3'
MS	MSFrt 5'-CCT-CCT-TTC-TTA-CGG-AGT-ACA-3'
	MSRrt 5'-CTA-AAT-ACA-ATA-GCC-CAA-GGC-AA-3'
	MSPrt 5'-FAM-ATT-CTA-AAA-GCG-GTT-GTG-TAT-CGC-T-BHQ1-3

A commercial IAC DNA kit could be included into the amplification mix to reduce the likelihood of false negatives results.

ii) Polymerase chain reaction

The amplification is conducted in a 96-well real-time thermocycler. A tube reaction is prepared for each sample to be tested for MG or MS. Each reaction contains a total of 25 µl comprising 5 µl of target DNA, 12.5 µl of 2× of universal real-time PCR master mix, 5 µM of each final primer and 0.2 µM final probe. An IAC reagent could be added in each reaction following manufacturer's instructions.

A common amplification protocol is used:

1 cycle	95°C	10 minutes	(single denaturation step)
45 cycles:	95°C	15 seconds	
	60°C	30 seconds	

Fluorescence signal for specific *Mycoplasma* probe and IAC is acquired in the appropriate detector channel during the extension step.

The cycle threshold value (Ct = Cq quantification cycle) automatically calculated by the software should be used. Cq values of 35 or lower are considered as positive, IAC should amplify in negative samples with Cq between 30 and 40, otherwise it could be set as inhibited.

1.3.4. Molecular typing

Different molecular methods are also available for differentiation of MG and MS strains, but their use tends to be restricted at present to specialist laboratories. Sequence-based methods have been developed to identify circulating strains to understand better the epidemiology of mycoplasma and to support control measures. MS strains can be identified and classified with methods such as MLST (Dijkman *et al.,* 2016; El-Gazzar *et al.,* 2017), sequencing of *vlh*A 5' and number of proline-rich repeats (Hammond *et al.,* 2008), differentiation of MS-H with *obg* and *opp*F-1 mutations (Shahid *et al.,* 2013; Zhu *et al.,* 2017). Vaccine strains can be differentiated with DIVA (detection of infection in vaccinated animals) analysis (Dijkman *et al.,* 2017). MG and MS

strains can be discriminated using core genome MLST (Ghanem *et al.,* 2017; Ghanem & El-Gazzar, 2018). Novel approaches such as MLVA (multilocus variable tandem array; Kreizinger *et al.,* 2018) and MAMA (mismatch amplification mutation assay) are very promising for discriminating field isolates from vaccine strains, but are not yet widely available.

These new methods are rapidly replacing the other molecular typing techniques, such as restriction endonuclease analysis, pulsed-field gel electrophoresis, amplified fragment length polymorphism and random amplified polymorphic DNA analysis because they are too labour intensive and costly for large-scale typing. Based on the high speed of improvement of the new analytical methods as well as the greater availability of increasingly sophisticated equipment, the methods currently available may be replaced in a short time. Furthermore, avian mycoplasmas strain nomenclature shall be reviewed in the near future for a more usable classification of circulating mycoplasma strains and better of disease control policies.

2. Serological tests

The serological tests in common use lack specificity and/or sensitivity. It is strongly recommended that their use is limited to flock monitoring rather than testing individual birds. Diagnosticians wishing to use such tests are advised to establish the test sensitivity and specificity (Chapter 1.1.6) under their own laboratory conditions. It should also be noted that these tests have not been validated for use with sera from day-old birds or from game birds (Bradbury, 2005).

The most commonly used tests are RSA, ELISA and HI. Several others have been described such as radioimmunoassay, micro-immunofluorescence, immunoblotting (Welchman *et al.,* 2013) and IP assay, but are rarely used. The number of sera to be tested within a flock depends on the level of detection and the confidence limits required. Minimal requirements, including frequency of testing for international trade within the European Union, have been described, for example for MG in Council Directive 2009/158/EC. Minimal requirements and approved tests are also set out for members of the National Poultry Improvement Plan (NPIP) of the USA.

Poultry companies using ELISA for screening large numbers of sera for virus antibodies may find this type of assay convenient also for mycoplasma testing. The ELISA will not be described in detail here because several MG and MS kits are available commercially. Instead, the details of the HI test are provided as the reagents needed for this test are not widely available commercially.

2.1. Rapid serum agglutination test

Sera are collected from a sample of the flock and, if not tested immediately, are stored at 4°C and not frozen. The test should be carried out at room temperature (20–25°C) within 72 hours of serum collection using reagents kept at room temperature. Prior centrifugation of sera will reduce nonspecific reactions. The RSA antigens are available commercially, but they may vary in specificity and sensitivity from different manufacturers and from batch to batch. They must be stored according to the manufacturer's instructions. Suitable RSA-stained antigens may also be prepared 'in-house' using culture methods as described in Section B.1.; these are then stained with crystal violet dye. Quality control standards for mycoplasma antigens for serological tests are described below.

2.1.1. Test procedure (Allan & Gough, 1974)

i) Drop one volume (approximately 0.02 ml) of serum on to a clean white tile or glass plate followed by one volume of stained MG or MS antigen. Do not allow the serum to dry out before addition of the antigen. It is important to shake the antigen bottle vigorously and frequently during use to keep the correct amount of antigen in suspension.

ii) Use a stirring rod to spread the mixture over a circular area of approximately 1.5 cm diameter. Rock the tile or plate for 2 minutes. Agglutination is indicated by flocculation of the antigen within 2 minutes.

iii) Include known positive and negative controls in the test.

iv) Retest serial dilutions of any sera that agglutinate after heating at 56°C for 30 minutes. If they still react strongly, they are considered to be positive on dilution (1/4 or more).

In the USA, MG and MS positive reference antisera can be obtained from the USDA National Veterinary Services Laboratories (NVSL), and in Europe from Anses Ploufragan[1], France. MG, MS and control sera produced in chickens or in turkeys, with a range of titres, can be purchased. Sets of antisera can be purchased also from the Department of Avian Medicine, University of Georgia, subject to availability.

There are no international standards for interpreting these tests, but a high proportion of positive sera in a flock (10% or more) suggests MG infection, especially if confirmed by HI test or ELISA. For further confirmation, the flock should be retested within a month. Inconclusive results make it necessary to attempt to isolate the organism or to demonstrate the presence of its DNA. Doubtful results for MG or MS should be investigated by performing tests with MS antigen (and *vice versa*) as infection with these organisms sometimes causes cross-reactions.

Tests can be conducted on yolk as well as sera although the yolk must first be diluted or extracted.

2.2. Haemagglutination inhibition test

MG and MS are capable of haemagglutinating avian red blood cells (RBCs), which can be inhibited by specific antibodies in sera. A strain that grows well and reliably haemagglutinates should be selected. The HI test requires a satisfactory haemagglutinating MG and MS antigens, washed fresh chicken or turkey RBCs, as appropriate, and the test sera. The antigen can be either a fresh broth culture or a concentrated washed suspension of the mycoplasma cells in PBS. It may be difficult to sustain a supply of high-titred broth culture antigen; however, the use of concentrated antigen (usually containing 25–50% glycerol and stored at –70°C), increases the likelihood of nonspecific reactions. In the USA, MG and MS haemagglutination (HA) antigens can be purchased from the NVSL.

The HI test follows well-known procedures (Allan & Gough, 1974). The HA titre of the antigen is first determined in doubling dilutions, the HA unit being defined as the least amount of antigen giving complete HA in the test system employed. The HI test should be performed using 4 HA units by the following method or a method having equivalent sensitivity as determined by tests with known positive sera.

All HA titrations and HI tests are best performed in multiwell plastic plates with V-shaped wells and using constant volumes of 50 µl. A positive and a negative control serum are incorporated into each test. One row of eight wells is required for each serum under test.

2.2.1. Test procedure

i) Add 50 µl of PBS to the first well in each row.

ii) Add 8 HA units of antigen in 50 µl volumes to the second well in each row and add 50 µl of 4 HA units of antigen to each of wells 3 to 8.

iii) Add 50 µl of a previously-prepared 1/5 dilution of the serum under test to the first well, mix, and transfer 50 µl to the second well, and so on, and discard 50 µl from the last well. The first well is the serum control well.

iv) Six wells are required for the antigen control. Add 50 µl of PBS to wells 2 to 6, inclusive, and add 50 µl of the 8 HA unit antigen to wells 1 and 2. Mix the contents of well 2 and transfer 50 µl to well 3, mix and repeat up to well 6, and discard 50 µl.

v) Two wells are required for the RBC control. Add 50 µl of PBS to each of these.

vi) Add 50 µl of a 0.5% suspension of RBCs (chicken cells for chicken serum and turkey for turkey serum) to all wells.

vii) Shake the plate lightly to ensure thorough mixing of the well contents, and read after standing for approximately 50 minutes at room temperature or when the antigen titration is reading 4 HA units. For reading, the plate should be tilted and only those wells in which the RBCs 'stream' at the same time as those in the RBC control wells should be considered to be inhibited. The serum control should show a clear button of RBCs and the positive and

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negative controls should react as expected. The HI titre is the highest serum dilution exhibiting complete inhibition of HA.

Sera giving nonspecific HA must be adsorbed to remove all nonspecific haemagglutinins so that a clear button is obtained in the control well without HA antigen. The adsorption is carried out by incubating 1 ml of the serum dilution with 6–8 drops of packed washed chicken or turkey RBCs. The cells are removed after incubation at 37°C for 10 minutes, and the supernatant is tested for haemagglutinating activity.

There is no recognised definition of positive and negative results for international trade.

2.3. Enzyme-linked immunosorbent assay

Several commercial MG and MS antibody ELISA kits are marketed and widely used in diagnostic laboratories. These ELISAs use different cut-offs and mathematical formulas to convert the ELISA result into a titre value. This means that every ELISA has its own interpretation and the titre results of different ELISAs on the same serum could differ.

2.4. Quality control of *Mycoplasma gallisepticum* and *M. synoviae* antigens

2.4.1 *Mycoplasma gallisepticum* antigens

Antigens are usually prepared from the S6 strain or the A5969 strain of MG. Antigens prepared from other strains may also be used when necessary.

i) MG antigen for the RSA test

The methods of quality control described below apply solely to suspensions of MG stained with a suitable dye containing preservative and intended for use in the rapid plate agglutination test with serum. Such antigens are available commercially.

On microscopic examination, the antigen should appear as a homogeneous suspension without floccules or precipitates and the suspending liquid should be free from residual dye. It must be sterile with a pH of between 6.5 and 7.0 and stored at 5±3°C. It must be warmed to room temperature before use.

The sensitivity and specificity of the antigen is determined with respect to its reaction with known positive sera of high and low titre and known negative sera. A positive reaction is recognised by the formation of coloured floccules and the clearing of the suspending medium. The criteria described above must continue to apply until the expiry date declared by the manufacturer.

ii) MG antigen for the HI test

The test is preferably performed with live, actively growing cultures. The antigen must be free from contamination with bacteria and fungi.

iii) MG antigen for the ELISA

It may be difficult to prepare satisfactory antigen for use in the indirect ELISA without considerable prior experimentation and confirmation of sensitivity and specificity. Use of a validated commercial kit is the best approach for most diagnostic laboratories.

2.4.2. *Mycoplasma synoviae* antigens

Antigens prepared from the WVU 1853 strain or other suitable strains should be used.

i) MS antigen for the RSA test

The specifications apply as for MG antigen for the RSA test.

ii) MS antigen for the HI test

The same specifications apply as for MG antigen for the HI test.

2.4.3. Additional comments

Sera giving nonspecific reactions to the RSA test do not usually give a positive reaction in the HI test using live HA antigen. Positive RSA reactions can be confirmed by the HI test with sera taken after the first 2–3 weeks of infection (the time taken for HI antibodies to develop). However, the HI test tends to be strain specific (Kleven *et al*., 1988) and therefore may lack sensitivity. ELISA may be a useful alternative.

Samples of serum should not be frozen before use in RSA tests. They should be free from haemolysis and contamination to avoid nonspecific reactions. The use of inactivated vaccines for other diseases may result in nonspecific reactions. Samples should be tested as soon as possible (within 72 hours) because mycoplasma antibodies may deteriorate on storage. Sera may be inactivated in a water bath at 56°C for 30 minutes.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The preferred method of control is to maintain MG- and MS-free flocks, to increase the biosecurity measures and to avoid or to limit mycoplasma production by the infected breeder groups, containing the spread of these pathogens through vertical transmission (Kleven, 2008). Vaccination should be considered only in situations where field exposure is inevitable, or in specific cases, such as on multi-age sites. Potential exposure of neighbouring poultry flocks should also be carefully considered.

Two types of vaccines, live and killed, are available for the control of MG and MS: mild to avirulent live MG or MS strains and inactivated oil-emulsion bacterins. Several scientific papers have been published on this issue, providing evidence of the effect of vaccination on reducing drop of egg production, on limiting respiratory signs, airsacculitis, and in reducing egg transmission.

Although antigenic variability is present among MG or MS strains, it is thought that vaccination with a single MG or MS strain is sufficient to achieve a good level of protection against the homologous species. Live vaccines can be considered a good and effective tool for the management and containment of mycoplasmoses. However, until recently, it was not easy to distinguish the vaccine strain from the wild strains using the available tests (Catania, 2016b). However, the newer biomolecular techniques, while not simple or easy, have been validated for differentiating the strains (Dijkman *et al.,* 2017; Kreizinger *et al.,* 2017), though they are usually based on single-point mutations.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Bacterin-based vaccines are used to prepare the immune system for exposure to the disease to reduce the clinical signs, such us egg production drops that occur as a consequence of MG infection in layers. Their use at present is limited mainly to MG.

The use of live vaccines is equivalent to a 'controlled exposure'. The objective is to infect the flock with a mild, immunogenic MG or MS strain at an age when little or no significant damage occurs. Such exposure results in resistance to challenge later in life, particularly on multi-age commercial sites. Successfully vaccinated birds should be resistant to respiratory disease, airsacculitis, egg production drops or other specific lesions caused by MG or MS. Vaccination should also reduce transmission of the pathogen through the egg in breeders.

1. Outline of production and minimum requirements for vaccines

1.1. Characteristics of the seed

1.1.1. Live vaccine

The vaccine strain should be immunogenic, must readily colonise the upper respiratory tract, and cause minimal damage to the respiratory system. A strong antibody response does not necessarily correlate with immunity.

The seed culture should be free from all extraneous agents. The culture should be cloned to ensure purity. If desired, restriction endonuclease patterns of the mycoplasmal DNA, or other methods such as 16s PCR DGGE, can be run to be sure of the identity and purity of the strain.

The seed culture should be stable with no tendency to revert to virulence. This can be confirmed with ten back passages in susceptible chickens. Contact chickens can be introduced at weekly intervals. If necessary, tracheal swabs can be taken from infected chickens and can then be inserted into the trachea of contact chickens. Transmission of the organism should be proven. The resulting isolate can then be used to challenge susceptible chickens.

1.1.2. Killed vaccine

For killed vaccines the most important characteristics are high yield and good antigenicity. It is assumed, but not proven, that virulent strains are desirable. The seed culture should be free from all extraneous organisms.

1.2. Method of culture

The seed culture may be propagated in a medium similar to that described above (Section B.1) for live vaccines, the broth culture is lyophilised or frozen at –70°C or colder. For bacterins the culture must be concentrated and resuspended in a small volume of saline or PBS before the emulsion is prepared.

1.3. Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. Chickens should be vaccinated by the same route that will be used in the field. Vaccinated birds should be challenged, and protection should be determined against respiratory signs, nasal discharge, and/or airsacculitis. Ideally, protection against egg-production losses should be evaluated, but such challenge trials are expensive and cumbersome.

Efficacy test: Groups of 20 specific pathogen free (SPF) chickens or at least mycoplasma-free chickens, 2 weeks of age or older, are vaccinated by eyedrop or other route of administration with one field dose of live vaccine, or subcutaneously or intramuscularly with one dose (usually 0.5 ml) of bacterin. A similar group of unvaccinated chickens is maintained separately as controls. All chickens should be challenged with a 24-hour broth culture of a virulent strain of MG, 2–3 weeks post-vaccination. A simple challenge method is inoculation of 0.1 ml of the challenge culture into the posterior thoracic air sac. All birds are necropsied 7–10 days post-challenge, and air sac lesions are scored. Alternative methods are to challenge by inoculating 0.1 ml into the infraorbital sinus and examining the birds for nasal discharge from 7 to 14 days post-challenge or to challenge by aerosol and measure the thickness of the tracheal mucosa on microscopic sections at four to six equidistant predetermined points (Whithear, 1996).

1.4. Method of manufacture

1.4.1. Procedure

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry. Special care must be taken to avoid MG contamination of other products manufactured in the same facility.

Production of vaccine should be on a seed-lot system, using a suitable MG strain of known origin, passage history, and purity. The growth medium is similar to that given above. The serum used in the growth medium should be inactivated at 56°C for 1 hour to prevent contamination with any mycoplasma that may be present, and filter sterilised. A source of SPF serum is desirable.

Broth medium is inoculated, with a rapidly growing inoculum, at a rate of approximately 5% (v/v). Incubation is at 37°C. Production can be in batches using large flasks or in a fermenter. In batch cultures, harvest is approximately 24 hours after inoculation. Live vaccines are preserved by lyophilisation or by freezing at –70°C, in liquid nitrogen, or on dry ice.

For bacterin production, the antigen must be concentrated, usually by centrifugation, ultrafiltration, or other suitable method. Bacterins are made as water-in-oil emulsions, typically 80% mineral oil, 20% aqueous, with suitable emulsifying agents.

1.4.2. Requirements for ingredients

See Chapter 1.1.8 with special focus on products of biological origin originating from a country with negligible risk for transmissible spongiform encephalopathies.

1.4.3. In-process control

i) Antigen content

At harvest, the titre should be from 10^8 to 10^9 CFU/ml. The antigen concentration of bacterins is difficult to standardise but may be based on packed cell volume, which is typically 1% (v/v) packed cells in the final product.

ii) Inactivation of killed vaccines

Inactivation is frequently done with either beta-propiolactone or formaldehyde. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine organism and potential contaminants.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation should be carried out by culture in mycoplasma broth on each batch of both the bulk harvest after inactivation and the final product. No evidence of growth of mycoplasma should be observed.

iii) Sterility of killed vaccines

Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the British Pharmacopoeia (Veterinary) 1985.

1.4.4. Batch control

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9.

ii) Safety

a) Live vaccine safety test

The birds vaccinated in the efficacy test given above can be used to evaluate the safety of the vaccine.

b) Killed vaccine safety test

Birds vaccinated in the efficacy test described above may be observed for adverse local or systemic effects.

iii) Batch potency

Potency tests for both live and killed vaccine can be conducted by the procedures given above for the efficacy test. The titre of live vaccines should be sufficient to induce infection by the route recommended by producers per dose per bird to last until the expiry date.

iv) Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.

1.5. Requirements for regulatory approval

1.5.1. Manufacturing process

For vaccine approval, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 and 2) should be submitted to the Authorities. Information should be provided from three consecutive vaccine batches to demonstrate consistency of production.

1.5.2. Safety requirements

i) Precautions (hazards)

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident, the person should go at once to a hospital, taking the vaccine package with him or her. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injection. Such wounds should be treated by the casualty doctor as a 'grease gun injury'.

Personnel vaccinating birds with live virus vaccines by aerosol spray should wear protective clothes and masks.

1.5.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s). Each batch of live vaccine should contain sufficient live mycoplasmas per dose per bird to last until the expiry date.

Vaccine efficacy (protection) should be estimated in vaccinated animals directly by evaluating their resistance to challenge.

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NB: There are WOAH Reference Laboratories for avian mycoplasmosis (*Mycoplasma gallisepticum* and *M. synoviae*) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for avian mycoplasmosis (*Mycoplasma gallisepticum* and *M. synoviae*)

NB: FIRST ADOPTED IN 1991 AS MYCOPLASMOSIS (*MYCOPLASMA GALLISEPTICUM*).
MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.3.6.

AVIAN TUBERCULOSIS

SUMMARY

Description of the disease: *Avian tuberculosis, or avian mycobacteriosis, is an important disease that affects companion, captive exotic, wild and domestic birds and mammals. The disease is most often caused by* Mycobacterium avium *subsp.* avium (M. a. avium). *However more than ten other mycobacterial species have been reported to infect birds. The most significant cause of poultry disease is* M. a. avium.

Clinical signs of the disease vary depending on the organs involved. The classical presentation is characterised by chronic and progressive wasting and weakness. Diarrhoea is common. Some birds may show respiratory signs and occasionally sudden death occurs. Some birds may develop granulomatous ocular lesions.

Mycobacterium tuberculosis (*gene IS*6100*1*) *is less commonly the cause of infection in birds, often as a result of transmission from pet bird owners.*

Members of M. avium *complex:* M. avium *subsp.* hominissuis (*serotypes 4–6, 8–11 and 21; lacking gene segment IS*901 *and containing segment IS*1245) *and* M. intracellulare (*serotypes 7, 12–20 and 22–28; lacking both IS*901 *and IS*1245) *can also infect an extensive range of mammals such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species. In humans, all members of* M. avium *complex and* M. genavense *are capable of inducing a progressive disease that is refractory to treatment, mostly in immunocompromised patients.*

All manipulations involving the handling of open live cultures or of material from infected birds must be carried out with appropriate biorisk management.

Diagnosis of avian tuberculosis in birds depends on the demonstration of the above-mentioned mycobacterial species *in live or dead birds, or the detection of an immune response, cellular or humoral, culture examination, or gene segments IS*6110*, IS*901 *and IS*1245 *by polymerase chain reaction* (*PCR*) *in the excretions or secretions of live birds.*

Detection of the agent: *Where clinical signs of avian tuberculosis are seen in the flock, or typical tuberculous lesions are present in birds at necropsy, the demonstration of acid-fast bacilli in smears or sections made from affected organs is sufficient for a quick positive diagnosis. If acid-fast bacilli are not found, but typical tuberculous signs or lesions are present in the birds, culture of the organism must be attempted. PCR could also be carried out directly on tissue samples. Any acid-fast organism isolated should be identified by nucleic-acid-based tests, or chromatographical* (*e.g. high performance liquid chromatography* [*HPLC*]) *criteria; serotyping of isolates of* M. avium *complex members or PCR for IS*6110*, IS*901 *and IS*1245 *could be performed.*

Tuberculin test and serological tests: *These tests are normally used to determine the prevalence of disease in a flock, or to detect infected birds. When used to detect the presence of avian tuberculosis in a flock, they should be supported by the necropsy of any birds that give positive reactions.*

In domestic fowl, the tuberculin test in the wattle is the test of choice. This test is less useful in other species of bird. A better test, especially in waterfowl, is the whole blood stained-antigen agglutination test. It is more reliable and has the advantage that it will give a result within a few minutes, while the bird is still being held.

Requirements for vaccines and diagnostic biologicals: *No vaccines are available for use in birds. Avian tuberculin purified protein derivative* (*PPD*) *is the standard preparation for use in the tuberculin test of domestic poultry. Avian PPD is also used as a component in the comparative intradermal tuberculin test in cattle* (*see Chapter 3.1.13 Mammalian tuberculosis* [*infection with* Mycobacterium tuberculosis *complex*]).

A. INTRODUCTION

Several mycobacterial species can be involved in the aetiology of avian tuberculosis and avian mycobacteriosis. Avian tuberculosis is most commonly produced by infection with *Mycobacterium avium* subsp. *avium* (serotypes 1, 2 and 3; containing specific gene segment IS*901* and nonspecific segment IS*1245*) and less frequently by *M. genavense* (Guerrero *et al.*, 1995; Pavlik *et al.*, 2000; Tell *et al.*, 2001). Avian mycobacteriosis is caused by other two members of *M. avium* complex: *M. avium* subsp. *hominissuis* (serotypes 4–6, 8–11 and 21; lacking gene segment IS*901* and containing segment IS*1245*) and *M. intracellulare* (serotypes 7, 12–20 and 22–28; lacking both gene segments IS*901* and IS*1245*) and by *M. intracellulare*, *M. scrofulaceum*, *M. fortuitum* and other potentially pathogenic mycobacterial species. Under some circumstances, an extensive range of mammalian species, such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic animals can be infected (Dvorska *et al.*, 2004; Mijs *et al.*, 2002; Shitaye *et al.*, 2009; Tell *et al.*, 2001; Thorel *et al.*, 1997; 2001). *Mycobacterium tuberculosis* and *M. bovis* are less common as causal agents of tuberculosis in birds (Tell *et al.*, 2001).

Mycobacterium avium species consists of four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *paratuberculosis* (Mijs *et al.*, 2002; Thorel *et al.*, 1990). The latter is the causal agent of Johne's disease, or paratuberculosis, in ruminants and other mammalian species (see Chapter 3.1.16 *Paratuberculosis* [*Johne's disease*]). *Mycobacterium a. silvaticum,* which like *M. avium* subsp. *paratuberculosis* grows *in-vitro* only on media with Mycobactin, can cause avian tuberculosis in wood pigeon (Thorel *et al.*, 1990).

All *M. a avium* isolates from birds and mammals, including humans, have a multiple repetitive sequence IS*901* in their genome and produce a characteristic three-band pattern in IS*1245* restriction fragment length polymorphism (RFLP) as described and standardised previously (Dvorska *et al.*, 2003; Ritacco *et al.*, 1998). This repetitive sequence is also present in *M. a. silvaticum* and RFLP analysis can help with identification. IS*901* has only been detected in *M. avium* strains with serotypes 1, 2 and 3 (Pavlik *et al.*, 2000; Ritacco *et al.*, 1998) that are apparently more pathogenic to birds than other serotypes (Tell *et al.*, 2001). On the basis of genetic and phenotypic differences it has recently been proposed to differentiate *M. a. avium* into two subspecies based on the target organism: *M. a. hominissuis* for human and porcine isolates and *M. a. avium* for bird-type isolates (Mijs *et al.*, 2002). *Mycobacterium a. hominissuis* has polymorphic multiband IS*1245* RFLP patterns and is able to grow between 24 and 45°C (Mijs *et al.*, 2002; Van Soolingen *et al.*, 1998). It is worth noting that the typical features of bird-isolates, the three-band pattern in IS*1245* RFLP and presence of IS*901*, have also been found in cervine and bovine isolates of *M. a. avium* (O'Grady *et al.*, 2000).

Avian tuberculosis in birds is most prevalent in gallinaceous poultry and in wild birds raised in captivity. Turkeys are quite susceptible, but duck, geese and other water birds are comparatively resistant. The practices of allowing poultry to roam at large on the farm (free range) and of keeping the breeders for several years are conducive to the spread of the causal agent of avian tuberculosis among them. Infected individuals and contaminated environment (water and soil) are the main source of infection. The above-mentioned mycobacterial species causing avian tuberculosis can survive for several months in the environment (Dvorska *et al.*, 2007; Kazda *et al.*, 2009; Shitaye *et al.*, 2008; Tell *et al.*, 2001).

In most cases, infected birds show no clinical signs, but they may eventually become lethargic and emaciated. Many affected birds show diarrhoea, and comb and wattles may regress and become pale. Affected birds, especially gallinaceous poultry, are usually older than 1 year. Some show respiratory signs and sudden death may occur, dyspnoea is less common, and granulomatous ocular lesions (Pocknell *et al.*, 1996) as well as skin lesions have been reported. Under intensive husbandry conditions, sudden death may occur, often associated with severe lesions in the liver; such lesions are easily observed at post-mortem examination (Tell *et al.*, 2001).

The primary lesions of avian tuberculosis in birds are nearly always in the intestinal tract. Such lesions take the form of deep ulcers filled with caseous material containing many mycobacterial cells, and these are discharged into the lumen and appear in the faeces. Before the intestinal tract is opened, the ulcerated areas appear as tumour-like masses attached to the gut wall, but when the intestine is opened, the true nature of the mass becomes evident. Typical caseous lesions are nearly always found in the liver and spleen, and these organs usually are greatly enlarged because of the formation of new tuberculous tissue. The lungs and other tissues are ordinarily free from lesions even in advanced cases (Tell *et al.*, 2001; Thorel *et al.*, 1997).

Among domestic animals (mammals), domestic pigs (*Sus scrofa* f. *domesticus*) are the most susceptible to avian tuberculosis. Usually, no clinical manifestations are observed in such animals. Avian tuberculosis is suspected when tuberculous lesions are found in the head and mesenteric lymph nodes on meat inspection after slaughter. Findings of tuberculous lesions that involve other organs (liver, spleen, lungs, etc.) are rare, usually occurring at the advanced stage of the disease. *Mycobacterium a. avium* accounted for up to 35% of the *Mycobacteria* isolated from such tuberculous lesions (Dvorska *et al.,* 1999; Pavlik *et al.,* 2003, 2005; Shitaye *et al.,* 2006). Unlike the other species mentioned previously, cattle are highly resistant to the causative agent of avian tuberculosis, and tuberculous lesions are detected in head lymph nodes, or occasionally in liver lymph nodes, only on meat inspection. *Mycobacterium a. avium* can be successfully isolated from tuberculous lesions in mesenteric lymph nodes from juvenile cattle: the isolation rate from cattle under 2 years of age was 34.4% in contrast to 13.0% from cattle over 2 years of age (Dvorska *et al.,* 2004).

It is essential to bear in mind that all members of *M. avium* complex and *M. genavense* are capable of giving rise to a progressive disease in humans that is refractory to treatment, especially in immunocompromised individuals (Pavlik *et al.*, 2000; Tell *et al*., 2001). Members of *Mycobacterium avium* complex are classed in Risk Group 2 for human infection and should be handled with appropriate measures as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian tuberculosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Ziehl–Neelsen staining	–	–	–	++	–	–
Culture	–	–	–	++	–	–
Haemagglutination (stained antigen)	+	+++	+	–	++	–
PCR	++	+	–	+++	–	–
Detection of immune response						
Tuberculin test	++	+++	+	–	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

If there is a characteristic history of avian tuberculosis in a flock and typical lesions are found in birds at post-mortem, the detection of acid-fast bacilli (AFB) in smears or sections from affected organs, stained by the Ziehl–Neelsen method, is normally sufficient to establish a diagnosis. Occasionally a case will occur, presumably as a result of large infecting doses giving rise to acute overwhelming disease, in which affected organs, most obviously the liver, have a 'morocco leather' appearance with fine greyish or yellowish mottling. In such cases AFB may not be found, but careful inspection will reveal parallel bundles of brownish refractile bacilli. Prolongation of the hot carbol-fuchsin stage of Ziehl–Neelsen staining to 10 minutes will usually reveal that these are indeed AFB, with unusually high resistance to penetration of the stain. DNA probes and polymerase chain reaction (PCR) techniques have been

used to identify the agent at the species and subspecies level. Traditionally, *M. a. avium* is separated from common nonchromogenic slow-growing organisms by their ability to grow at 42°C (*M. a. avium*). *Mycobacterium genavense* is particularly fastidious and has special requirements for growth and identification (Shitaye *et al.,* 2010).

1.1. Culture

If there is a characteristic flock history and suggestive lesions are found at necropsy, but no AFB are seen in smears or sections, an attempt must be made to isolate the causative organism from the necropsy material. Liver or spleen is usually the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. As with culture of *M. bovis*, non-sterile specimens need to be processed with detergent, alkali or acid to eliminate rapidly growing microorganisms before culture (see Chapter 3.1.13 *Mammalian tuberculosis* [*infection with* Mycobacterium tuberculosis *complex*]). *Mycobacterium a. avium* grows best on media such as Lowenstein–Jensen, Herrold's medium, Middlebrook 7H10 and 7H11 or Coletsos, with 1% sodium pyruvate added. It may occasionally be necessary to incorporate mycobactin, as it is used for the isolation of *M. a. paratuberculosis* and *M. a. silvaticum.* Growth may be confined to the edge of the condensation water. Cultures should be incubated for at least 8 weeks. Typically *M. a. avium* produces 'smooth' colonies within 2–4 weeks; rough variants do occur. Shorter incubation times can be achieved using the liquid culture BACTEC system or automated fluorescent MGIT 960 culture system. *Mycobacterium a. avium* can also be detected in massively infected tissue by a conventional PCR, which also allows acceleration of the pathogen identification (Moravkova *et al.,* 2008). Currently, direct detection and quantification of *M. a. avium* using IS*901* quantitative real-time PCR can be considered as the best method (despite its rather high cost per test) (Kaevska *et al.,* 2010; Slana *et al.,* 2010).

For *M. genavense*, the optimal solid medium is Middlebrook 7H11 medium acidified to pH 6 and supplemented with blood and charcoal (Realini *et al*., 1999). The incubation period at 37°C should be extended for at least 6 months (Shitaye *et al.*, 2010).

Typing of mycobacteria to the species and subspecies level requires a specialised laboratory. Conventional biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and *M. intracellulare*. Thus, a miscellaneous group of mycobacteria that includes both species is usually classified under the denomination of *M. avium* complex (MAC). Seroagglutination, which is based on sugar residue specificity of surface glycopeptidolipids, allows classification of *M. avium* complex (MAC) organisms into 28 serovars (Wolinsky & Schaefer, 1973). More sophisticated typing methods directed at cell-wall-specific targets are currently available, such as enzyme-linked immunosorbent assays with monoclonal antibodies to major serovars, and high performance liquid chromatography (HPLC). Serovars 1 to 6, 8 to 11 and 21 are currently ascribed to *M. a. avium* and *M. a. hominissuis*, and serovars 7, 12 to 20 and 25 to *M. intracellulare*. However, no consensus was achieved on other serovars, and some isolates cannot be typed (Inderlied *et al*., 1993). Avian tuberculosis in birds is caused by *M. a. avium* types 1, 2, or 3. If the isolate is not one of these three serotypes, further identification tests (IS*901* PCR) must be carried out. However, it should be borne in mind that superficial tuberculous lesions in caged birds, especially psittacines, may be caused by *M. tuberculosis* and IS*6110* PCR should be used for precise identification.

1.2. Nucleic acid recognition methods

Specific and reliable genetic tests for speciation are currently available (Saito *et al*., 1990). Commercial nucleic acid hybridisation probes have become a reference method for distinction between *M. avium* and *M. intracellulare* cultures. *M. genavense* can also be distinguished with these tests. A further probe that covers the whole MAC was also developed, as genuine MAC strains have been described that fail to react with specific *M. avium* and *M. intracellular* probes (Soini *et al*., 1996). Nevertheless identification errors were reported due to the cross-reactivity, which may have serious consequences (van Ingen *et al.,* 2009). Various in-house molecular methods have been reported for the identification of mycobacterial cultures, including MAC. A multiplex PCR method for differentiating *M. avium* from *M. intracellulare* and *M. tuberculosis* complex has some advantages (Cousins *et al*., 1996). 16S rRNA sequencing (Kirschner *et al*., 1993) may also be used. Culture-independent in-house molecular tests have been developed for the detection and identification of species belonging to the *M. avium* complex directly from samples (Keavska *et al.,* 2010).

Mycobacterium a. avium, the causative agent of avian tuberculosis (Thorel *et al.,* 1990), previously designated as *M. avium* species only, is assigned to serotypes 1 to 3 within the *M. avium* complex of 28 serotypes (Wolinsky & Schaefer, 1973). As revealed by molecular biological studies, the detected insertion sequence IS*901* (Kunze *et al.,* 1992) is possessed not only by the isolates of the above-named serotypes, but also by isolates, virulent for birds, that could not be typed because agglutination occurred (Pavlik *et al.,* 2000). In epidemiological studies, a standardised IS*901* RFLP methods replaced serotyping (Dvorska *et al.,* 2003).

2. Immunological methods

Tests used for export depend on the importing requirement of individual countries. In the main, the tuberculin test or the haemagglutination (stained antigen) test are most frequently used for export testing of poultry.

2.1. Tuberculin test

The tuberculin test is most widely used test in domestic fowl, and the only test for which an international standard for the reagent exists. The tuberculin is the standard avian purified protein derivative (PPD). Birds are tested by intradermal inoculation in the wattle with 0.05 ml or 0.1 ml of tuberculin (containing approximately 2000 International Units [IU]), using a very fine needle of approximately 10 mm × 0.5 mm. The test is read after 48 hours and a positive reaction is any swelling at the site, from a small firm nodule approximately 5 mm in diameter to gross oedema extending into the other wattle and down the neck. With practice, even very small wattles on immature birds can be inoculated successfully. However, in immature birds the comb may be used, although results are not so reliable. Tuberculin testing of the wattle in turkeys is much less reliable than in the domestic fowl. Inoculation in the wing web has been recommended as being more efficient, but this is still not as good as for domestic fowl. Other birds may also be tested in the wing web, but results are not generally satisfactory. The bare ornamental skin areas on Muscovy ducks and some species of pheasant can be used, but reliability is doubtful and interpretation difficult. Testing in the foot web of waterfowl has also been described; the test is not very sensitive and is often complicated by infections of the inoculation site.

In pheasants, the tuberculin test can be performed in either of two ways. In the first, 0.05 ml or 0.1 ml of tuberculin is injected into the skin of the lower eyelid. A positive result is indicated by marked swelling at the site of injection after 48 hours. Alternatively, 0.25 ml of tuberculin is injected into the thoracic muscles and the birds are observed for 6–10 hours. Infected birds will show signs of depression and keep aside from the flock, and there may be cases of sudden death. No clinical signs will be provoked in uninfected birds.

2.2. Stained antigen test

2.2.1. Preparation of the antigen

An antigen stained with 1% malachite green is used for the rapid whole blood plate agglutination test (Rozanska, 1965). The strain used for preparation of the stained antigen must be smooth and not autoagglutinate in saline suspension. It must conform to the characteristics of *M. a. avium*.

A strain that will detect infection with any serotype is recommended to be used instead of the specific serotype that is most likely to be encountered (in Europe serotype 2 for domestic fowl, serotype 1 for waterfowl, and birds and swine in the USA). It may be preferable to use a strain that is highly specific for the serotype it detects. The specificity of strains can be determined only by testing them as antigens, although in general, a serotype 2 antigen will always detect serotype 3 infection and vice versa. Serotype 1 strains appear to detect more often a wide spectrum of infection, and will frequently also detect infections with mycobactin-dependent mycobacteria or *M. a. silvaticum*. There is no reason not to use a culture containing more than one strain of *M. a. avium*, provided that it shows the desired properties of sensitivity and specificity. Consistency of results between batches will be easier with the use of pure cultures.

The organism should be grown in a suitable liquid medium, such as Middlebrook 7H9 containing 1% sodium pyruvate for better growth. Good growth should be obtained in approximately 7 days. The liquid culture is used as seed for bulk antigen preparation.

Antigen for agglutination tests is best grown on solid medium, such as Löwenstein–Jensen or 7H11, containing 1% sodium pyruvate instead of glycerol, using Roux flasks or large bottles. The use of solid medium maximises the chance of detecting any contamination, and antigens grown in some liquid media are not agglutinated by specific antibody. Liquid seed culture should be diluted (on the basis of experience) to give discrete colonies on the solid medium. This will usually give the best yield, and again increases the chance of detecting contamination. About 10 ml of inoculum will usually be enough to allow it to wash over the whole surface, and provide sufficient moisture to keep the air in the bottle near 100% humidity.

The bottles are incubated at 37°C, and good growth should be obtained in 14–21 days with most strains. The antigen is harvested by the addition of sterile glass beads and twice the volume of sterile normal saline (containing 0.3% formalin) as was used to inoculate the bottle. The bottle is then shaken gently to wash off all the growth and the washing is collected into a sterile bottle and re-incubated at 37°C for 7 days. The killed bacilli are then washed twice in sterile normal saline with 0.2% formalin by centrifugation and re-suspension. This sequence is safer than the original method in which the washing was carried out before the incubation that kills the organisms. Finally bacilli are again centrifuged and re-suspended in sterile normal saline containing 0.2% formalin and 0.4% sodium citrate, to a concentration of about 10^{10} bacteria per ml. This corresponds to a concentration ten times that which matches tube No. 4 on McFarland's scale.

Cultures for antigen should be inspected for contamination daily for the first 5 days of incubation. The suspension made from the culture washings is also re-examined microscopically (for likely contaminants such as yeasts), and rechecked by culture to ensure that the formalin has killed the mycobacteria.

2.2.2. Validation of the antigen

Cultures should be checked by Gram staining for the presence of organisms other than mycobacteria.

One or more batches for agglutinating antigen must be tested for efficacy in naturally or artificially infected tuberculous birds by comparison with a standard preparation of known potency. The potency relative to that of the standard preparation must not differ significantly from that declared on the label. Each bottle of antigen must be tested with normal chicken serum (to detect autoagglutination) and *M. a. avium* positive chicken serum of low and high antibody content. This should be done, where possible, alongside a previous batch of stained antigen. Those bottles that give satisfactory agglutination reactions with the antisera can now be pooled and the antigen stained. This is done by the addition of 3 ml of 1% malachite green solution per 100 ml of suspension. If possible, the stained antigen should now be checked using whole blood just as the unstained antigen was tested with serum. The agglutinating antigen should keep for at least 6 months in the refrigerator at 4°C, and much longer if frozen at –20°C or below. If a batch has not been used for a long time it should be rechecked, especially for autoagglutination.

The only safety test needed is the culture test of the unwashed antigen after 7 days of incubation, to ensure that all the bacilli are dead.

2.2.3. Test procedure

The stained-antigen agglutination test has been used with good results, especially in both domestic and ornamental waterfowl. A drop (0.05–0.1 ml) of the antigen is mixed with the same volume of fresh whole blood, obtained by venipuncture, on a white porcelain or enamel tile. The mixture is rocked for 2 minutes and examined for agglutination. The agglutination may be coarse, in which case it is obvious, or quite fine, in which case it may be most clearly seen as an accumulation of the malachite-green-stained antigen around the edge of the drop, leaving the centre a normal blood-red colour. This test is especially useful for screening large flocks for immediate culling, and therefore has advantages over the tuberculin test for the control of the disease, even in domestic fowl. It has also been claimed that in domestic fowl it is more reliable than the tuberculin test.

Note on limitation of use

Neither the tuberculin test with avian tuberculin nor the stained-antigen agglutination test is likely to be of any value in cases of *M. tuberculosis* infection in caged birds.

C. REQUIREMENTS FOR DIAGNOSTIC BIOLOGICALS

1. Background

No vaccines are available.

Avian tuberculin is a preparation of purified protein derivatives (PPD) made from the heat-treated products of growth of *M. a. avium*. It is used by intradermal injection to reveal delayed hypersensitivity as a means of identifying birds infected with or sensitised to the same species of tubercle bacillus. It is also used as an aid to differential diagnosis in the comparative intradermal tuberculin test for bovine tuberculosis (see Chapter 3.1.13).

The general principles as given in Chapter 1.1.8 *Principles of veterinary vaccine production* should be followed for injectable diagnostic biologicals such as tuberculin. The standards set out here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for tuberculin production

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Strains of *M. a. avium* used to prepare seed cultures should be identified as to species by appropriate tests. The strains recommended by the European Union (EU), for example, are D4ER and TB56. Reference may also be made to the World Health Organization (1987).

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Seed cultures should be shown to be free from contaminating organisms and to be capable of producing tuberculin with sufficient potency. The necessary tests are described below.

2.2. Method of manufacture

2.2.1. Procedure

The seed material is kept as a stock of freeze-dried cultures. If the cultures have been grown on solid media, it will be necessary to adapt the organism to grow as a floating culture. This is most easily accomplished by incorporating a piece of potato in the flasks of liquid medium (e.g. Watson Reid's medium). When the culture has been adapted to liquid medium, it can be maintained by passage at 2–4-week intervals (Angus, 1978; Haagsma & Angus, 1995).

The organism is cultivated in modified Dorset-Henley's synthetic medium, then killed by heating in flowing steam and filtered to remove cells. The protein in the filtrate is precipitated chemically (ammonium sulphate or trichloroacetic acid [TCA] are used), washed and resuspended. An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Mercurial derivatives should not be used. Glycerol (not more than 10% [w/v]) or glucose (2.2% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile neutral glass containers, which are then sealed to prevent contamination. The product may be freeze-dried.

2.2.2. Requirements for ingredients

The production culture substrate must be shown to be capable of producing a product that conforms to the standards of the European Pharmacopoeia (2000) or other international standards. It must be free from ingredients known to cause toxic or allergic reactions.

2.2.3. In-process controls

The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate time period. Any flasks showing contamination or grossly abnormal growth should be discarded after autoclaving. As incubation proceeds, the surface growth of many cultures becomes moist and may sink into the medium or to the bottom of the flask. In PPD tuberculins, the pH of the dissolved precipitate (the so-called concentrated tuberculin) should be pH 6.6–6.7. The protein level of the PPD concentrate is determined by the Kjeldahl method. Total nitrogen and trichloroacetic acid precipitable nitrogen are usually compared.

2.2.4. Final product batch tests

i) Sterility

Sterility testing is generally performed according to the European Pharmacopoeia (2000) or other guidelines (see also Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

ii) Identity

One or more batches of tuberculin may be tested for specificity together with a standard preparation of bovine tuberculin by comparing the reactions produced in guinea-pigs sensitised with *M. bovis* using a procedure similar to that described in Section C.2.2.4.iv. In guinea-pigs sensitised with *M. bovis*, the potency of the preparation of avian tuberculin must be shown to be not more than 10% of the potency of the standard preparation of bovine tuberculin used in the potency test.

iii) Safety

Tuberculin PPD can be examined for freedom from living mycobacteria using the culture method described previously. This culture method, which does not require use of animals, is used in many laboratories and its use is encouraged over the use of animals for this purpose. The following is the previously described method, using experimental animals, to evaluate safety of PPD. Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and this must be injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days, and are examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture. Each filled container must be inspected before it is labelled, and any showing abnormalities must be discarded.

A test for the absence of toxic or irritant properties must be carried out according to the specifications of the European Pharmacopoeia (2000).

To test for lack of sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test are each injected intradermally on each of three occasions with the equivalent of 500 IU of the preparation under test in a 0.1 ml volume. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different, when measured 24–28 hours later.

iv) Batch potency

The potency of avian tuberculin is determined in guinea-pigs sensitised with *M. a. avium*, by comparison with a standard preparation calibrated in IU.

Use no fewer than nine albino guinea-pigs, each weighing 400–600 g. Sensitise the guinea-pigs by administering to each, by deep intramuscular injection, a suitable dose of inactivated or live *M. a. avium*. The test is performed between 4 and 6 weeks later as follows: Shave the guinea-pigs' flanks so as to provide space for three-to-four injections on each side. Prepare at least three dilutions of the tuberculin under test and at least three dilutions of the standard preparation in isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (Tween 80). Choose the dilutions so that the reactions produced have diameters of not less than 8 mm and not more than 25 mm. Allocate the dilutions to the injection sites randomly according to a Latin square design. The dilutions correspond to 0.001, 0.0002 and 0.00004 mg of protein in a final dose of 0.2 ml, injected intradermally.

At 24 hours, the diameters of the reactions are measured and the results are calculated using standard statistical methods, taking the diameters to be directly proportional to the logarithms of the concentrations of the tuberculins. The estimated potency must be not less than 75% and not more than 133% of the potency stated on the label. The test is not valid unless the fiducial limits of error ($p = 0.95$) are not less than 50% and not more than 200% of the estimated potency. If the batch fails a potency test, the test may be repeated one or more times provided that the final estimate of potency and of fiducial limits is based on the combined results of all the tests.

It is recommended that avian tuberculin should contain the equivalent of at least 25,000 IU/ml or approximately 0.5 mg protein per ml, giving a dose for practical use of 2500 IU/0.1 ml.

3. Requirements for authorisation/registration/licensing

3.1. Manufacturing process

The manufacturing process should follow the requirements of European Pharmacopoeia (2000) or other international standards.

3.2. Safety requirements

3.2.1. Target and non-target animal safety

Antimicrobial preservatives or other substances that may be added to a tuberculin, must have been shown not to impair the safety and effectiveness of the product. The maximum permitted concentrations for phenol is 0.5% (w/v) and for glycerol it is 10% (v/v). The pH should be between 6.5 and 7.5.

3.2.2. Precautions (hazards)

Experience both in humans and animals led to the observation that appropriately diluted tuberculin injected intradermally results in a localised reaction at the injection site without generalised manifestations. Even in very sensitive persons, severe, generalised reactions are extremely rare and limited.

3.3. Stability

During storage, liquid avian tuberculin should be protected from the light and held at a temperature of 5°C (±3°C). Freeze-dried preparations may be stored at higher temperatures (but not exceeding 25°C) protected from the light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a minimum.

Provided the tuberculins are stored at a temperature of between 2°C and 8°C and protected from light, they may be used up to the end of the following periods subsequent to the last satisfactory potency test:

Liquid PPD tuberculins: 2 years; lyophilised PPD tuberculins: 8 years; HCSM (heat-concentrated synthetic-medium) tuberculins diluted: 2 years.

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NB: There is currently (2022) no OIE Reference Laboratory for avian tuberculosis
(please consult the OIE Web site for the current list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989 AS TUBERCULOSIS IN BIRDS. MOST RECENT UPDATES ADOPTED IN 2014.

CHAPTER 3.3.7.

DUCK VIRUS ENTERITIS

SUMMARY

***Description and importance of the disease**: Duck virus enteritis (DVE) or duck plague is an acute contagious infection of ducks, geese and swans (order* Anseriformes*) caused by an alpha-herpesvirus. Diagnosis is based on a combination of assessing the clinical signs, gross pathology and histopathology supported by identification of the virus by either isolation or polymerase chain reaction.*

***Identification of the agent:** The virus may be isolated from the liver, spleen and kidneys of birds dying from this infection. Virus may be recovered by inoculating embryonated Muscovy duck eggs on the chorioallantoic membrane; or by inoculating primary cell cultures of duck embryo or Muscovy duck embryo origin. The identity of the virus can be confirmed by neutralisation tests using specific antiserum to inhibit pathological changes in the duck embryos or the cytopathological effects in the cell cultures, or by direct or indirect immunofluorescence tests on infected cell cultures. Alternatively the viral DNA may be detected by the polymerase chain reaction from oesophagus, liver and spleen of DVE virus infected birds as well as from Muscovy duck embryos or cells used for virus isolation.*

***Serological tests:** Immunological tests have little value in the diagnosis of acute infection. Serum neutralisation tests* in ovo *and* in vitro *have been used to monitor exposure to the virus in wildfowl.*

***Requirements for vaccines:** A live attenuated virus vaccine is available to control DVE in birds over 2 weeks of age. Ducks are vaccinated subcutaneously or intramuscularly for active immunity. Vaccine virus is not thought to spread from vaccinated to unvaccinated stock. An inactivated vaccine has been reported to be efficacious in laboratory tests, but has not been developed or licensed for large-scale use.*

A. INTRODUCTION

Duck virus enteritis (DVE) is an acute, sometimes chronic, contagious virus infection that occurs naturally only in ducks, geese and swans, all members of the family *Anatidae* of the order *Anseriformes*. The disease is a potential threat to commercially reared, domestic and wild waterfowl. The aetiological agent, *Anatid alphaherpesvirus-1* or DVE virus (DVEV), is a member of the *Alphaherpesvirinae* subfamily of the *Herpesviridae,* genus *Mardivirus*. DVE may also be referred to as duck plague, anatid herpes, eendenpest, entenpest and peste du canard. The infection has not been reported in other avian species, mammals or humans.

In domestic ducks and ducklings, DVE has been reported in birds ranging from 7 days of age to mature breeders. In susceptible flocks the first signs are often sudden, high and persistent mortality with a significant drop in egg production in laying flocks. In domestic ducks the incubation period ranges from 3 to 7 days. Mortality usually occurs 1–5 days after the onset of clinical signs and is often more severe in susceptible adult breeder ducks. In chronically infected partially immune flocks only occasional deaths occur. Recovered birds may be latently infected carriers and may shed the virus in the faeces or on the surface of eggs over a period of years (Richter & Horzinek, 1993; Shawky & Schat, 2002). DVE limited solely to Muscovy ducks has been observed in the USA (Campagnolo *et al.*, 2001; Davison *et al.,* 1993).

Clinical signs and gross pathology associated with a DVE outbreak vary with the species, immune status, age and sex of the affected birds, and with the virulence of the virus. Similarly, as infection progresses within a flock, more clinical signs are typically observed. In breeder ducks the range of signs include sudden deaths, photophobia associated with partially closed, pasted eye-lids, polydipsia, loss of appetite, ataxia, and nasal discharge. Birds often have ruffled feathers, watery diarrhoea and soiled vents. Sick birds may maintain an upright stance by using their

wings for support, but their overall appearance is one of weakness and depression. In ducklings 2–7 weeks of age, losses may be lower than in older birds, and the signs associated with DVE include dehydration, loss of weight, conjunctivitis and serous ocular discharge, a blue colouration of the beaks and blood-stained vents.

At necropsy, adult ducks that have died are typically in good body condition. In mature males, prolapse of the penis may occur. In mature females, haemorrhages may be observed in ovarian follicles. The gross lesions are characterised by vascular damage, with tissue haemorrhages, free blood in the body cavities and intestinal lumen and a range of lesions affecting the digestive tract mucosa. Specific digestive mucosal lesions may be found in the oral cavity, oesophagus, caeca, rectum and cloaca. Lesions undergo alterations as the disease progresses, from initial macular surface haemorrhages, to yellow-white crusty plaques, then green superficial scabs. Lesions may coalesce and be covered with a diphtheritic membrane. These latter lesions progress with the course of the disease and include initial mucosal haemorrhages and eruptions and intense annular congestion, leading to pseudo-membranous or diphtheritic mucosal lesions. Necrotic degenerative changes are evident in the lymphoid and parenchymatous organs. In the liver this manifests as irregularly distributed pinpoint haemorrhages and white foci giving a speckled appearance. In ducklings lesions of the lymphoid tissues tend to be more prominent than visceral haemorrhages. Collectively, these lesions are pathognomonic for DVE. The pathology and histopathology of DVE in white Pekin ducks has been reviewed (Sandhu & Metwally, 2008). Microscopic lesions are characterised by vascular damage and its consequences in visceral organs. Eosinophilic intranuclear inclusions and cytoplasmic inclusions in epithelial cells of the digestive tract are typically present. Birds that recover from natural infection are suggested to be immune to re-infection, but latency (in the trigeminal ganglion) and reactivation of virus is recognised.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of duck virus enteritis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[a]						
Virus isolation	+	–	++	+++	+	–
Antigen detection	+	+	++	++	+	–
Real-time PCR	++	++	++	+++	++	–
Conventional PCR	++	++	++	+++	++	–
LAMP	++	++	++	+	++	–
Detection of immune response[b]						
Microtitre plate VN	+++	+++	+++	+	+++	+++
VN in duck embryos	+++	+++	+++	++	+++	+++
ELISA	+	+	+	+	++	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; LAMP = loop mediated isothermal amplification
ELISA = enzyme-linked immunosorbent assay; VN = Virus neutralisation.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]Antibody response of little value in acute DVE infection. Humoral response may be low in natural infection and antibodies may be short lived.

1. Identification of the agent

Primary isolation of the virus is best achieved from samples of liver, spleen or kidney tissue, which have been homogenised in buffered saline containing antibiotics and clarified by low-speed centrifugation (1800 ***g***). Isolation may be attempted by inoculating such homogenates into cell cultures or duck embryos.

1.1. Cell cultures

Cell culture is reported as the method of choice for isolation of DVEV, but may not always be successful. If attempted, isolations may be made in primary duck embryo fibroblasts (DEF) (Wolf *et al.*, 1976) or, preferably primary Muscovy duck embryo fibroblasts (MDEF) (Gough & Alexander, 1990; Kocan, 1976). Muscovy duck embryo liver (MDEL) cells are thought to be even more sensitive. Cell monolayers grown in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin are washed with serum-free MEM and then inoculated with the clarified sample homogenate suspected to contain DVEV. After incubation for 1 hour at 37°C to allow for virus adsorption, the cultures are maintained on MEM containing 2% FCS, 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin, and incubated in an atmosphere containing 5% CO_2. The cytopathic effect (CPE) is characterised by the appearance of rounded clumped cells that enlarge and become necrotic 2–4 days later. Cultures should be stained with a labelled antibody conjugate using a direct or indirect method to identify the virus (see Section B.1.3). Cells can also be fixed and then stained with haematoxylin and eosin to show syncytial formation, intranuclear inclusions and marked cytoplasmic granulation. It has been reported (Burgess & Yuill, 1981) that the isolation of DVEV in MDEF cells is favoured by incubation at temperatures between 39.5°C and 41.5°C. However, an elevated temperature does not appear to be essential for isolation, which is often carried out at 37°C. More than one passage in cell culture may be necessary to isolate the virus. This virus isolation method in cell cultures may be modified to a plaque assay by overlaying the cell monolayer with maintenance medium containing 1% agarose. As the virus can be cell associated, sequential passaging should be carried out by trypsinising potentially infected cells and replanting them, as well as inoculating fresh cells with infected culture supernatant from the previous passage.

1.2. Duck embryos

Primary virus isolations can be made by inoculation on to the chorioallantoic membrane (CAM) of 9–14 day embryonated Muscovy duck eggs. The inoculated embryos should be monitored daily and those that die within 72 hours should be removed. Virus may be harvested from embryos that survive for 72–120 hours. Before harvest, inoculated embryonated eggs should be chilled at 4°C for 4 hours or overnight to kill the embryos before further manipulations. The embryos may die, showing characteristic extensive haemorrhages 4–10 days after inoculation. Two to four serial blind passages of the homogenised CAMs may be necessary before isolation can be effected.

Embryonated chicken eggs are not very susceptible to infection with field strains of DVEV. The virus can nevertheless be adapted to chicken embryos by serial passages. Pekin duck embryos vary in their susceptibility to strains of DVEV.

1.3. Immunological methods

Serological methods used to confirm the identity of newly isolated virus include neutralisation assays performed in either embryonated eggs or cell cultures. A plaque assay for DVEV in duck embryo cell cultures has been described (Dardiri & Hess, 1968). A microtitre assay using primary MDEF or MDEL cells can be used. Provided a hyperimmune antiserum of sufficiently high titre is used, a fluorescent antibody test (direct or indirect) for DVEV in DEF, MDEF or MDEL cells is the next most sensitive assay after isolation in 1–9-day old ducklings (Erickson *et al.*, 1974). A reverse passive haemagglutination test for DVEV has been described (Deng *et al.*, 1984), but it is reported to be less sensitive than immunofluorescence and plaque assays. An avidin–biotin–peroxidase method of immunoperoxidase staining to detect DVEV antigen in formalin-fixed, paraffin-embedded sections of liver and spleen from experimentally infected birds has been described (Islam *et al.*, 1993). The identity of the virus may also be confirmed by negative stain electron microscopy, but this alone is not positive confirmation that the herpesvirus observed is DVEV. Immunoelectron microscopy has been developed recently using DVE hyperimmune serum (Pearson & Cassidy, 1997).

1.4. Nucleic acid recognition methods: polymerase chain reaction

Several protocols for the detection of DVEV by conventional polymerase chain reaction (PCR) have been reported (Hansen *et al.*, 1999; 2000; Plummer *et al.*, 1998; Pritchard *et al.*, 1999). Real-time quantitative PCR protocols for the detection of DVEV have also been reported (Qi *et al.*, 2009; Wu *et al.,* 2011a; Yang *et al.*, 2005).

A loop-mediated isothermal amplification (LAMP)-based method for the detection of DVEV DNA has been published (Ji *et al.*, 2009; Woźniakowski & Samorek-Salamonowicz, 2014). Primers have been identified that are able to amplify DNA from DVEV present in various tissues, including oesophagus, liver and spleen, from an original outbreak and after passage from Muscovy duck embryos. Tissues are preferred to cloacal swabs as DVEV shedding may be intermittent in infected waterfowl.

The following is an example protocol for conventional PCR for detection of DVEV (Hansen *et al.,* 2000); other protocols exist.

1.4.1. Extraction of viral DNA

This DNA extraction procedure can be used on disrupted cell suspensions from DVEV-infected tissue culture, 10% ground tissue suspensions, or cloacal swab material in transport medium. This method is used to prepare DVEV DNA for the known positive PCR controls.

Note: All product transfers in steps i to v are performed in a biological safety cabinet.

i) For a 10% ground tissue suspension, add 400 µl to a 1.5 ml microfuge tube and microfuge at 16,000 ***g*** for 5 minutes. Transfer the supernatant to a new tube and go to step ii.

ii) For tissue culture suspensions and cloacal swab material, add 400 µl of the sample, or supernatant from step i above, to a 1.5 ml tube and microfuge at 16,000–20,000 ***g*** for 45 minutes to pellet the virus.

iii) Discard the supernatant and resuspend the pellet with 200 µl of Tris/ethylene diamine tetra-acetic acid (EDTA) buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA).

iv) Add 10 µl of a 5 µg/µl proteinase K solution to give a final concentration of 0.2 µg/µl, mix thoroughly, and incubate at 56°C for 1 hour.

v) Add 25 µl of 10% sodium dodecyl sulfate (SDS) solution to give a final SDS concentration of 1%, mix thoroughly, and incubate at 37°C for 1 hour.

vi) Add 15 µl of 5 M NaCl to give a final concentration of 0.3 M and mix thoroughly.

vii) Add 300 µl of fresh phenol buffered with Tris/HCl, pH 8.0, to the tube, and mix by inverting 50 times.

viii) Microfuge the tube at 16,000 ***g*** for 5 minutes and transfer the top aqueous phase (sample) to a new tube.

ix) Repeat the phenol extraction steps vii and viii once more.

x) Add 500 µl of ether to the tube, mix thoroughly, and microfuge at 16,000 ***g*** for 1 minute.

xi) Discard the top aqueous phase (ether) and repeat the ether extraction step (step x) once more.

xii) Heat the tube with the lid open at 56°C for about 15 minutes or until the smell of ether is gone.

xiii) Split the tube contents in two and add 2.25 times the sample volume of 100% ethanol to each tube, mix the tube contents by inverting the tube several times, and leave at room temperature (22°C) for 30 minutes.

xiv) Microfuge the tube at 16,000 ***g*** for 45 minutes and discard the supernatant.

xv) Add 200 µl of 70% ethanol to gently wash the pellet and then microfuge at 16,000 ***g*** for 15 minutes.

xvi) Discard the supernatant and dry the pellet at 56°C for 30–45 minutes with the tube lid open.

xvii) Resuspend the DNA in 30 µl distilled water that is RNAase and DNAase free.

xviii) Store the sample tube at 4°C until tested (few days) or at –20°C for long-term storage.

1.4.2. Test method

Lower reaction mixtures for the DVEV PCR and the lambda control are prepared in advance in a biosafety cabinet using the kit manufacturer's recommended methods for a hot start PCR. The lower reaction mixture is dispensed into tubes, sealed with wax at 80°C, as recommended by the manufacturer, and stored at 4°C for 1–2 months.

PCR primers for DVEV DNA-directed DNA polymerase gene
Primer 1 sequence: 5'-GAA-GGC-GGG-TAT-GTA-ATG-TA-3' (forward)
Primer 2 sequence: 5'-CAA-GGC-TCT-ATT-CGG-TAA-TG-3' (reverse)

i) The upper reaction mixture is prepared according to the kit manufacturer's recommendations the day of the test, and distributed to each sample tube including DVEV and lambda control tubes.

ii) Add 10 µl of DNA suspension from the stored sample tubes to the PCR lower reaction tubes with corresponding labels.

iii) Place known DVEV DNA diluted to 1 pg/10 µl into one control tube and 10 µl of distilled water into the no DNA control tube. Add 10 µl of lambda DNA supplied in the kit and 10 µl of water to the corresponding lambda control tubes.

iv) Place all the tubes in a thermal cycler that is programmed as follows:

One cycle: Hold 94°C for 2 minutes
Hold 37°C for 1 minute
Hold 72°C for 3 minutes

35 cycles: Hold 94°C for 1 minute
Hold 55°C for 1 minute
Hold 72°C for 2 minutes

One cycle: Hold 72°C for 7 minutes
Hold 4°C until stored

PCR tubes are stored at 4°C until the samples are examined for amplification products.

1.4.3. Electrophoretic analysis of PCR products

i) A fresh 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) is prepared from a 10× stock for agarose preparation and for use in the electrophoresis chamber.

ii) A 1% agarose solution is prepared in TAE buffer, heated to dissolve the agarose, and, when cool, poured into a gel former with a comb.

iii) The solidified gel is placed into the electrophoresis chamber and TAE running buffer is added.

iv) PCR test samples, including the DVEV and lambda controls, are mixed 1/10 with 1 µl of loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 0.01 M Tris/HCl, pH 8.0, and 50% [v/v] glycerol) and 10 µl of each is added to individual wells of the gel. The 100 bp molecular size markers are added to each side of the gel.

v) Run the gel for 1 hour at 120 volts and then stain in a 1% ethidium bromide solution for 20 minutes (alternative, safer stains should preferably be used to visualise PCR products). De-stain the gel for 45 minutes in deionised water and view the gel on a UV-illuminated light box. Photograph the gel to record results.

1.4.4. Interpretation of the results

A 500 bp amplification band in the lambda control sample indicates the PCR ran successfully. A 446 bp band in the DVEV known DNA control indicates the DVEV primers are working. A 446 bp

band in the unknown test sample indicates DVE viral DNA was present. No amplification products will be present in the DVEV or lambda no DNA controls. If bands appear in these negative control products, cross-contamination occurred during the test set-up and the test must be repeated.

1.4.5. Real-time PCR

The following is an example protocol for a real-time PCR for detection of DVEV (Y*ang et al*; 2005); other protocols exist.

PCR primers targeting a 124-bp fragment of the DVE DNA polymerase gene

Primer 1 sequence: 5'-CTC-TAC-GCA-GCT-TTT-GAC-GAT-TT-3' (forward)

Primer 2 sequence: 5'-AGA-AAC-ATA-CTG-TGA-GAG-TGA-CGA-3' (reverse)

The labelled probe (5'-CCT-CCT-CCT-CGC-TGA-GTG-GCA-TCC-3') is complementary to a 24 bp region between the upstream and downstream primer pair labelled with 6-carboxy-fluorescein at the 5' end and 6-carboxy-tetramethyl-rhodamine at the 3' end.

i) The DVEV DNA extraction can be performed using a suitable tissue/cell DNA extraction kit or the extraction procedure described in Section B.1.4.1. The PCR can be performed using a suitable real-time PCR kit and PCR detection system and software.

ii) The amplification is performed in a total of 25 µl PCR mixture containing 1 µl of DNA solution, 13PCR buffer, 10 mM $MgCl_2$, 0.4 mM dNTP, 0.2 µM concentration of each primer, 0.24 µM fluorogenic probe, and 1.25 U of Taq polymerase.

iii) The PCR conditions consisted of:

a) one cycle of 5 minutes at 95 C

b) 40 two-step cycles of 5 seconds at 94°C and 20 seconds at 65°C.

iv) If quantification is desired, the number of target copies in the reaction can be deduced from the threshold cycle (CT) values corresponding to the fractional cycle number at which the released fluorescence exceeds 15 times the standard deviation of the mean baseline emission.

1.5. Strain variation

Although strains of DVEV differ considerably in virulence, there is little reported evidence of serological variation.

2. Serological tests

Serological tests have little value in the diagnosis of acute DVE infections, but assays based on serum neutralisation in embryonated eggs and cell cultures have been used to monitor antibodies following exposure to DVE in wild waterfowl. The humoral response to natural infection with DVEV is often low and antibodies may be short-lived (Docherty & Franson, 1992); it is assumed that cell-mediated immunity also plays a role in the infection (Richter & Horzinek, 1993). However, detection of neutralising antibodies to DVEV in serum is possible. Virus neutralisation (VN) (Thayer & Beard, 1998) assays using a constant-serum/varying-virus method may be performed in chicken or duck embryos by using embryo-adapted virus, or in cell cultures. For laboratories lacking duck embryos, serological diagnosis is possible by virus neutralisation, using a chicken embryo fibroblast adapted DVEV strain and primary chicken embryo fibroblasts (CEF). Neutralisation indices (NI) (Thayer & Beard, 1998) between 0 and 1.5 were detected in domestic and wild waterfowl that had not been exposed to DVEV; a NI of 1.75 or greater was considered to be evidence of prior exposure to DVEV (Dardiri & Hess, 1967). Alternatively, sera may be screened using a constant-virus/varying-serum method. In the author's laboratory a microtitre neutralisation assay using primary MDEF or DEF is used. Serial twofold dilutions of each serum sample (heat-inactivated at 56°C) are prepared in 50 µl of serum-free MEM in microtitre plates. Approximately $10^{2.0}$ $TCID_{50}$ (50% tissue culture infective dose) of DVEV in 50 µl of MEM is added to each well and the mixtures are allowed to react at 37°C for 1 hour. A suspension of primary MDEF or DEF in MEM supplemented with 2 mM L-glutamine, 0.17% sodium bicarbonate and 10% FCS, are adjusted

to contain 3×10^5 cells per ml. Cells are next added to the plates at 100 µl per well and the plates are then incubated for up to 96 hours at 37°C in a humidified 5% CO_2 atmosphere. Following incubation, cells are observed daily by light microscopy and finally fixed with 10% formol-buffered saline and stained with 1% crystal violet. The plates are read macroscopically. The titre for virus neutralising activity is expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e. there is no evidence of CPE and therefore complete virus neutralisation has occurred. A titre of less than 3 $\log_2$ is usually considered to be negative. A VN titre of 8 or greater is considered to be significant and is evidence of exposure to DVEV (Docherty & Franson, 1992). VN antibody may also be detected using cell cultures by mixing sera at a single dilution, e.g. 1/10, with 100–200 $TCID_{50}$ virus and then testing inoculated cell cultures for non-neutralised virus by immunofluorescence. Although this method is not quantitative, it can be useful for screening large numbers of sera. These latter methods, using constant-virus/varying-serum, are much more economical on sera than the NI methods.

Immunochromatographic (ICS) strip tests have been developed for DVE antibody detection and may have promise for use in the field (Shen *et al.,* 2010). The ICS strip test is based on membrane chromatography and uses recombinant UL51 protein as the capture antigen. This test is reported to have a sensitivity comparable to ELISA and much higher than VN tests. A dot-ELISA and passive haemagglutination tests have been reported for the detection of DVE antibodies but the sensitivity and specificity of these tests are moderate (Malmarugan & Sulochana, 2002). Several indirect ELISAs (Wu *et al.,* 2011b) have been described for the serological detection of DVE. Indirect ELISA using the entire DVEV virion as coated antigen has been described for DVE antibody detection and is commercially available (Xuefeng *et al.,* 2007). Several indirect ELISAs that use recombinant DVE proteins that act as coating antigen have also been developed. Wu *et al.* described an indirect ELISA using a recombinant UL55 protein of DVE expressed in *E. coli* (UL55-ELISA). Compared with a commercial indirect ELISA based on whole DVE virions and VN tests, the UL55-ELISA was found to be intermediate in sensitivity and specificity (Wu *et al.,* 2011b). Another indirect ELISA using thymidine kinase fusion protein expression in *E. coli* as coating antigen was reported to detect post-vaccine DVE antibodies 5 days earlier compared with conventional assays (Wen *et al.,* 2010). While ELISAs based on recombinant DVE proteins are reported to be rapid, simple and more economical alternatives for DVE serological detection, further testing of their stability is needed before widespread use.

C. REQUIREMENTS FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1.1. Rationale and intended use of the product

A live attenuated virus vaccine can be used to control DVE in birds over 2 weeks of age (Richter & Horzinek, 1993). The live vaccine virus is not thought to spread by contact from vaccinated to unvaccinated ducklings. Fattening or breeding ducks may be vaccinated subcutaneously or intramuscularly to produce an active immunity. Maternally derived immunity in ducklings is reported to decline rapidly and progeny of breeders vaccinated with a live attenuated vaccine are fully susceptible.

A live attenuated vaccine propagated in a duck embryo fibroblast cell line has been reported to be successful (Mondal *et al.*, 2010).

An inactivated vaccine has been reported to be as efficacious as modified live vaccine (Shawky & Sandhu, 1997). This vaccine has been tested only under laboratory conditions; it has not been tested on a large scale and is not licensed.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

DVE vaccine can be prepared from a strain of the virus that has been attenuated by serial passage in embryonated chicken eggs. In the USA the vaccine strain seed was originally imported from Holland and has been serially passaged 41–46 times.

The seed virus should be prepared in 8- to 11-day-old specific pathogen free (SPF) embryonated chicken eggs by inoculating on to the CAM followed by incubation at 37°C. The seed may be stored at –70°C or lower in the form of a homogenate of the embryo CAM in buffered saline

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The seed virus should be shown to be free from extraneous viruses pathogenic to ducks, chickens and turkeys. It should also be free from bacterial, fungal and mycoplasmal contaminants.

The identity of the virus should be confirmed by a VN test conducted with specific antiserum using the constant-serum/varying-virus method. This test should be performed in embryonated chicken eggs. The antiserum should reduce the virus titre by at least $10^{1.75}$ ELD_{50} (50% embryo lethal dose).

2.2. Method of manufacture

2.2.1. Procedure

The vaccine is produced in 8–11-day-old SPF embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. Most embryo deaths occur between 48 and 96 hours after inoculation. The embryos, their CAMs and chorioallantoic fluids are harvested, pooled and homogenised in buffered saline and clarified by low-speed centrifugation (1800 ***g***). The preparation is diluted as appropriate, and a stabiliser is incorporated. It is then dispensed into vials and preferably frozen rapidly to –70°C or lower.

2.2.2. Requirements for substrates and media

All reagents should be sterile and eggs obtained from a specific pathogen-free source.

2.2.3. In-process controls

Any embryo dying within the first 24 hours of inoculation should be discarded as nonspecific deaths.

2.2.4. Final product batch tests

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in the chapter 1.1.9.

ii) Safety

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

A group of 1-day-old ducklings susceptible to DVE should be inoculated subcutaneously or intramuscularly with the vaccine at 10 times the recommended dose, and observed for 7–14 days for any signs of adverse reactions.

iii) Batch potency

The virus titre of the vaccine should be determined in 9- to 11-day-old embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. The vaccine should contain a minimum of $10^{3.0}$ ELD_{50} per dose at time of use.

The immunogenicity of the vaccine can be assessed in DVE-susceptible ducks or ducklings by inoculating the recommended vaccine dose intramuscularly and challenging intramuscularly 21 days later with virulent DVEV. The vaccinated birds should survive challenge while unvaccinated control birds should die. This test should be carried out on the master seed but need not be done routinely on each vaccine batch produced. For release of subsequent batches, the titre of the virus should be a sufficient indication of vaccine potency.

2.3. Requirements for authorisation

2.3.1. Safety requirements

A group of 1-day-old ducklings susceptible to DVE should be inoculated subcutaneously or intramuscularly with the vaccine at 10 times the recommended dose, and observed for 7–14 days for any signs of adverse reactions.

i) Target and non-target animal safety

The vaccine is intended solely for use to protect ducklings and ducks against DVEV.

ii) Reversion-to-virulence for attenuated/live vaccines

There are no reports of reversion to virulence by the DVE vaccine

iii) Environmental consideration

None.

2.3.2. Efficacy requirements

i) For animal production

Immunity in vaccinated ducks should last throughout a breeding season. Annual re-vaccination is recommended (Sandhu & Metwally, 2008).

ii) For control and eradication

The vaccine virus is not thought to spread by contact from vaccinated to unvaccinated ducks, as the unvaccinated birds remain susceptible to infection.

2.3.3. Stability

When stored at –70°C or lower the vaccine is stable for at least 1 year. Potency testing should be repeated after this time on an aliquot of vaccine to determine whether virus titre has been maintained. Once thawed the vaccine should not be refrozen, it should be maintained at 4°C in a refrigerator but for no longer than 1 week. Lyophilised vaccine should be stored at 4–8°C and used before the stated expiry date.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Research has been published on the development and efficacy of recombinant DVE vaccines in SPF ducks. In 2011, Liu *et al.* reported the use of a DVEV-vectored live bivalent vaccine in which the haemagglutinin gene of H5N1 avian influenza virus was inserted between the unique short (US) 7 and US8 genes of the DVEV genome. This bivalent vaccine was reported to be efficacious against both DVEV and H5N1 avian influenza viral infection in SPF ducks under experimental conditions (Liu *et al.,* 2011). DVE

vaccines based on biotechnology show promise under experimental conditions but are currently not commercially available for widespread use.

3.2. Special requirements for biotechnological vaccines, if any

None.

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NB: At the time of publication (2018) there were no WOAH Reference Laboratories for duck virus enteritis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.3.8.

DUCK VIRUS HEPATITIS

SUMMARY

Duck virus hepatitis (DVH) is typically associated with an acute, contagious infection in susceptible ducklings less than 6 weeks of age and frequently under 3 weeks of age. It does not occur in older birds. The causal viruses of DVH are not considered zoonotic. The disease, DVH, has traditionally been subdivided into types I, II and III.

***DVH type I** can be caused by at least three different genotypes of duck hepatitis A virus (DHAV) virus, a member of the genus* Avihepatovirus*, of the family* Picornaviridae*. The most pathogenic and widespread is DHAV type 1 (DHAV-1), which was formerly designated as duck hepatitis virus type 1. DHAV-2 and DHAV-3 are two additional genotypes within the genus Avihepatovirus that have subsequently been identified as additional aetiological agents of DVH in ducklings.*

***DVH type II** is caused by duck astrovirus type 1 (DAstV-1), a member of the family Astroviridae. DAstV-1 has been reported primarily in the United Kingdom. It has been reported in ducklings from 10 days to 6 weeks of age, and causes pathological changes similar to those of DHAV-1.*

***DVH type III** is caused by duck astrovirus type 2 (DAstV-2), a member of the Astroviridae. It is considered distinct from DAstV-1 and has been reported only in the United States of America. It causes similar liver lesions in young ducklings, but is less virulent than DHAV.*

Diagnosis of hepatitis in ducklings is based on the characteristic disease pattern in the flock, gross pathological changes, the recovery of virus from dead ducklings, and the reproduction of the disease in susceptible ducklings.

The causal viruses of DVH are not considered zoonotic.

***Identification of the agent:** It is not possible to distinguish among DVH types I, II and III on the basis of clinical findings and pathology, but distinctions can be made based on the responses of ducklings, embryonated eggs and cell cultures to the isolated viruses. Alternatively, DHAV RNA may be detected by a one-step reverse-transcriptase polymerase chain reaction (RT-PCR) from duckling liver, and also from allantoic fluid and embryo liver from inoculated duck eggs. Molecular tests have also been described for the detection of DAstV-1 and DAstV-2.*

***Serological tests:** Serological tests have little value in the diagnosis of acute infections caused by DHAV, DAstV-1 and DAstV-2.*

Serum neutralisation tests in ovo *have been used with all three viruses and* in-vitro *tests have been developed for DHAV-1. These tests have been used for virus identification, assay of immune responses to vaccination and epidemiological surveys.*

***Requirements for vaccines:** DHAV-1 infections can be controlled by the use of live attenuated virus vaccines and an inactivated virus vaccine. They are administered to breeder ducks to confer passive immunity to ducklings. Live attenuated virus vaccines may also actively immunise DHAV-1 susceptible day-old ducklings.*

Ducklings susceptible to DHAV-1 may be passively protected with a chicken egg yolk antibody preparation.

DAstV-2 infections can be controlled by the use of a live attenuated virus vaccine given to breeder ducks to confer passive immunity to ducklings.

A. INTRODUCTION

The disease, duck virus hepatitis (DVH) has traditionally been categorised as DVH types I, II or III. It is caused by at least three different small RNA viruses that are of no known public health significance.

1. DVH type I (DHAV-1, DHAV-2, DHAV-3)

DVH type I is caused by the virus species, *Duck hepatitis A virus* (DHAV) *Avihepatovirus* within the *Picornaviridae* family the International Committee on Taxonomy of Viruses created the novel genus, *Avihepatovirus*[1]. This genus contains the species, *Duck hepatitis A virus* (DHAV). Three antigenically unrelated genotypes have been identified, DHAV-1, DHAV-2 and DHAV-3 (Kim *et al.,* 2006; Tseng & Tsai, 2007; Wang *et al.,* 2008). The most prevalent and internationally widespread is DHAV-1. Until recently DHAV-1 had only been associated with disease in mallard and Pekin ducklings, but it has now been reported to cause pancreatitis and encephalitis in Muscovy ducks (Guerin *et al.,* 2007). DHAV-2 is also documented as N-DHV (Tseng & Tsai, 2007). DHAV-2 was originally isolated from a mule duckling and a gosling in Chinese Taipei (Tseng & Tsai, 2007). Mixed DHAV-1 and DHAV-2 infections are common in Chinese Taipei (Tseng & Tsai, 2007). DHAV-3 isolates have been reported from Korea (Rep. of) (Kim *et al.,* 2007a) and China (People's Rep. of) (Fu *et al*., 2008). The limited information on the pathogenicity of DHAV-2 and DHAV-3 indicates that the clinical presentation is similar with DHAV-1.

DHAV causes a highly contagious infection of ducks. The disease is an acute, rapidly spreading, often fatal virus infection of young ducklings. It usually affects ducklings under 6 weeks of age and often much younger. The clinical disease is characterised by lethargy and ataxia followed by opisthotonos and death. Ducklings lose their balance, fall on their sides and kick spasmodically prior to death. The whole disease sequence is rapid and can take as little as 1–2 hours. Practically all mortality in a flock will occur within 3–4 days, with most deaths on the second day. Gross pathological changes appear chiefly in the liver, which is enlarged and displays distinct petechial and ecchymotic haemorrhages. Spleen enlargement and swelling of the kidneys with some congestion of renal blood vessels may also be apparent. Microscopic changes in the liver are characterised by extensive hepatocyte necrosis and bile duct hyperplasia, together with varying degrees of inflammatory cell response and haemorrhage.

2. DVH type II (duck astrovirus type 1 [DAstV-1])

DVH type II is caused by the virus, duck astrovirus-1 (DAstV-1), in the family *Astroviridae* (Gough *et al.,* 1985; Koci & Schultz-Cherry, 2002). Virions have the typical astrovirus morphology and are 28–30 nm in diameter.

Infection of ducklings with DAstV-1 has only been reported from the United Kingdom (Asplin, 1965; Gough *et al.,* 1985). It is an acute, fatal infection of ducklings producing clinical and pathological signs similar to DHAV. Affected birds may show signs of polydypsia and usually die within 1–2 hours of appearing sick.

Gross pathological changes include multiple haemorrhages with both punctate and confluent bands in the liver, swollen pale kidneys with congested blood vessels, and enlarged spleens. The alimentary tract is often empty although the small intestine may contain mucus, and haemorrhagic areas are occasionally seen. Petechial haemorrhages are occasionally seen on the heart. Histologically, changes in the liver are similar to those seen in DHAV infections; the extent of bile duct hyperplasia may be somewhat greater than with DHAV.

3. DVH type III (duck astrovirus type 2 [DAstV-2])

The causal virus of DVH type III is classified as duck astrovirus-2 (DAstV-2), distinct from DAstV-1 (Todd *et al.,* 2009). DAstV-2 has been reported in the USA only. Losses of up to 20% occur in ducklings immune to DHAV-1 (Haider & Calnek, 1979; Toth, 1969). DAstV-2 causes an acute infection of young ducklings with clinical signs similar to those seen in type I infections.

The gross pathology induced by DAstV-2 is also similar to DHAV infection. The liver surface is pale and mottled with many red bands and some petechial haemorrhages. The spleen is paler, but not noticeably enlarged, and the kidneys may show patchy congestion.

1 http://www.ictvonline.org/taxonomyHistory.asp?taxnode_id=20140971&taxa_name=Avihepatovirus

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of duck hepatitis A virus (DHAV) and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[a]						
Virus isolation	+	++	+	++	++	–
Antigen detection	+	++	+	++	++	–
RT-PCR[b]	++	+++	+++	+++	++	–
Detection of immune response						
ELISA	++	++	++	–	+	++
VN	+++	+++	+++	–	+	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]The use of RT-PCR (reverse-transcription polymerase chain reaction) is most frequently reported for testing clinical suspect samples; typically livers. Use of RT-PCR for detection of DHAV using faecal specimens requires further validation.
ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.

Table 2. Test methods available for the diagnosis of duck astrovirus type 1 (DAstV-1) and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Virus isolation	+	++	+	++	++	–
Antigen detection	++	++	++	++	++	–
RT-PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
ELISA	++	+	++	–	+	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

Table 3. Test methods available for the diagnosis of duck astrovirus type 2 (DAstV-2) and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Virus isolation	+	++	+	++	++	–
Antigen detection	++	++	++	++	++	–
RT-PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
ELISA	++	+	++	–	+	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

1.1. DVH type I (DHAV-1, DHAV-2, DHAV-3)

The clinical and pathological observations in ducklings can be highly indicative of DHAV infection. DHAV can readily be recovered from liver tissue by homogenisation as a 20% (w/v) suspension in buffered saline. The suspension is clarified, and can then be treated further (if desired) with 5% chloroform (v/v) for 10–15 minutes at ambient temperature. DHAV is resistant to this treatment.

The presence of DHAV is usually confirmed by one or more of the procedures listed in Section B.1.1.1.

1.1.1. Confirmatory procedures

i) By inoculation of primary cultures of duck embryo liver (DEL) cells, which are particularly sensitive (Woolcock, 1986). Dilutions of the liver homogenate containing DHAV type 1 cause a cytopathic effect (CPE), which is characterised by cell rounding and necrosis. When overlaid with a maintenance medium containing 1% agarose (w/v), the CPE gives rise to plaques approximately 1 mm in diameter.

ii) By inoculation of serial dilutions of the liver homogenate into the allantoic sac of embryonated duck eggs (10–14 days) or chicken eggs (8–10 days). Duck embryos die between 24 and 72 hours post-inoculation, whereas chicken embryos are more variable and erratic in their response and death usually occurs 5–8 days post-inoculation. Gross pathological changes in the embryos include stunting and subcutaneous haemorrhages over the whole body, with oedema particularly of the abdominal and hind limb regions. The embryo livers may be red and yellowish, swollen and may show some necrotic foci. In embryos that take longer to die, a greenish-yellow colour of the allantois is more pronounced, and both the liver lesions and stunting become more evident.

1.1.2. Immunological tests

Antigenic variation among DHAV isolates inducing hepatitis in ducklings exists. Serologically, there is no cross neutralisation between DHAV-1 and DHAV-2 (Tseng & Tsai, 2007) and limited cross neutralisation between DHAV-1 and DHAV-3 (Kim *et al.,* 2007a). A variant, DHAV-1a, isolated in the United States of America (USA) only partially reacts with the classical DHAV-1 virus in cross serum neutralisation tests (Sandhu *et al.,* 1992; Woolcock, 2008b). Other variants have been reported from India and Egypt, but nothing further is known about them. Reports of disease

in Muscovy ducks from France (Guerin *et al.,* 2007; Sandhu *et al.,* 1992; Woolcock, 2008b) also suggest a greater diversity among DHAV than was originally thought.

Immunological tests have not been used extensively for the routine identification of DHAV infection. Various virus neutralisation (VN) assays have been described, which may assume greater significance if DVH types II and III infections become more widespread. The tests that have been described for DHAV-1 (Chalmers & Woolcock, 1984; Woolcock, 1986; 1991; 2008a) include:

i) Serial tenfold dilutions of the virus isolate are mixed with equal volumes of DHAV-1-specific hyperimmune serum diluted between 1/5 and 1/10. The mixtures are allowed to react at room temperature for 1 hour and are then inoculated (0.2 ml) subcutaneously into susceptible ducklings, also via the allantoic cavity (0.2 ml) of embryonated duck eggs and on to primary DEL cell monolayer cultures. Controls in each case consist of the virus isolate mixed with control serum.

1.1.3. Nucleic acid recognition methods

Several publications on the molecular structure of DHAV have been published emphasising genotypic variation among isolates (Tseng *et al.,* 2007; Wang *et al*., 2008). Based on phylogenetic analyses, DHAV known isolates are subdivided into 3 genotypes (Wang *et al*., 2008). DHAV-1 is the most prevalent genotype (Asplin, 1965; Ding & Zhang, 2007; Jin *et al*., 2008; Kim *et al.,* 2006; Liu *et al.,* 2008). DHAV-2 has been reported in Chinese Taipei (Tseng & Tsai, 2007). DHAV-3 has been reported in South Korea (Kim *et al.,* 2007a; 2008) and China (Fu *et al*., 2008).

A one-step reverse-transcription polymerase chain reaction (RT-PCR) assay using primers to the conserved 3D gene has been described for DHAV-1 (Kim *et al.,* 2007b). Anchun *et al*. 2009 also report an RT-PCR to detect Chinese isolates, but it is not clear whether these are DHAV-1 or DHAV-2. The development of a one-step real-time Taqman RT-PCR assay again based on primers to a conserved region in the 3D gene has been reported (Yang *et al.,* 2008), but it is also not clear whether this is for DHAV-1 or DHAV-2; this report does not provide a clear step-by-step protocol used for the method they developed. Several multiplex RT-PCR tests have been developed for the simultaneous detection and differentiation of DHAV genotypes.

i) Polymerase chain reaction

This method has been extracted from Kim *et al.* (2007b). It is based on primers specific to amplifying a region of the 3D gene of DHAV-1.

ii) Detection of DHAV-I from duck and chicken embryo organs and nucleic acid extraction

Supernatants prepared from duckling livers infected with DHAV-1 are collected and filtered (0.2 μm). The allantoic cavities of each of five 11-day-old duck and 9-day-old chicken embryonated eggs are inoculated with 0.2 ml viral supernatant. The allantoic fluid and liver samples are collected from embryos inoculated with two reference strains and each liver sample is ground in a tissue grinder and phosphate buffered saline is added to make 10% suspensions. Liver sample suspensions and allantoic fluid are centrifuged at 2000 ***g*** for 30 minutes, the supernatants are treated with a suitable viral DNA/RNA extraction kit following the manufacturer's instructions. The nucleic acids are used for one-step RT-PCR. After measuring RNA, the samples are stored at –20°C.

iii) Oligonucleotide primers

DHAV-1 ComF (5'-AAG-AAG-GAG-AAA-ATY-[C or T]-AAG-GAA-GG-3') and

DHAV-1 ComR (5'-TTG-ATG-TCA-TAG-CCC-AAS- [C or G]-ACA-GC-3')

Flanked by a 467 bp DNA sequence in the 3D gene.

Alternative primers proposed by Fu *et al.* (2008) are:

Antisense 501-519: 5'-CCT-CAG-GAA-CTA-GTC-TGG-A-3'

Sense 270-285: 5'-GGA-GGT-GGT-GCT-GAA-A-3'

iv) One-step RT-PCR

Times and temperatures should be optimised according to reagents or kits used. The following are given as examples. The one-step RT-PCR is conducted using a suitable kit containing 1 U reverse transcriptase, 2.5 mM dNTPs, 2.5 U DNA polymerase, and RT-PCR buffer (50 mM Tris/HCl and 75 mM KCl). In addition, the following components are included in the reaction: 4 µl (50 ng) RNA or DNA template, 1 µl (10 pmol/µl) of each specific primer (DHAV-1 ComF and DHAV-1 ComR), and diethyl pyrocarbonate (DEPC)-treated dH_2O to a total reaction volume of 20 µl.

A T-gradient thermal cycler is used for one-step RT-PCR. Reverse transcription is performed at 45°C for 30 minutes, after which the enzyme is inactivated at 94°C for 5 minutes. PCR amplification is conducted using an initial denaturation for 20 seconds at 94°C; followed by 40 cycles of annealing for 30 seconds at 52°C, extension for 30 seconds at 72°C, and denaturation for 20 seconds at 94°C; and a final extension for 5 minutes at 72°C. Reaction products are stored at 4°C.

v) Detection of one-step RT-PCR products

PCR products (10 µl) are separated by electrophoresis (100 V) in horizontal 1.5% agarose gels and Tris-acetate buffer (40 mM Tris-acetate, 1 mM ethylenediamine tetra-acetic acid). Gels are stained with nucleic acid stain (0.5 ug/ml), visualised under ultraviolet or blue light, and photographed.

vi) Interpretation of results

A DNA fragment of 467 bp is amplified by one-step RT-PCR using RNA extracted from the livers of ducklings infected with reference DHAV-1 strains. Negative control RNA is obtained from an uninfected duckling liver and does not amplify under the same conditions.

1.2. DVH type II (DAstV-1)

For DAstV-1 detection, homogenised liver suspensions can be evaluated by electron microscopy for DAstV-like particles.

DAstV-1 may be recovered in 20% (w/v) homogenised liver suspensions in buffered saline. This can be used to inoculate:

i) Embryonated chicken or duck eggs, either via the amniotic cavity or yolk sac. These may respond erratically, after four passages, but no deaths may be seen during earlier passages. Embryos take 6–10 days to show evidence of infection; when this occurs there is stunting with green necrotic livers.

Growth of DAstV-1 in primary chicken embryo liver cell cultures has been reported (Baxendale & Mebatsion, 2004); plaque formation was detected at 5 days post-infection after 4 or 5 serial passages. Cell culture techniques are not routinely used as propagation of DAstV-1 in tissue culture typically does not result virus propagation to sufficient levels for diagnostic tests.

1.2.1. Immunological tests

Immunological tests have not been employed routinely for detection of DAstV-1 antigen. However, a neutralisation assay has been applied (Gough *et al.,* 1985) for DAstV-1 identification by inoculating chicken embryos via the amniotic cavity with constant-serum/varying-virus mixtures.

1.2.2. Molecular characterisation

The complete genome of DAsTV-1 consists of 7752 nucleotides and 3 ORF, ORF1a, ORF 1b and ORF2 (Chen *et al.,* 2012).

Confirmation of the identity of putative DAstV-1 isolates can been made using RT-PCR followed by nucleic acid sequence determination and analysis of the amplified fragment (Todd *et al.,* 2009). This degenerate primer based RT-PCR amplifies approximately 434 nt in ORF 1b. Since

this RT-PCR method can amplify other duck astroviruses in samples, sequence analysis of the amplified product is required.

i) RT-PCR detection of DAstV-1 (method extracted from Todd *et al.,* 2009)

Extract viral RNA from 200 µl samples using a suitable RNA extraction kit. Degenerate oligonucleotide primers are used with a suitable One-Step RT-PCR kit. Forward primer 5'-GAY-TGG-ACI-MGI-TAY-GAY-GGI-ACI-ATI-CC-3' and reverse primer 5'-YTT-IAC-CCA-CAT-ICC-RAA-3 amplifies a fragment of approximately 434 nt, which corresponds to nucleotides 3799 to 4233 in the genome of G4260 (accession number: AB033998). The RT-PCR conditions were initial denaturation, 94°C for 5 minutes, then 45 cycles of denaturation, 94°C for 1 minute; annealing, 45°C for 1 minute; and extension, 72°C for 90 seconds followed by a final extension step, 72°C for 5 minutes. Examine the amplified product by electrophoresis in a 1% agarose gel, treated with nucleic acid stain and visualised by ultraviolet or blue light transillumination. Sequencing and sequence comparison of other ORF1b sequences of astroviruses should be performed.

1.3. DVH type III (DAstV-2)

DAstV-2 can be recovered from homogenised liver suspensions and is resistant to treatment with 5% chloroform. The virus can be isolated by:

i) Inoculating the isolate on to the chorioallantoic membrane (CAM) of 10-day-old embryonated duck eggs. The response is erratic, but some embryo mortality always occurs within 7–10 days. The membranes assume a dry crusty appearance, beneath which they are oedematous. The embryos may be stunted and oedematous with skin haemorrhages. The liver, kidneys and spleen are enlarged.

Attempts to cultivate the virus in hens' eggs have not been successful.

Attempts to induce a CPE with the virus in tissue cultures have not been successful, but the virus has been detected by direct immunofluorescence in experimentally infected DEL and duck embryo kidney (DEK) cell monolayer cultures (Haider & Calnek, 1979).

1.3.1. Molecular characterisation

DVH type III has now been identified as an astrovirus (DAstV-2) by nucleic acid sequence data. It is considered distinct from DVH type II (DAstV-1) (Todd *et al.,* 2009). The RT-PCR and sequencing molecular method described for detection of DAstV-1 can also be used to identify DAstV-2 (Todd *et al.,* 2009).

2. Serological tests

These antibody detection tests are not useful for the diagnosis as the clinical disease is too acute for detection of early infection. Serological tests are useful for virus identification, epidemiological disease investigations and titration of antibody response to vaccination. A comparative study assessing DHAV-1 VN, ELISA and agar gel immunodiffusion (AGID) tests, suggest that VN and ELISA have comparable sensitivity but the sensitivity of AGID was very low (Zhao *et al*., 1991). A VP1-ELISA utilising the VP1 of DHAV-1 as a coating antigen has been described for the detection of DHAV-1 antibodies in sera. In comparison with VN, the VP1-ELISA had a reported specificity and sensitivity of 92.5% and 96.7%, respectively (Liu *et al.,* 2010). The level of detectable antibodies after DAstV-1 challenge were shown to be low. While an indirect ELISA expressing the C-terminal ORF2 protein of DAstV-1 has been reported, the exact sensitivity and specificity of this test is unclear.

2.1. ELISA for DHAV-1 Detection (procedure extracted from Zhao *et al.*, 1991)

Add 100 µl of coating antigen diluted in carbonic acid-buffered saline (0.5 M, pH 9.6) to each well of an ELISA plate. Incubate overnight at 4°C. Wash plate with 0.01 M PBS (pH 7.4) containing 0.05% Tween-20 (PBST) three times. Add 100 µl of each test serum diluted in PBST containing 0.1% bovine serum albumin (PBST-BSA) to duplicate wells in the sensitised plate. Incubate for 2 hours at 37°C in a humid chamber. Wash plate as described above. Add 100 µl of rabbit anti-duck IgG HRP conjugate diluted in PBST-BSA to each well. Incubate for 2 hours at 37°C. Wash plate as described above. To each well add 100 µl

substrate solution (24.3 ml of 0.1 M citric acid + 25.7 ml of 0.2 M phosphate + 50 ml of distilled water, mix and add 40 mg o-phenylenediamine-free base or dihydrochloride, as well as 40 µl of 30% H_2O_2, and use immediately). After 30 minutes, add 25 µl 2.5 M H_2SO_4 to each well. Read absorbance at 490 nm with an ELISA reader. Alternative chromogens may be used in a suitable diluent with appropriate detection systems.

2.2. Virus neutralisation

DHAV, DastV-1 and DastV-2 have been used in VN tests *in ovo*. Their success depends on the expression of the virus in the assay system used; with DAstV-1 and DAstV-2, this can be a problem. *In-vitro* tests have been developed for DHAV-1; these include a plaque reduction assay and a microtitre assay (Woolcock, 1986; 1991). The plaque reduction assay may be performed using either primary DEK or DEL cells. Primary cell culture monolayers are prepared in Eagle's minimal essential medium (MEM) containing 5–10% fetal calf serum (FCS), 2 mM glutamine, 0.17% sodium bicarbonate and gentamicin. Trypsinised cells are seeded into 5 cm diameter Petri dishes, then incubated at 37°C in a 5% CO_2 atmosphere. Monolayers should be nearly confluent at 24–48 hours post-seeding. The monolayers are washed twice with serum-free MEM or Hank's balanced salt solution to remove all traces of FCS before infecting with DHAV-1. Equal volumes of DHAV-1 suspended in serum-free MEM, adjusted to 200 plaque-forming units (PFU) per 0.1 ml, are mixed with equal volumes of serially diluted duck sera (twofold dilutions in MEM). The serum samples should be heat inactivated at 56°C for 30 minutes before testing. The virus/serum mixtures are incubated at 37°C for 1 hour; then 0.1-ml aliquots are added to the confluent cell monolayers, three dishes per dilution. The plates are left for 30 minutes at room temperature (20–22°C), then overlaid with agarose maintenance medium (MEM containing 2% chicken serum and 0.1–0.2% FCS to which agarose had been added to a final concentration of 1% [w/w]). The plates are then placed at 37°C in a 5% CO_2 atmosphere. The number of plaques produced is recorded after 48 hours' incubation. Plaques may be observed using an oblique light source, or alternatively monolayers may be fixed with 10% formol-buffered saline and stained with 1% crystal violet. Serum antibody titres are expressed as the reciprocal of the highest serum dilution that reduces the plaque count by 50%.

A microtitre neutralisation assay may be performed using primary DEK cells. Serial twofold dilutions of each serum sample (heat-inactivated) are prepared in 50 µl of serum-free Eagle's basal medium (BME) in microtitre plates. Approximately $10^{2.0}$ $TCID_{50}$ (50% tissue culture infective dose) of DHAV-1 in 50 µl of BME is added to each well and the mixtures are allowed to react at 37°C for 1 hour. Primary DEK cells are suspended in BME supplemented with 10% tryptose phosphate broth, 2 mM L-glutamine, 0.17% sodium bicarbonate and 2–4% chicken serum, and are adjusted to contain 3×10^5 cells/ml. Cells are next added to the plates at 100 µl per well and the plates are then incubated for up to 96 hours at 37°C in a humidified 5% CO_2 atmosphere. Following incubation, cells are fixed with 10% formol-buffered saline and stained with 1% crystal violet. The plates are read macroscopically. The titre for virus neutralising activity is expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e., there is no evidence of CPE and therefore complete virus neutralisation has occurred. A titre of less than 4 log_2 is considered to be negative.

These neutralisation tests have been used to assay humoral immune responses to vaccination and for epidemiological surveys, as well as for virus identification.

C. REQUIREMENTS FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1.1. Rationale and intended use of the product

DHAV-1 can be controlled by the use of a live attenuated virus vaccine. This is given to breeder ducks so that immunity is transferred via the yolk to newly hatched birds. Live vaccine virus can also be used to

actively immunise newly hatched DHAV-1-susceptible ducklings (Crighton & Woolcock, 1978). An inactivated DHAV-1 vaccine is also effective when administered to breeder ducks that have been primed with live vaccine or previously field exposed to live DHAV-1; progeny from these breeders have maternal immunity (Woolcock, 1991). Ducks may also be passively protected by inoculation of antibodies in chicken egg-yolk.

The development and evaluation of a vaccine to protect ducklings against DHAV-3 in Korea has been described (Kim *et al.,* 2009). The methods used are comparable to those described in this text for DHAV-1.

An attenuated live virus DVH type II (DAstV-1) vaccine has been used to protect ducklings only under experimental conditions (Gough *et al.,* 1985).

DAstV-2 infections have been controlled by the use of attenuated live virus vaccines given to breeder ducks, so that the immunity is transferred via the yolk sac to the hatching ducklings.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The DHAV-1 virus vaccine seed used most commonly in Europe is derived from an isolate passaged in embryonated chicken eggs 53–55 times; the DHAV-1 virus vaccine seed used in the USA for live and inactivated vaccines has been passaged 84–89 times.

The DAstV-1 virus vaccine seed originated from an isolate attenuated by 25 serial passages in embryonated chicken eggs (Asplin, 1965), and has been employed only experimentally under field conditions (R.E. Gough, pers. comm.).

The DAstV-2 vaccine seed has been attenuated by 30 serial passages in embryonated duck eggs inoculated via the CAM.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

All seed viruses should be shown to be free from extraneous viruses that are pathogenic for ducks, chickens or turkeys. The seeds should be free from all microbiological and fungal contamination.

The identity of the virus type should be confirmed by a VN test conducted with specific antiserum by a constant-serum/varying-virus method. In the case of DHAV-1 and DAstV-1, the tests are performed in embryonated chicken eggs; with DAstV-2 the tests are done in embryonated duck eggs. The antiserum should reduce the titre of the respective virus by at least $10^{2.0}$ ELD_{50} (50% embryo lethal dose).

2.2. Method of manufacture

2.2.1. Procedure

DHAV-1 and DAstV-1 viruses are treated similarly. The vaccine is produced in 9- to –10-day-old specific-pathogen free (SPF) embryonated chicken eggs inoculated via the allantoic route, and incubated at 37°C. Most embryo deaths occur within 2–3 days in the case of DHAV-1, but with DAstV-1, the deaths do not occur until 6–10 days after inoculation, although they are harvested at 3–5 days for maximum virus yield. The embryo harvests are homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at –70°C or below. Subsequently, they may be stored satisfactorily between –20°C and –40°C. DHAV-1 attenuated vaccine is also available as a lyophilised preparation that may be stored at 2–8°C. The reconstituted vaccine may be used with or without the incorporation of aluminium hydroxide in the diluent.

In the case of inactivated DHAV-1 vaccine, the embryo harvests are homogenised and clarified by low-speed centrifugation and then further purified by treatment with chloroform (final concentration 10% [v/v]). This preparation is then inactivated with freshly prepared binary ethylenimine (BEI). The inactivated virus is then blended with a suitable adjuvant; 0.2 % (v/v) formalin is added as a preservative (Woolcock, 1991).

The DAstV-2 vaccine is prepared in 10-day-old SPF duck eggs inoculated via the CAM with attenuated DAstV-2 and incubated at 37°C. Most embryo deaths occur between 6 and 10 days. Eggs containing dying embryos, together with their CAMs, are harvested and homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at –70°C or below.

2.2.2. Egg yolk antibody

Virulent DHAV-1 prepared from duckling livers or attenuated virus may be used to hyperimmunise SPF chickens for egg-yolk antibody production. Eggs are collected from the hyperimmunised birds and stored at 4°C until time of production. The yolks are separated, pooled and blended with an antifoaming agent. The mixture is diluted with buffered saline containing no more than 0.2% (v/v) formalin as a preservative. The dispensed product is stored at 4°C and has a shelf life of 1 year. Tests are carried out for sterility in the usual way for the absence of contaminants.

2.2.3. Requirements for substrates and media

All reagents should be sterile and eggs obtained from a specific pathogen-free source.

2.2.4. In-process controls

Any embryo deaths within the first 24 hours of inoculation should be discarded as nonspecific deaths.

2.2.5. Final product batch tests

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in the chapter 1.1.9.

ii) Safety

A group of 1- to 3-day-old ducklings susceptible to the type of virus concerned, should be inoculated subcutaneously or intramuscularly (in the case of DHAV-1 and DAstV-1), or subcutaneously (in the case of DAstV-2), with the attenuated vaccine at ten times the recommended dose, and kept under observation for between 10 and 21 days for any adverse reactions. Attenuated live vaccines should be stable and not revert to virulence on back passages in susceptible ducklings.

A safety test on the inactivated DHAV-1 vaccine is performed by inoculating the recommended dose (0.5 ml) intramuscularly into a group of day-old ducklings; no adverse effects should be observed during the period of testing.

Safety tests on egg-yolk antibody are done by inoculating 1 ml subcutaneously into each of a group of ducklings, which are then kept under observation for 3 days for signs of adverse effects.

iii) Batch potency

For DHAV-1 and DAstV-1 viruses, the virus titre of the vaccine should be determined in 9- to 10-day-old embryonated chicken eggs inoculated into the allantoic cavity and incubated at 37°C. The immunogenicity, of the vaccine for ducklings susceptible to DHAV-1 and DAstV-1 virus can be assessed by inoculating subcutaneously a minimum of $10^{3.3}$ ELD_{50} per duckling of the vaccine virus and challenging subcutaneously 72 hours later with $10^{3.0}$ LD_{50} per duckling of virulent DHAV-1 and DAstV-1 (Crighton & Woolcock, 1978). At least 80% of the

vaccinated birds should survive. In the case of DHAV-1, at least 80% of the controls should die and in the case of DAstV-1, a 20% mortality in the controls is more realistic.

The immunogenicity of the inactivated vaccine is considered to be satisfactory if a four-fold or greater increase in neutralising antibody titre can be demonstrated following administration to ducklings that have been previously primed with live attenuated DHAV-1.

For DAstV-2, the titre of the vaccine should be determined in 10-day-old embryonated duck eggs inoculated onto the CAM. Immunogenicity tests in ducklings have proved difficult because of the variable pathogenicity of the challenge virus for ducklings.

Potency tests on yolk antibody are done by determining the neutralising index (NI) for the product in embryonated hens' eggs using the constant-yolk/varying-virus method. A minimum NI of $10^{3.0}$ is considered to be satisfactory. The efficacy of the product is determined by inoculating a group of susceptible ducklings with the recommended dose of egg yolk antibody. A second group is left untreated. After 24 hours, each group is challenged with virulent DHAV-1. The product is adjudged efficacious if at least 80% of the treated ducklings survive and at least 80% of the controls die.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

A group of 1–3-day-old ducklings susceptible to the type of virus concerned, should be inoculated subcutaneously or intramuscularly (in the case of DHAV-1 and DAstV-1), or subcutaneously (in the case of type DAstV-2), with the attenuated vaccine at ten times the recommended dose, and kept under observation for between 10 and 21 days for any adverse reactions. Attenuated live vaccines should be stable and not revert to virulence on back passages in susceptible ducklings.

A safety test on the inactivated DHAV-1 vaccine is performed by inoculating the recommended dose (0.5 ml) intramuscularly into a group of day-old ducklings; no adverse effects should be observed during the period of testing.

Safety tests on yolk antibody are done by inoculating 1 ml subcutaneously into each of a group of ducklings, which are then kept under observation for 3 days for signs of adverse effects.

i) Target and non-target animal safety

The vaccines and egg yolk are intended solely for use to protect ducklings against DVH and to immunise breeder ducks so that maternal antibodies may be transferred to progeny.

ii) Reversion-to-virulence for attenuated/live vaccines

Reversion to virulence on serial passage in ducklings has been reported (Woolcock & Crighton, 1979; 1981).

iii) Environmental consideration

None.

2.3.2. Efficacy requirements

i) For animal production

In the case of newly hatched ducklings, attenuated live DHAV-1 replicates rapidly and results in an immunity within 48–72 hours of vaccination. This immunity persists throughout the susceptible period of life (Crighton & Woolcock, 1978). However in ducklings protected by vaccination of their parents, the level of maternally derived immunity decreases over the first 2 weeks of life, but such ducklings can be actively re-immunised with attenuated virus given subcutaneously or orally at about 7–10 days of age (Hanson & Tripathy, 1976; Tripathy & Hanson, 1986). Alternatively, the immunity can be enhanced by the administration of either specific hyperimmune serum or egg yolk antibody prepared from eggs laid by chickens actively hyperimmunised against DHAV-1.

Breeder ducks primed with live DHAV-1 at 12 weeks of age and then given, intramuscularly, a single dose of inactivated DHAV-1 vaccine at 18 weeks of age should produce maternally immune progeny through a complete laying cycle (Woolcock, 1991).

ii) For control and eradication

Breeder ducks given live attenuated DHAV-1 vaccine two or three times at 12, 8 and 4 weeks before coming into lay, and breeder ducks given live attenuated DAstV-2 vaccine twice at 12 and 4 weeks before coming into lay should produce passively immune progeny throughout a breeding season. However, it is usually recommended to revaccinate every 3 months with DHAV-1 vaccine and every 6 months with DAstV-2 vaccine after the onset of lay. DHAV-1 attenuated vaccine can also be supplied as a lyophilised preparation that is blended with a diluent containing aluminium hydroxide, just before administration. This is given at 7 weeks of age with a second dose 2 weeks before onset of lay. This should provide maternally immune progeny throughout a complete laying cycle. No information on the use of DAstV-1 vaccine in breeder ducks is available.

Live attenuated DHAV-1 and DAstV-1 vaccine given subcutaneously or intramuscularly to 1-day-old ducklings protects against the disease for the duration of their susceptibility. No information is available on the use of DAstV-2 vaccine to actively immunise 1-day-old ducklings.

Breeder ducks primed with live DHAV-1 at 12 weeks of age and then given a single dose of inactivated DHAV-1 vaccine intramuscularly at 18 weeks of age, should produce maternally immune progeny through a complete laying cycle (Woolcock, 1991).

Egg-yolk antibody offers passive immunisation in the face of an outbreak. The duration of its efficacy is short-lived.

2.3.3. Stability

Aqueous preparations of live attenuated DHAV-1, DAstV-1 and DAstV-2 vaccines when stored frozen at –70°C or lower should remain stable for at least 1 year. Once thawed these vaccines should be held at 4°C and used within 1 week. Live lyophilised vaccines may be stored at 2–8°C and should retain their potency for at least 1 year.

The inactivated DHAV-1 vaccine is blended with adjuvant and can be stored at 4°C for at least 20 months without loss of immunogenicity.

Egg-yolk antibody can be stored for up to 1 year at 4°C.

3. Vaccines based on biotechnology

Not applicable at present.

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NB: At the time of publication (2022) there were no OIE Reference Laboratories for duck virus hepatitis (please consult the OIE Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 3.3.9.

FOWL CHOLERA

SUMMARY

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is distributed world-wide. Fowl cholera outbreaks often manifest as an acute fatal septicaemia, primarily in adult birds. Chronic and subclinical infections also occur. Diagnosis depends on isolation and identification of the causative bacterium, Pasteurella multocida*. Presumptive diagnosis may be based on the occurrence of typical signs and lesions and/or on the microscopic demonstration of myriad bacteria in blood smears, or impression smears of tissues such as liver or spleen. Mild or chronic forms of the disease also occur where the disease is endemic, with localised infection primarily of the respiratory and skeletal systems.*

Identification of the agent: Pasteurella multocida *is readily isolated, often in pure culture, from visceral organs such as lung, liver and spleen, bone marrow, gonads or heart blood of birds that succumb to the acute bacteraemic form of the disease, or from the caseous exudate characteristic of chronic fowl cholera lesions. It is a facultative anaerobic bacterium that grows best at 37°C. Primary isolation is usually accomplished using media such as dextrose starch agar, blood agar, and trypticase–soy agar. Isolation may be improved by the addition of 5% heat-inactivated serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation and are discrete, circular, convex, translucent, and butyraceous. The cells are coccobacillary or short rod-shaped, 0.2–0.4 × 0.6–2.5 μm in size, stain Gram negative, and generally occur singly or in pairs. Bipolar staining is evident with Wright or Giemsa stains.*

Identification of P. multocida *has been traditionally based on the results of biochemical tests, which include carbohydrate fermentation, enzyme production, and selected metabolite production. In recent times, polymerase chain reaction (PCR)-based assays have been widely adopted.*

Serological characterisation of strains of P. multocida *includes capsular (Carter) serogrouping and somatic (Heddleston) serotyping. A PCR-based method is now the accepted method for Carter capsular typing. A PCR method that recognises eight genotypes (L1-L8) based on the lipopolysaccharide (LPS) outer core biosynthesis locus is now preferred to conventional Heddleston serotyping. DNA fingerprinting and, most recently, whole genome sequencing can differentiate among* P. multocida *having the same capsular serogroup and somatic serovar. These characterisations require a specialised laboratory with appropriate diagnostic reagents.*

Serological tests: *Serological tests are rarely used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis through isolation and identification of the causative organism generally precludes the need for serodiagnosis.*

Requirements for vaccines: *The* P. multocida *vaccines in general use are bacterins, containing aluminium hydroxide or oil as adjuvant, prepared from multiple serovars. Two doses of the killed vaccine are typically required. It is now known that a killed vaccine provides protection only against isolates with the identical or near to identical LPS structure, with multiple structures being possible within a Heddleston serovar. Live culture vaccines tend to impart greater protective immunity and do not have the same requirement of an exact LPS structure to provide protection and can even provide cross serovar protection. Some live vaccines have been associated with potential post-vaccinal sequelae such as pneumonitis and arthritis. Multivalent vaccines typically incorporate somatic serovars 1, 3, and 4 as they are among the more commonly isolated avian serovars. Safety and potency testing of bacterins usually use the host animal. Final containers of live cultures are tested for potency by bacterial counts.*

A. INTRODUCTION

Fowl cholera (also known as avian cholera, avian pasteurellosis and avian haemorrhagic septicaemia) is a contagious bacterial disease of domesticated and wild avian species. The disease typically occurs as a fulminating disease with massive bacteraemia and high morbidity and mortality in older birds. Chronic infections also occur with clinical signs and lesions related to localised infections. The pulmonary system and tissues associated with the musculoskeletal system are often the seats of chronic infection. The causative agent is *Pasteurella multocida,* a member of the family *Pasteurellaceae.* While *P. multocida* is a pathogen of humans, this association is typically linked with animal bites. Fowl cholera isolates of *P. multocida* are not considered to have zoonotic potential as avian isolates are generally nonpathogenic in mammals exposed by the oral or subcutaneous routes. However, laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Other bacterial diseases, including salmonellosis, colibacillosis, and listeriosis in chickens, and pseudotuberculosis, erysipelas, and chlamydiosis in turkeys, may present with clinical signs and lesions similar to fowl cholera. Differentiation is traditionally based on isolation and identification, as *P. multocida* is readily cultured from cases of fowl cholera. Direct testing of clinical material with molecular tools has also been validated.

B. DIAGNOSTIC TECHNIQUES

Fowl cholera (avian pasteurellosis) is a commonly occurring avian disease that can affect all types of birds and is often fatal (Blackall & Hofacre, 2020). In the peracute form, fowl cholera is one of the most virulent and infectious diseases of poultry. Diagnosis depends on identification of the causative bacterium, *P. multocida*, following isolation from birds with signs and lesions consistent with this disease or direct detection of the organism with specific molecular assays. Presumptive diagnosis may be based on the observance of typical signs and lesions and/or on the microscopic demonstration of bacteria showing bipolar staining in smears of tissues, such as blood, liver, or spleen. Mild forms of the disease may occur.

All avian species are susceptible to *P. multocida*, although turkeys may be the most severely affected. Birds older than 16 weeks are primarily affected. Often the first sign of disease is dead birds. Other signs include: fever, anorexia, depression, mucus discharge from the mouth, diarrhoea, ruffled feathers, drop in egg production coupled with smaller eggs, increased respiratory rate, and cyanosis at the time of death. Lesions that are often observed include: congested organs with serosal haemorrhages, enlarged liver and spleen, multiple small necrotic areas in the liver and/or spleen, pneumonia, and mild ascites and pericardial oedema. Birds that survive the acute septicaemic stage or those infected with organisms of low virulence may develop chronic fowl cholera, characterised by localised infections. These infections often involve joints, foot pads, tendon sheaths, sternal bursa, conjunctivae, wattles, pharynx, lungs, air sacs, middle ears, bone marrow, and meninges. Lesions resulting from these infections are usually characterised by bacterial colonisation with necrosis, fibrino-suppurative exudate, and degrees of fibroplasia.

Traditionally, diagnosis has depended on the isolation and identification of the causative organism.

Table 1. Test methods available for the diagnosis of fowl cholera and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Culture	–	–	–	+++	–	–
PCR methods	–	–	–	+++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
ELISA	–	–	–	–	–	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

1.1. *In-vitro* culture

Pasteurella multocida is a facultative anaerobic bacterium that grows best at 35–37°C. Primary isolation is usually accomplished using media such as blood agar, trypticase–soy agar or dextrose starch agar, and isolation may be improved by supplementing these media with 5% heat-inactivated serum. Maintenance media usually do not require supplemental serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation. They usually are discrete, circular, convex, translucent, and butyraceous. Capsulated organisms usually produce larger colonies than those of noncapsulated organisms. Watery mucoid colonies, often observed with mammalian respiratory tract isolates, are very rare with avian isolates. The cells are coccobacillary or short rod-shaped, usually 0.2–0.4 × 0.6–2.5 μm in size, stain Gram negative, and generally occur singly or in pairs. Recently isolated organisms or those found in tissue smears show bipolar staining with Wright or Giemsa stains or methylene blue, and are usually encapsulated.

Isolation of the organism from visceral organs, such as liver, bone marrow, spleen, or heart blood of birds that succumb to the acute form of the disease, and from exudative lesions of birds with the chronic form of the disease, is generally easily accomplished. Isolation from those chronically affected birds that have no evidence of disease other than emaciation and lethargy is often difficult. In this condition or when host decomposition has occurred, bone marrow is the tissue of choice for isolation attempts. The surface of the tissue to be cultured is seared with a hot spatula and a specimen is obtained by inserting a sterile cotton swab, wire or plastic loop through the heat-sterilised surface. Alternatively the sterilised surface can be cut with sterile scissors/scalpel and the swab or loop inserted into the cut without touching the outer surface. The specimen is inoculated directly on to agar medium or into tryptose or another broth medium, incubated for 2–3 hours, transferred to agar medium, and incubated again.

Identification has traditionally been based primarily on the results of biochemical tests. Carbohydrate fermentation reactions are essential. Those carbohydrates that are fermented include: glucose, mannose, galactose, fructose, and sucrose. Those not fermented include: rhamnose, cellobiose, raffinose, inulin, erythritol, adonitol, m-inositol, and salicin. Mannitol is usually fermented. Arabinose, maltose, lactose, and dextrin are usually not fermented. High quality maltose with minimal glucose contamination must be used to avoid false positive reactions from the glucose contamination present in lower quality maltose preparations. Variable reactions occur with xylose, trehalose, glycerol, and sorbitol. Avian isolates of *Pasteurella multocida* do not cause haemolysis on sheep blood agar, are not motile and only rarely grow on MacConkey agar. The organism produces catalase, oxidase, and ornithine decarboxylase, but does not produce urease, lysine decarboxylase, ß-galactosidase, or arginine dihydrolase. Phosphatase production is variable. Nitrate is reduced; indole and hydrogen sulphide are produced, and methyl red and Voges–Proskauer tests are negative. Full details are provided by Glisson *et al*. (2008; 2013).

Phenotypic differentiation of *P. multocida* from other similar avian organisms can usually be accomplished using the tests and results indicated in Table 2. Laboratory experience has shown that *P. multocida* is most easily identified by its colony morphology and appearance in Gram stains. Positive reactions to oxidase, indole and ornithine decarboxylase are the useful biochemical indications.

Commercial biochemical test kits are available but have not been recommended (Blackall & Norskov-Lauritsen, 2008). The use of matrix-assisted adsorption ionisation – time of flight (MALDI-TOF) mass spectrometry has been evaluated in just two studies to date with both studies indicating that the technology correctly identified all tested *P. multocida* isolates (Kuhnert *et al.,* 2012; Zangenah *et al.*, 2013).

Table 2. Tests used to differentiate* Pasteurella multocida *from other similar organisms found in avian hosts*

Test*	*Pasteurella multocida*	*Avibacterium gallinarum*	*Gallibacterium anatis* biovar haemolytica	*Riemerella anatipestifer*
Haemolysis on sheep blood agar	–	–	+	V
Growth on MacConkey's agar	–	–	v	–
Indole production	+	–	–	–
Gelatin liquefaction	–	–	–	+u
Catalase production	+	+	+	+
Urease production	–	–	–	v
Glucose fermentation	+	+	+	–
Lactose fermentation	–u	–	V	–
Sucrose fermentation	+	+	+	–
Maltose fermentation	–u	+	V	–
Ornithine decarboxylase	+	–	–	–

**Avibacterium gallinarum* was once known as [*Pasteurella*] *gallinarum*, *Gallibacterium anatis* biovar haemolytica was once known as [*Pasteurella*] *haemolytica* and *Riemerella anatipestifer* was once known as [*Pasteurella*] *anatipestifer*. Test reaction results: – = no reaction; + = reaction; v = variable reactions; –u = usually no reaction; +u usually a reaction.

1.2. Animal inoculation

Mouse inoculation has been a traditional approach for isolating *P. multocida,* particularly from non-sterile sites and a full description of the methodology is provided by Muhairwa *et al.* (2001). This approach selectively isolates those clones that are pathogenic for mice. Most importantly, the technique is no longer regarded as a suitable alternative given the issues of animal welfare and the need to replace the use of animals in diagnostic techniques.

1.3. Antigenic characterisation

Antigenic characterisation of *P. multocida* has traditionally been accomplished by capsular serogrouping and somatic serotyping. Capsular serogroups are determined by a passive haemagglutination test (Carter, 1955; 1972). Capsular serogroups are A, B, D, E, and F. All but serogroup E have been isolated from avian hosts. A nonserological disk diffusion test that uses specific mucopolysaccharidases to differentiate serogroups A, D, and F has been developed (Rimler, 1994). A multiplex polymerase chain reaction (PCR) that allows rapid and specific capsule typing has been developed (Townsend *et al.,* 2001) and thus the traditional serological-based capsule typing has been largely replaced.

Somatic serovars have been traditionally determined by an agar gel immunodiffusion (AGID) test and focus on the lipopolysaccharide (LPS) antigens (Heddleston, 1962; Heddleston *et al*., 1972). Serovars 1 through 16 have been reported with all 16 serovars having been isolated from avian hosts (Blackall & Hofacre, 2020). However, a comparison of typing achieved by a full chemical characterisation of the LPS structure, the Heddleston somatic serovar as determined by AGID and a new molecular assay that focuses on the LPS biosynthetic loci (discussed below) revealed that traditional serotyping has a high error rate (Harper *et al*., 2015). When combined with the difficulty in producing high titre antisera, the traditional Heddleston somatic serotyping scheme can no longer be recommended as a suitable typing methodology.

1.4. Molecular methods – detection of nucleic acids

A number of PCR assays developed for the confirmatory identification of suspect *P. multocida* isolates have been compared (Adhikary *et al.,* 2013). The PCR developed by Townsend *et al.* (1998) was shown to have a sensitivity of 100% and a specificity of 92% when tested with 85 *P. multocida* isolates and 13 strains of related taxa (Adhikary *et al.,* 2013) and is the recommended molecular assay for the identification of *P. multocida* isolates. This assay targets a cloned sequence known as KMT1. The Townsend *et al.* (1998) PCR uses the following primers:

5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3' (KMT1SP6)

5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3' (KMT1T7)

The PCR amplification mix contains 1.65 μM of each primer, dNTPs (each at 200 μM), 1× Expand High Fidelity buffer with 1.5 mM $MgCl_2$, and 1 U of *Taq* polymerase. The cycle conditions are as follows: initial denaturation at 95°C for 4 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 9 minutes. A 460 bp product is produced by isolates of *P. multocida.*

Few assays have been validated for direct detection of *P. multocida* in clinical material. The real-time PCR described by Corney *et al.* (2007) has been shown to perform at least as well as culture when used directly on swabs from birds. The Corney *et al.* (2007) assay targets the 16S rRNA gene and uses the following primers and a minor groove binder (MGB) probe:

forward primer PMA2f, 5'-ATA-ACT-GTG-GGA-AAC-TGC-AGC-TAA-3'

reverse primer PMA2r, 5'-GGT-CCC-ACC-CTT-T(A/C)-CTC-CTC-3'

MGB probe PMA2, 5'-6FAM-CCG-CGT-A(A/T)-TCT-CT-MGBNFQ-3'

The assay uses a primer concentration of 0.2 μM for the primers and 0.3 μM concentration of probe and a commercial real-time mastermix. The cycle conditions are 15 seconds at 95°C, 60 seconds at 60°C for 50 cycles.

A specific multiplex capsular PCR assay has been developed that allows for rapid and specific capsular typing (Townsend *et al.,* 2001). This assay is now widely used in place of the traditional capsular serotyping assay or the non-serological methods. Details of the primers are provided in Table 3.

***Table 3. Primers and target gene details of the* Pasteurella multocida *multiplex capsular PCR typing assay of Townsend* et al. (*2001*)**

Serogroup	Gene	Primer	Sequence (5' → 3')	Amplicon size (bp)
All	KMT1	KMT1T7	ATC-CGC-TAT-TTA-CCC-AGT-GG	460
		KMT1SP6	GCT-GTA-AAC-GAA-CTC-GCC-AC	
A	*hyaD-hyaC*	CAPA-FWD	TGC-CAA-AAT-CGC-AGT-CAG	1044
		CAPA-REV	TTG-CCA-TCA-TTG-TCA-GTG	
B	*bcbD*	CAPB-FWD	CAT-TTA-TCC-AAG-CTC-CAC-C	760
		CAPB-REV	GCC-CGA-GAG-TTT-CAA-TCC	
D	*dcbF*	CAPD-FWD	TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC	657
		CAPD-REV	CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG	
E	*echJ*	CAPE-FWD	TCC-GCA-GAA-AAT-TAT-TGA-CTC	511
		CAPE-REV	GCT-TGC-TGC-TTG-ATT-TTG-TC	
F	*fcbD*	CAPF-FWD	AAT-CGG-AGA-ACG-CAG-AAA-TCA-G	851
		CAPF-REV	TTC-CGC-CGT-CAA-TTA-CTC-TG	

The multiple PCR mixture contained each primer (3.2 μM) of the six primer sets, 1 U of *Taq* DNA polymerase, 2 mM $MgCl_2$, and dNTPs (each at a concentration of 200 μM). The cycling conditions are as follows: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes (Townsend *et al.*, 2001).

As noted in the previous section, the molecular characterisation of the LPS genotype is now recognised as being far more reliable than the traditional serological typing scheme based on the LPS antigens – the Heddleston scheme (Harper *et al.*, 2015). The LPS multiplex PCR developed by Harper *et al.* (2015) targets the LPS outer core biosynthesis locus and recognises eight LPS genotypes – termed L1 to L8. The original 16 Heddleston LPS serovar reference strains are assigned as follows to the eight LPS genotypes: serovars 1 and 14 – LPS L1; serovars 2 and 5 – LPS L2; serovars 3 and 4 – LPS L3; serovars 6 and 7 – LPS L4; serovar 9 – LPS L5; serovars 10, 11, 12 and 15 – LPS L6; serovars 8 and 13 – LPS L7; serovar 16 – LPS L8. The details of the primers used in the LPS genotyping assay are shown in Table 4.

***Table 4. Primers and target gene details of the* Pasteurella multocida *multiplex LPS genotyping assay of Harper* et al. (*2015*)**

LPS Locus	Primer location	Primer*	Sequence (5' → 3')	Amplicon size (bp)
L1	*pcgD*	BAP6119 (f)	ACA-TTC-CAG-ATA-ATA-CAC-CCG	1307
	pcgB	BAP6120 (r)	ATT-GGA-GCA-CCT-AGT-AAC-CC	
L2	*nctA*	BAP6121 (f)	CTT-AAA-GTA-ACA-CTC-GCT-ATT-GC	810
		BAP6122 (r)	TTT-GAT-TTC-CCT-TGG-GAT-AGC	
L3	*gatF*	BAP7213 (f)	TGC-AGG-CGA-GAG-TTG-ATA-AAC-CAT-C	474
		BAP7214 (r)	CAA-AGA-TTG-GTT-CCA-AAT-CTG-AAT-GGA	
L4	*latB*	BAP6125 (r)	TTT-CCA-TAG-ATT-AGC-AAT-GCC-G	550
		BAP6126 (f)	CTT-TAT-TTG-GTC-TTT-ATA-TAT-ACC	
L5	*rmlA*	BAP6129 (f)	AGA-TTG-CAT-GGC-GAA-ATG-GC	1175
	rmlC	BAP6130 (r)	CAA-TCC-TCG-TAA-GAC-CCC-C	
L6	*nctB*	BAP7292 (f)	TCT-TTA-TAA-TTA-TAC-TCT-CCC-AAG-G	668
		BAP7293 (r)	AAT-GAA-GGT-TTA-AAA-GAG-ATA-GCT-GGA-G	
L7	*ppgB*	BAP6127 (f)	CCT-ATA-TTT-ATA-TCT-CCT-CCC-C	931
		BAP6128 (r)	CTA-ATA-TAT-AAA-CCA-TCC-AAC-GC	
L8	*natG*	BAP6133 (f)	GAG-AGT-TAC-AAA-AAT-GAT-CGG-C	255
		BAP6134 (r)	TCC-TGG-TTC-ATA-TAT-AGG-TAG-G	

* f = forward primer, r = reverse primer

The multiplex LPS PCR was performed in a 50 μl volume and consisted of 0.4 μM of each primer, 0.2 mM dNTPs and 1.7 U *Taq* in a commercial buffer. The cycling conditions (when using a DNA extract) were 96°C for 5 minutes, followed by 30 cycles of 96°C for 30 seconds, 52°C for 30 seconds, and 72°C for 2.5 minutes, with a final extension at 72°C for 5 minutes. For PCR using direct colony material, the only change to the cycling conditions was that the initial denaturation step at 96°C was increased to 10 minutes.

A range of molecular methods have been used in epidemiological studies of fowl cholera outbreaks e.g. DNA fingerprinting of *P. multocida* by restriction endonuclease analysis (REA) (Wilson *et al*., 1992), enterobacterial repetitive insertion consensus (ERIC)-PCR (Singh *et al.,* 2014) and multi-locus sequence typing (MLST) (Singh *et al.*, 2013). However, these methods are now being replaced by whole genome sequencing and bioinformatic analysis (LeCount *et al.*, 2018; Omaleki *et al.,* 2020). It is now clear that the WGS/bioinformatic analysis provides a more in-depth and accurate strain tracking as well as providing *in silico* LPS typing and MLST (LeCount *et al.,* 2018; Omaleki *et al.,* 2020). The LPS loci can also be examined to identify variants within the LPS genotype. This sequence information can then be used to predict the LPS structure produced by these variants, e.g. due to introduced stop

codons and frame shifts (Omaleki *et al.*, 2020). This predicted LPS structure can then be compared with the predicted structure of the killed fowl cholera vaccine in use or planned for use. This ability to predict the LPS structure is critical as it is now known that a killed fowl cholera vaccine only provides protection against field isolates of the identical or near to identical LPS structure (Harper *et al.,* 2016).

2. Serological tests

Serological tests for the presence of specific antibodies are not used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis by isolation and identification of the causative organism precludes the need for serodiagnosis. Serological tests, such as agglutination, AGID, and passive haemagglutination, have been used experimentally to demonstrate antibody against *P. multocida* in serum from avian hosts; none were highly sensitive. Determinations of antibody titres using enzyme-linked immunosorbent assays have been used with varying degrees of success in attempts to monitor seroconversion in vaccinated poultry, but not for diagnosis.

C. REQUIREMENTS FOR VACCINES

C1. Inactivated vaccine

1. Background

1.1. Rationale and intended use of the product

Fowl cholera may be caused by any of 16 Heddleston serovars of *P. multocida*, although certain serovars appear to be more often associated with disease. The *P. multocida* vaccines in general use are inactivated, containing aluminium hydroxide or oil adjuvant, prepared from cells of serovars selected on the basis of epidemiological information. Commercial vaccines are usually composed of serovars 1, 3, and 4. Vaccination plays a significant role in the control of this disease. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Heddleston *et al.* (1970) showed that a killed fowl cholera vaccine could protect against the homologous strain but not a strain from a heterologous serovar. This finding has long been assumed to mean that killed fowl cholera vaccines provide protection that is limited to the somatic serovars of the strains present in the vaccine. However, the recently gained knowledge of the LPS biosynthetic genes has provided a far more subtle and informed understanding of the protection provided by killed fowl cholera vaccines.

An inactivated vaccine is normally administered by intramuscular injection in the leg or breast muscles, or subcutaneously at the back of the neck. Two doses are typically administered at 2- to 4-week intervals. As with most killed vaccines, full immunity cannot be expected until approximately 2 weeks after the second dose of a primary vaccination course. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

All strains of *P. multocida* to be incorporated into a vaccine must be well characterised, of known serovar, pure, safe and immunogenic. See chapter 1.1.8 for guidelines on master seeds.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Pasteurella multocida seeds must be pure culture and free from extraneous bacteria and fungi (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

2.1.3. Validation as a vaccine strain

Suitability as a vaccine strain is demonstrated in efficacy and safety trials.

2.2. Method of manufacture

2.2.1. Procedure

Production cultures of each bacterial isolate to be included in the final product are prepared separately. *Pasteurella multocida* cultures may be grown in a suitable broth media or initially grown on agar media and scaled up to broth media. Cultures are sub-passaged until the desired volume is prepared. Cultures are harvested when they reach a suitable density, frequently measured by spectrophotometry (optical density).

Cultures are then inactivated by formaldehyde or other suitable inactivating agent. The inactivated cells may be concentrated, typically by centrifugation or filtration, or diluted to reach the proper concentration for blending into completed product. All the standardised component cultures are mixed, and usually blended with an adjuvant, prior to filling sterile final containers.

2.2.2. Requirements for ingredients

See chapter 1.1.8.

2.2.3. In-process controls

The purity of the cultures is determined at each stage of production prior to inactivation. This may be achieved by microscopic examination (e.g. phase–contrast microscopy, Gram strain) or by culture. Killed cultures are tested for completeness of inactivation. Analytical assays to determine the levels of formaldehyde or other preservatives are done on bulk vaccine and must be within specified limits. During manufacturing, production parameters must be tightly controlled to ensure that all serials (batches) are produced in the same manner as that used to produce the serials used for efficacy studies.

2.2.4. Final product batch tests

i) Sterility/purity

Sterility tests are done on filled vaccine. Each lot must pass sterility requirements, for example those detailed in the 9 CFR Part 113.26 (CFR USDA, 2013). (See also Chapter 1.1.9.)

ii) Identity

The identity of the antigens in inactivated products is typically ensured through the master seed concept and good manufacturing controls. Separate identity testing on completed product batches is not required in the USA, but procedures may differ in other countries.

iii) Safety

Safety testing is conducted on each bulk or filled vaccine lot and may be assessed in birds vaccinated for batch potency tests.

Certain countries or regions, such as the European Union (EU), also may require the testing of each batch for endotoxin content.

iv) Batch potency

In the USA, inactivated vaccines are typically tested for batch potency in a vaccination–challenge trial, such as described in 9 CFR Parts 113.116-118 (USDA, 2013). Separate groups of birds (20 vaccinates, 10 controls) are challenged with each of the serovars of *P. multocida* for which protection is claimed. Vaccines are administered according to the dose and route recommended on the label, and all birds are challenged 2 weeks after the second dose. The birds are observed for 14 days after challenge. For a satisfactory test according to 9 CFR, at least 14 of 20 vaccinates must survive and at least 8 of 10 controls must die.

In the EU, a serological test or other validated method may be used for batch potency after a batch of minimum permissible potency is initially tested in a vaccination–challenge trial (European Pharmacopoeia, 2008).

v) Formaldehyde content

Vaccines inactivated with formaldehyde are tested for residual formaldehyde (VICH, 2003a).

2.3. Requirements for regulatory approval

The following section is based on the requirements for inactivated *P. multocida* vaccines in the USA. Other countries may have slightly different requirements.

2.3.1. Manufacturing process

The general method for production of manufacturers should demonstrate that the procedure used to inactivate bacteria is sufficient for complete inactivation. A test should be developed to confirm inactivation of each bacterial culture.

2.3.2. Safety requirements

i) Target and non-target animal safety

Inactivated vaccines should pose no hazard to non-target species. Safety in target animals may be evaluated according to harmonised requirements in VICH GL44 (VICH, 2009). The EU and USA recommend vaccinating at least 20 non-immune, unexposed birds according to label recommendations and evaluating daily for adverse reactions. The EU monitors for 21 days. In the USA, target animal safety is evaluated during the pre-challenge period of the efficacy study, which is typically 5 weeks.

Safety also should be evaluated in a field setting prior to regulatory approval. This evaluation typically involves multiple geographical locations or husbandry conditions and much larger numbers of birds.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Not applicable.

iii) Precautions (hazards)

Vaccines prepared with aluminium-based adjuvants may cause temporary nodules at the site of injection. Operator self-injection poses no immediate problems, but medical advice should be sought as there is a risk of infection via a contaminated needle.

Vaccines prepared with oil-based adjuvants may cause more severe reactions at the site of injection, which may manifest as large nodules. Care should be taken to administer these vaccines correctly. Operator self-injection requires immediate medical attention, involving prompt incision and irrigation of the site.

2.3.3. Efficacy requirements

Products prepared from candidate master seeds should be shown to be effective against challenge infection. Efficacy should be demonstrated in each animal species (e.g., chickens, turkeys) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. Birds used in efficacy studies should be immunologically naïve to fowl cholera and at the minimum age recommended for product use. The lot of product used to demonstrate efficacy should be produced from the highest allowable passage of master seed.

In the USA and EU, 20 vaccinates and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 (USA) or 21 (EU) days after vaccination and are observed for 14 days after challenge. In the USA, mortality is measured, and a satisfactory test requires that at least eight of the controls die and at least 16 of the vaccinates survive (USDA, 2013). In the EU, birds are expected to remain free from severe signs of disease, and a satisfactory test requires at least 70%

of the control birds to be affected while at least 70% of the vaccinates remain free from disease (European Pharmacopoeia, 2008).

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable to this disease.

2.3.5. Duration of immunity

Formal duration of immunity studies are not typically required, although it is important to check the requirements of individual countries. Revaccination recommendations, beyond the primary vaccination series, are more often determined empirically.

2.3.6. Stability

Vaccine stability should be confirmed by testing the product for potency at periodic intervals through the dating period. In the USA, at least three lots of vaccine are tested and must pass established potency requirements at the end of dating. Vaccines are typically stored at 2–7°C and protected from freezing. Partly used containers should be discarded at the end of a day's operations.

C2. Live vaccine

1. Background

1.1. Rationale and intended use of the product

Live vaccines containing modified *P. multocida* are used in some regions of the world, e.g. North America and Australia. Live vaccines are typically administered in the drinking water or wing web. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances.

A key feature of live fowl cholera vaccines is that the protective efficacy of these vaccines is independent of the LPS outer core structure (Harper *et al.*, 2016).

2. Outline of production and minimum requirements for vaccines

Guidelines for the production of the veterinary vaccines are given in chapter 1.1.8.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

All strains of *P. multocida* to be incorporated into a vaccine must be well characterised, of known serotype, pure, safe and immunogenic. See chapter 1.1.8 for guidelines on master seeds.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Pasteurella multocida seeds must be pure culture and free from extraneous bacteria and fungi.

2.1.3. Validation as a vaccine strain

Suitability as a vaccine strain is demonstrated in efficacy and safety trials. In addition, seeds used in live vaccines must be genetically and phenotypically stable upon repeated *in-vivo* passage. Ideally, they should not persist in the vaccinated animal and any shedding of the vaccine organism from vaccinated birds should be of limited magnitude and duration.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic

Many countries have mechanisms for provisional acceptance in the event of an epizootic in which commercially available vaccines are not effective. As inactivated fowl cholera vaccines are

typically effective and pose less safety risk, however, it is more likely that an inactivated vaccine would be considered for a fowl cholera epizootic.

2.2. Method of manufacture

2.2.1. Procedure

Production cultures of each bacterial isolate to be included in the final product are prepared separately. *Pasteurella multocida* cultures may be grown in a suitable broth media or initially grown on agar media and scaled up to broth media. Cultures are sub-passaged until the desired volume is prepared. Cultures are harvested when they reach a suitable density, frequently measured by spectrophotometry (optical density).

Each component culture may be standardised, by concentration or dilution, to a desired concentration. All of the standardised component cultures are mixed prior to filling sterile final containers. Live vaccines are typically lyophilised, to be reconstituted with sterile diluent immediately prior to use.

2.2.2. Requirements for ingredients

See chapter 1.1.8.

2.2.3. In-process controls

The purity of the cultures is determined at each stage of production. This may be achieved by microscopic examination (e.g. phase–contrast microscopy, Gram strain) or by culture. During manufacturing, production parameters must be tightly controlled to ensure that all serials (batches) are produced in the same manner as that used to produce the serials used for efficacy studies.

2.2.4. Final product batch tests

i) Sterility

Sterility tests are done on filled vaccine. Each lot must pass sterility requirements, for example those detailed in the 9 CFR Part 113.27 (CFR USDA, 2013). (See also chapter 1.1.9.)

ii) Purity

Each batch shall pass a test for purity carried out using sold media and ignoring the growth of the vaccinal bacterium, for example as detailed in the 9 CFR Part 113.27 (CFR USDA, 2013). (See also chapter 1.1.9.)

iii) Identity

Each batch of live vaccine in the USA is tested for identity. Requirements of other countries may vary. This is most commonly accomplished by characterising the bacteria *in vitro*.

iv) Safety

Live vaccines may be tested according to the method described in Section C1.2.3.2.i, except that frequently only one representative animal species is required.

Certain countries (e.g. EU) also may require testing each batch for endotoxin content (European Pharmacopoeia, 2008).

v) Batch potency

The potency of live vaccine lots is determined by a bacterial count performed on reconstituted lyophilised product in its final container. In the USA, the mean bacterial count of any vaccine lot at the time of preparation must be sufficiently high to ensure that at any time prior to product expiration, the count is at least twice the immunogenicity standard. The EU requires a count that is at least equal to the immunogenicity standard.

vi) Moisture content

Lyophilised vaccine is tested for moisture content. Harmonised requirements for testing moisture by a gravimetric method are found in VICH GL26 (VICH, 2003b). Typically moisture is expected to be less than 5%.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

See chapter 1.1.8.

2.3.2. Safety requirements

i) Target and non-target animal safety

The safety of master seeds used in the production of live vaccines must be evaluated prior to regulatory approval. Safety must be tested in each animal species (chickens, turkeys, ducks, psittacines) for which the product is recommended. Harmonised VICH GL44 (VICH, 2007) is available for target animal safety.

Overdose studies are typically required for live vaccines. For example, each of 10 birds is given an equivalent of 10 vaccine doses and observed for 10 days. If unfavourable reactions are seen, this finding should be included in a risk assessment, and it may be appropriate to designate maximum permissible serial potency requirements.

The master seed is also tested in representative non-target species (e.g. rodents or non-target avian species) that may be expected to come into contact with vaccine bacteria shed by vaccinated birds. Master Seed bacteria should be administered to the most sensitive species at the most sensitive age, by the route (e.g. oral) expected to occur in the field.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Master seed bacteria for live vaccines should be evaluated for their stability with repeated passage in vivo. The seed should remain avirulent and genotypically stable after multiple passages. Harmonised requirements for reversion to virulence studies are described in VICH GL41 (VICH, 2007).

Seeds for live vaccines also should be tested for their potential to shed from vaccinated animals and persist and spread in the environment. Ideally vaccine organisms should shed no more than briefly and should not persist in the environment. Exceptions from the ideal should be addressed in a risk assessment for the product.

iii) Precautions (hazards)

Inadvertent human exposure to the vaccine organism should be reported to a physician.

2.3.3. Efficacy requirements

Products prepared from candidate master seeds should be shown to be effective against challenge infection. Efficacy should be demonstrated in each animal species (e.g. chickens, turkeys) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. Birds used in efficacy studies should be immunologically naïve to fowl cholera and at the minimum age recommended for product use. The lot of product used to demonstrate efficacy should be produced from the highest allowable passage of master seed.

For live avian *Pasteurella* vaccines in the USA, 20 vaccinates and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 days after vaccination and are observed for 10 days after challenge. A satisfactory test requires that at least eight of the controls die and at least 16 of the vaccinates survive.

The arithmetic mean count of colony-forming units in the lot of product that is used to demonstrate efficacy is used as the minimum standard (immunogenicity standard) for all subsequent production lots of vaccine.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable

2.3.5. Duration of immunity

Formal duration of immunity studies are not typically required, although it is important to check the requirements of individual countries. Revaccination recommendations, beyond the primary vaccination series, are more often determined empirically.

2.3.6. Stability

Vaccine stability should be confirmed by testing the product for potency at periodic intervals through the dating period. In the USA, batches of vaccine are tested until a statistically valid stability record is established. Each lot must pass established potency requirements at the end of dating. Live vaccines should be used promptly upon opening.

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NB: At the time of publication (2022) there was no WOAH Reference Laboratory for fowl cholera (please consult the WOAH Web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.3.10.

FOWLPOX

SUMMARY

Description of the disease: *Fowlpox is a disease of chickens and turkeys caused by a DNA virus of the genus* Avipoxvirus *of the family* Poxviridae*. Its distribution is world-wide. It is slow-spreading and characterised by the formation of proliferative lesions and scabs on the skin, and diphtheritic lesions in the upper parts of the digestive and respiratory tracts. In the case of the cutaneous form, the mortality rate is usually low, unless lesions develop around the eyes, and affected birds are more likely to recover than those with the diphtheritic form. In the diphtheritic form, proliferative lesions involving the nasal passages, tongue, larynx or trachea can result in respiratory distress and death from suffocation. Transmission of the virus is generally associated with contamination of open wounds and from biting insects such as mosquitos and mites.*

Fowlpox causes a transient drop in egg production and a reduced growth rate in young birds.

Detection of the agent: *Fowlpox should be suspected where skin eruptions occur on exposed areas. Histological examination of cutaneous or diphtheritic lesions reveals epithelial hyperplasia with intracytoplasmic inclusions in affected cells. Elementary bodies may be detected in smears from lesions by the use of the Gimenez method. Electron microscopy of lesions will detect virus particles with the characteristic poxvirus morphology by negative staining or in ultrathin sections of the lesion.*

The diphtheritic form of fowlpox involving the trachea must be differentiated from infectious laryngotracheitis, which is caused by gallid herpesvirus-1 and is characterised by the presence of intranuclear inclusion bodies.

Virus isolation is done by inoculation on to chorioallantoic membranes of 9- to 12-day-old developing chicken embryos or avian cell cultures. Eggs from specific pathogen free flocks should be used for virus isolation.

Serological tests: *Immune responses to fowlpox virus (FPV) may be demonstrated by the use of virus neutralisation, agar gel immunodiffusion, immunofluorescence, or passive haemagglutination tests, enzyme-linked immunosorbent assay and by immunoblotting.*

Requirements for vaccines: *Modified live FPV or pigeon pox virus vaccines of chicken embryo or avian cell culture origin are available commercially. Recombinant vaccines using FPV as a vector are also available for use. The use of vaccines is indicated in areas where the disease is endemic, or on premises where infection has been diagnosed.*

A. INTRODUCTION

The morphology of the fowlpox virus (FPV) is like that of other viruses of the *Poxviridae* family. The mature virus (elementary body) is brick shaped and measures about 330 × 280 × 200 nm. The outer coat is composed of random arrangements of surface tubules. The virion consists of an electron-dense centrally located biconcave core or nucleoid with two lateral bodies in each concavity and surrounded by an envelope. The 288 kbp FPV genome encodes for over 250 genes.

Fowlpox has a world-wide distribution and is caused by a DNA virus of the genus *Avipoxvirus* of the family *Poxviridae* (Tripathy, 1993; Tripathy & Reed, 2020). Its incidence is variable in different areas because of differences in climate, management and hygiene or the practice of regular vaccination. It can cause drops in egg production, or retarded growth in younger birds. Infection in mammals is considered non-significant.

Fowlpox is a slow-spreading virus disease of chickens and turkeys, characterised in the cutaneous form (dry pox) by the development of proliferative lesions, ranging from small nodules to spherical wart-like masses on the skin of the comb, wattle and other unfeathered areas. In the diphtheritic form (wet pox), slightly elevated white opaque nodules develop on the mucous membranes. They rapidly increase in size to become a yellowish diphtheritic membrane. Lesions occur on the mucous membranes of the mouth, tongue, oesophagus, larynx or trachea. The mortality rate is higher in the diphtheritic form than in the cutaneous form, sometimes nearing 50% particularly in young birds. Mortality from the cutaneous form can also be elevated in instances where lesions develop around the eyes. Infection is generally associated with contamination of open wounds and via biting insects such as mosquitos and poultry red mites (*Dermanyssus gallinae*) (Tripathy & Reed, 2020).

Integration of reticuloendotheliosis virus (REV) sequences has been observed in the genome of FPV (Singh *et al*., 2000; 2003). It is interesting that this insertion event occurred over 50 years ago (Kim & Tripathy, 2001). While most field strains of FPV contain REV provirus, vaccine strains have only remnants of long terminal repeats (Singh *et al*., 2003). Virulence is enhanced by the presence of REV provirus in the genome of field strains of FPV. Whole genome sequences have been reported for several poxviruses (Afonso *et al*., 2000; Banyai *et al.,* 2015; Laidlaw & Skinner, 2004; Sarkar *et al.,* 2021). The functions of the majority of the genes are not known at this time. However, it is interesting that the virus tends to persist in the poultry environment for extended periods of time where other viruses may not survive. In this regard, the presence of photolyase gene and A-type inclusion body gene in the virus genome appear to protect the virus from environmental insults (Srinivasan *et al*., 2001; Srinivasan & Tripathy, 2005). Antigenic cross-reactivity is observed among avipoxviruses and it appears that many genes are conserved. Limited studies on antigenic, genetic and biologic comparison of FPV with other avipoxviruses especially those that infect the wild birds are available. Complete genome sequences of a canarypox virus, as well as a pigeon and penguin poxvirus have been reported (Offerman *et al.,* 2014; Tulman *et al,* 2004).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of fowlpox and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Histopathology	–	–	–	+++	–	–
Virus isolation	–	+	–	+++	–	–
PCR	–	++	–	+++	–	–
Real-time PCR	–	++	–	+++	–	–
Detection of immune response						
AGID	–	–	–	–	–	++
ELISA	+++	+	–	–	+	++
VN	–	–	–	–	–	+++
MFIA	+++	–	–		–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion;
ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation;
MIFI = multiplex fluorometric bead-based immunoassay.

1. Detection of the agent

FPV multiplies in the cytoplasm of epithelial cells with the formation of large intracytoplasmic inclusion bodies (Bollinger bodies) that contain smaller elementary bodies (Borrel bodies). The inclusions can be demonstrated in sections of cutaneous and diphtheritic lesions by the use of haematoxylin and eosin (H&E), acridine orange or Giemsa stains (Tripathy *et al*., 1973). The elementary bodies can be detected in smears from lesions, for example by the Gimenez method (Tripathy & Hanson, 1976), which is described below. Electron microscopy can be used to demonstrate viral particles of typical poxvirus morphology by negative staining or in ultrathin sections of infected tissues (Doane & Anderson, 1987). Molecular detection methods designed to amplify FPV DNA by polymerase chain reaction (PCR) or real-time PCR have become routine in many avian diagnostic laboratories.

1.1. A smear technique for fowlpox

1.1.1. Stock solutions

i) *Stock solution for primary stain:* a solution of basic fuchsin (5 g) in 95% ethanol (100 ml) is slowly added to a second solution of crystalline phenol (10 g) in distilled water (900 ml). This stock solution, kept in a tightly screw-capped glass bottle, is incubated for 48 hours at 37°C, and then stored at room temperature.

ii) *Phosphate buffer, pH 7.5:* $NaH_2PO_4H_2O$ (2.47 g) and Na_2HPO_4 (11.65 g) are added to distilled water (1000 ml) and stored at 4°C.

1.1.2. Test procedure

i) Place a drop of distilled water and the lesion (cutaneous or diphtheritic) on a clean slide. Prepare a thin smear by pressing the lesion with another clean slide and rotating the upper slide several times.

ii) Air dry and gently fix the smear over a flame.

iii) Stain the smear for 5–10 minutes with freshly prepared primary stain (8 ml stock solution of basic fuchsin mixed with 10 ml of phosphate buffer, pH 7.5, and filtered through Whatman filter paper No. 1).

iv) Wash thoroughly with tap water.

v) Counterstain with malachite green (0.8% [w/v] in distilled water) for 30–60 seconds.

vi) Wash the smear with tap water and then dry.

vii) Examine the smear under oil immersion. The elementary bodies appear red and are approximately 0.2–0.3 µm in size.

1.2. Virus isolation

FPV can be isolated by the inoculation of suspected material into embryonated chicken eggs or in cell cultures of avian origin. Approximately 0.1 ml of tissue suspension of skin or diphtheritic lesions, treated with the appropriate concentration of antibiotics, is inoculated on to the chorioallantoic membranes (CAMs) of 9- to 12-day-old developing chicken embryos or in cell culture. It is advisable to check the inoculum for any residual contamination by inoculation of a blood agar and McConkey plate examined 24 hours after incubation. Following inoculation of the embryos with the contamination free sample the eggs are incubated at 37°C for 5–7 days, and then examined for focal white pock lesions or generalised thickening of the CAMs. Histopathological examination of the CAM lesions will reveal eosinophilic intracytoplasmic inclusion bodies following staining with H&E (Tripathy *et al*., 1973; Tripathy & Reed, 2020).

Primary chicken embryo fibroblasts, chicken embryo kidney cells, chicken embryo dermis cells, or the permanent quail cell line QT-35, can also be used to propagate FPV (Ghildyal *et al*., 1989; Schnitzlein *et al*., 1988). The adaptation of virus strains in cell cultures is an important requirement for plaque formation, as not all strains will form plaques initially.

1.3. Molecular methods

Tracheas, tracheal swabs, skin lesions and formalin-fixed paraffin-embedded tissues are the best samples for PCR/real-time PCR (Tripathy & Reed, 2020). However, prolonged fixation in formalin, especially unbuffered formalin, can reduce ability to detect nucleic acid of fowlpox virus and other pathogens (Crawford *et al.,* 1999). In addition, tissue impressions on commercially available cellulose paper cards can be used to collect and transport specimens while preserving the nucleic acid (Sanchez & Sellers, 2015) and in the absence of maintaining the cold chain.

PCRs have been described for detection of fowlpox DNA (Fallavena *et al*., 2002; Lee & Lee, 1997) and facilitate detection of the smallest amounts of viral DNA. In addition, real-time PCR with a hydrolysis probe is also used to detect fowlpox DNA (Hauck *et al.,* 2009). The use of real-time PCR has facilitated discrimination between FPV and infectious laryngotracheitis virus (ILTV) in tissues (Davidson *et al.,* 2015), as well as aiding the determination of reticuloendotheliosis virus (REV) provirus in FPVs (Hauck *et al.,* 2009). Diagnostic PCR primers used for detection and phylogenetic analyses of fowlpox virus DNA target conserved regions of the major core protein, P4b.

4b core potein (Lee & Lee, 1997)	Forward	5'-CAG-CAG-GTG-CTA-AAC-AAC-AA-3'	578 bp
	Reverse	5'-CGG-TAG-CTT-AAC-GCC-GAA-TA-3'	
4b core protein (Fallavena *et. al*., 2002)	Forward	5'-ACG-ACC-TAT-GCG-TCT-TC-3'	419 bp
	Reverse	5'-ACG-CTT-GAT-ATC-TGG-ATG-3'	
4b core protein (Hauck *et al.,* 2009)	Forward	5'-TCA-GCA-GTT-TGT-TAC-AAG-ACA-3'	109 bp
	Reverse	5'-CCA-TTT-CCG-TGA-ATA-GAA-TAG-TAT-3'	
	Probe	5'-Cyan5-ATC-TCC-GCC-GTC-GCA-ACT-TCC-A-BHQ1-3'	

As most of the field strains of FPV may contain insertion of reticuloendotheliosis virus (REV) in their genome, identification of such strains can be determined by using REV envelope (RENV) specific following primers for amplification of a 227 bp fragment or in a real-time PCR assay detecting REV-proviral DNA (gag).

REV LTR (Ottiger, 2010)	Forward	5'-CAT-ACT-GGA-GCC-AAT-GGT-T-3'	291 bp
	Reverse	5'-AAT-GTT-GTA-CCG-AAG-TAC-T-3'	
REV gag (Hauck *et al*., 2009)	Forward	5'-GTT-TTC-TAT-ACA-CAC-CAG-CCT-ACC-T-3'	111 bp
	Reverse	5'-TCC-TGA-CCT-CCC-GCC-TAC-T-3'	
	Probe	5'-FAM-CTG-TCC-TCA-CCC-TCT-CCC-TCT-CCT-CCA-BHQ1-3'	

Restriction fragment length polymorphism (RFLP) analysis has been reported for comparison of field isolates and vaccine strains of FPV (Ghildyal *et al*., 1989; Schnitzlein *et al*., 1988); however this procedure is not used routinely for detection or diagnosis.

2. Serological tests

Although both cell-mediated immunity (CMI) and humoral immunity play an important role in poxvirus infections, CMI tests are not routinely performed. Therefore, serological tests, such as virus neutralisation (VN), agar gel immunodiffusion (AGID), as well as the enzyme-linked immunosorbent assay (ELISA), are used to measure specific humoral antibody responses. Evidence of successful immunisation with vaccine can be determined by examining a flock 7–10 days after vaccination for 'takes'. A take consists of a swelling of the skin or a scab at the site where the vaccine was applied and its presence is evidence of successful immunisation.

2.1. Virus neutralisation

After virus/serum interaction, the residual virus activity may be assayed in embryonating chicken eggs or in cell cultures (Morita, 1973). This technically demanding test may not be convenient for routine diagnosis. Only some selected strains of the virus have plaque-forming ability in chicken embryo cells. Neutralising antibodies develop within 1–2 weeks of infection.

2.2. Agar gel immunodiffusion

Precipitating antibodies can be detected by reacting test sera against viral antigens. The antigen can be derived by sonication and homogenisation of infected skin or CAM lesions as well as by treatment of infected cell cultures as described in Section B.2.6 below. The lysed suspension is centrifuged and the supernatant is used as antigen. Gel-diffusion medium is prepared with 1% agar, 8% sodium chloride and 0.01% thiomersol. The viral antigen is placed in the central well and the test sera are placed in the peripheral wells. It is important to include a positive and negative control serum. The plates are incubated at room temperature. Precipitation lines develop in 24–48 hours after incubation of the antigen with antibody to homologous or closely related strains. The test is less sensitive than the ELISA (Buscaglia *et al*., 1985) or the passive haemagglutination test.

2.3. Enzyme-linked immunosorbent assay

ELISAs have been developed to detect humoral antibodies to FPV. They are capable of detecting antibody 7–10 days after infection (Buscaglia *et al*., 1985).

FPV antigens are prepared either from infected QT-35 cell monolayers or CAM lesions. Infected QT-35 cells are pelleted (700 ***g*** for 10 minutes at 4°C), washed with isotonic buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM ethylene diamine tetra-acetic acid [EDTA]) followed by lysis in hypotonic buffer (10 mM Tris, pH 8.0, 10 mM KCl, 5 mM EDTA) containing 0.1% Triton X-100 and 0.025% beta-mercaptoethanol. Nuclei and cellular debris are removed by low-speed centrifugation (500 ***g*** for 5 minutes at 4°C) and the resulting supernatant is used as a source of FPV antigens for ELISA. To isolate viral antigen from CAM lesions, initial grinding of the lesions with subsequent detergent treatment as described earlier would be required. Virus propagated in chicken embryo fibroblasts and chicken embryo dermis cells has also been used for antigen. The antigen preparation is as described for QT-35 cells.

Wells of microtitre plates are coated with 1 µg of soluble FPV antigen in 100 µl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4°C (Buscaglia *et al*., 1985; Tripathy *et al*., 1973). Each well is then rinsed once with wash solution (0.29 M NaCl, 0.05% Tween 20) and then blocked with phosphate buffered saline (PBS, pH 7.4) containing 3% bovine serum albumin (BSA) for 1 hour at 37°C. After one wash, serial dilutions of the test sera in PBS containing 1% BSA are added to the wells. After rocking for 2 hours at 37°C, the wells are washed three times prior to the addition of 100 µl/well horseradish-peroxidase-conjugated goat anti-chicken IgɣY (H+L) antibodies at a recommended dilution in PBS. After 2 hours' incubation at 37°C and three subsequent washes, 100 µl of the chromogen/substrate TMB (tetramethyl benzidine) is added to each well. Reactions are terminated by the addition of 1 M phosphoric acid and absorbance at 450 nm is recorded using an ELISA plate reader.

C. REQUIREMENTS FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements

Fowlpox and pigeonpox virus vaccines (Winterfield & Hitchner, 1965) of chicken embryo or cell culture origin are available from the majority of biological companies that produce poultry vaccines. The vaccines are used in susceptible flocks where the disease has been endemic or has been diagnosed in previous flocks. Several recombinant fowlpox vaccines are available for use by *in-ovo*, or 1-day-of-age subcutaneous or wing-web administration. The recombinant poxvirus vaccines include gene inserts for pox-ILTV, *Mycoplasma gallisepticum* (pox-MG), avian influenza H5 (Pox-AIV), or Newcastle disease virus (pox-NDV) (Tripathy & Reed, 2020). These recombinant vaccines provide protection against the FPV vector and the pathogen of the gene insert.

Passively acquired immunity should be taken into consideration during vaccination of progeny from flocks that have either had a recent natural infection or been recently vaccinated. As passive immunity (for 2–3 weeks) may interfere with vaccine virus multiplication, such progeny should be vaccinated only after the decline of passively acquired antibody.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

Procedures described in Chapter 1.1.8 *Principles of veterinary vaccine production* and in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* must be strictly followed.

2.1.1. Biological characteristics of the master seed

Live FPV vaccines of either fowlpox or pigeonpox virus origin are used for prevention of fowlpox in poultry. The virus is propagated either in specific pathogen free (SPF) chicken embryo or in cell culture of avian origin. A master seed virus (MSV) must be established and used according to a seed-lot system. Proper records must be kept of its origin, passage history and characteristics.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The MSV must be propagated in suitable premises with materials that meet approved standards, and must be tested for freedom from contamination as well as for identity and purity.

2.1.3. Validation as a vaccine

i) Purity

The MSV may be neutralised with a specific hyperimmune serum before testing for purity. Because of difficulty in neutralising avian pox virus, it is acceptable to centrifuge the MSV at 1000 ***g*** for 20 minutes, followed by filtration through a 0.2 μm filter. The neutralised or filtered MSV is then used in tests to demonstrate freedom from extraneous agents. These tests should be done in embryonating eggs or avian cell cultures, to demonstrate absence of extraneous virus replication, and in SPF chickens, to demonstrate freedom from antibodies to extraneous agents.

ii) Safety

Vaccines should be prepared only from virus that is a stable attenuated strain or a naturally occurring isolate of low virulence.

The vaccine must be shown to be safe by the recommended route of administration, which is wing web stab, in all ages of susceptible birds. A suitable test is to take ten SPF chickens and inoculate each by piercing the wing web with a needle dipped in the vaccine. The birds are observed for 7–10 days for evidence of 'takes' and for the absence of adverse effects attributable to the vaccine. A 'take' consists of swelling of the skin or a scab at the site where the vaccine was applied and is evidence of successful vaccination. The safety test should be repeated after at least four serial passages of the virus in SPF chickens to show that there has been no reversion to virulence.

iii) Efficacy

Data should be obtained using the highest passage level (fifth passage from the master seed) and the lowest titre of virus to be used in the final product: 20 SPF chickens of the minimum age indicated for vaccination should receive one dose of vaccine by the recommended method. The chickens, together with 20 unvaccinated chickens of the same age and source, should be challenged 3 weeks later by scarification with a virulent strain of FPV. The birds should be observed for 3 weeks. Ninety per cent of the control birds should develop lesions due to the challenge virus and at least 90% of the vaccinated birds should remain free from such lesions.

2.2. Method of manufacture

2.2.1. Procedure

Vaccine is manufactured on a seed-lot system from the validated MSV. This must be done in approved premises designed to avoid the risk of contamination. All media and cell cultures must be tested to ensure freedom from contamination.

2.2.2. Requirements for ingredients

The MSV may be propagated in SPF chicken embryos, using the CAMs, or in avian cell cultures, such as primary chicken embryo fibroblasts, chicken embryo kidney or chicken embryo dermis.

2.2.3. In-process control

During the process of validation as a vaccine, the efficacy data must be compared to the virus content of the vaccine. A suitable potency can thus be established. The vaccine should be filled into final containers to ensure that each container has sufficient virus to achieve the specified potency.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in the Chapter 1.1.9.

ii) Identify

Identity tests shall demonstrate that no other vaccine strain is present when several strains are propagated in a laboratory for multivalent vaccines.

iii) Safety

The safety test described in Section C.2.1.3 above, except the requirement for six passages in SPF chickens, should be used on each batch of vaccine.

iv) Potency

Virus content tests should be carried out using each of at least three containers. The dilutions should span 0–100% infection range, using five-fold dilution steps and at least seven replicates per dilution. Tests should be done in parallel with a standard vaccine, if available. Each lot of vaccine should be titrated in the diluent provided for its use. The virus titre should not normally be higher than 1/10 of the dose at which the vaccine has been shown to be safe and must not be lower than the release titre determined in the test for efficacy. A suitable potency for an attenuated live fowlpox vaccine is likely to be in the region of 10^5 EID_{50} (50% embryo infective dose) per ml. Alternative potency tests may be used in accordance with national regulatory requirements.

2.3. Requirements for regulatory approval

For regulatory approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

2.3.1. Safety requirements

Tests use single dose, overdose (for live vaccines only) and repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) containing the maximum permitted antigenic content and according to the case, the maximum number of vaccine strains.

2.3.2. Precautions (hazards)

It is usually recommended not to vaccinate birds that are in lay. Avoid human contact with the live vaccine. Standard fowlpox vaccine is not to be used in pigeons, though they can be vaccinated with pigeon pox vaccine. In many countries, pigeon pox vaccine has been superseded by attenuated live fowlpox vaccine designed for use in day-old chicks. These products have been safely used in pigeons in the absence of an available pigeon pox vaccine

2.3.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

Usually vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to live pathogen challenge, i.e. lack of local lesion at the site of inoculation in vaccinated animals and development of lesion in the control animals.

2.3.4. Duration of immunity

The efficacy test given in Section C.2.1.3 may be used to determine the duration of immunity (approximately 6–12 months) by testing at intervals after vaccination, using separate groups of birds for each test.

2.3.5. Stability

Evidence of stability must be presented to justify the shelf life. This should be based on virus titrations carried out at intervals until 3 months beyond the requested shelf life on at least four batches of vaccine kept under recommended storage conditions.

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NB: At the time of publication (2023) there was no WOAH Reference Laboratory for fowlpox (please consult the WOAH Web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.3.11.

FOWL TYPHOID AND PULLORUM DISEASE

SUMMARY

***Description and importance of the disease**: Pullorum disease of chickens is a bacterial infection caused by* Salmonella enterica *subspecies* enterica *serovar Gallinarum biovar Pullorum* (Salmonella *Pullorum*)[1]*. At this time the serovar is referred to as Gallinarum in some parts of the world and Pullorum in others; in this chapter the serovar will be referred to as Gallinarum or Pullorum according to the biovar under discussion as this is more meaningful from a clinical and epidemiological perspective.*

In its acute form, Pullorum disease is almost exclusively a septicaemic disease of young chickens. However, the organism may also be associated with disease in turkey poults and may be carried subclinically or lead to reduced egg production and hatchability, plus a range of atypical signs in older birds. Ovarian transmission is a major route by which the organism can spread. Game birds and 'backyard' poultry flocks may act as reservoirs of infection, and wild birds may act as vectors for the organism and as such are important in the epidemiology of the disease.

Fowl typhoid in chickens and turkeys is caused by S. Gallinarum biovar Gallinarum and is more often observed in the later growing period and in mature stock. Disease is often characterised by rapid spread with high morbidity and acute or subacute mortality. Red mites may be involved in the transmission of disease and persistence in poultry houses

Clinical signs in chicks and poults include anorexia, diarrhoea, dehydration, weakness and death. In mature birds, Pullorum disease is less severe but decreased egg production, poor hatchability and some increased mortality may occur. Fowl typhoid is a more acute septicaemic condition which mainly affects mature birds and may be particularly severe in commercial laying flocks.

***Identification of the agent:** Samples should not be taken from birds or eggs that have recently been treated with antimicrobial drugs. Swabs or aseptically collected samples from infected tissues, or intestinal and cloacal contents should be used for diagnostic testing. Other materials that may be sampled include eggs, embryos, faecal droppings and hatcher debris, especially fluff, dust and broken eggshells and chick box linings. Samples of tissues such as caecal tonsils, liver, gall bladder and spleen from infected birds are preferable to faecal and environmental samples. Tissue samples should be inoculated into non-selective and selective enrichment broths and on selective agar medium, such as brilliant green agar, as soon as possible after collection. In case of delay, samples should be stored at 4°C. Typical colonies can be identified by serological and biochemical tests. Molecular approaches can also be used to identify and differentiate* S. *Gallinarum and* S. *Pullorum. Final serological confirmation of suspect isolates can normally only be completed in a* Salmonella *Reference Typing Laboratory.*

***Serological tests:** These are satisfactory for identifying the presence and estimating the prevalence of infection within a flock. The test used in the field is the rapid whole blood plate agglutination test. This test is unreliable in turkeys and ducks as many uninfected birds may give positive reactions. In the laboratory a serum agglutination test is used, either as a rapid plate test or as a tube test. These can be applied as macro- or microagglutination tests, though the latter may be more likely to give false-positive results with turkey sera. Any positive reactors should be confirmed as being infected by culture at post-mortem examination. Enzyme-linked immunosorbent assays have been reported but no commercial test is available.*

1 See the note in Chapter 3.10.7 *Salmonellosis* for the principles followed concerning the nomenclature of *Salmonella*.

The use of vaccines to control S. Enteritidis or S. Gallinarum infections in chickens may cause problems in the interpretation of serological results.

***Requirements for vaccines*:** *Live and inactivated vaccines are available for fowl typhoid in some countries. The most commonly used vaccine is a commercial live vaccine derived from the stable rough strain of S. Gallinarum known as '9R'.*

A. INTRODUCTION

Fowl typhoid and pullorum disease, caused by *Salmonella enterica* subspecies *enterica* serovars Gallinarum biovars Gallinarum and Pullorum, respectively, are widely distributed throughout the world, especially in developing countries (Barbour *et al.,* 2015) where increasing antimicrobial resistance in these strains has also become a problem (Parvej *et al*., 2016). They have been eradicated from commercial poultry in many developed countries of Western Europe, the United States of America (USA), Canada, Australia and Japan. The move towards fee-range production in many countries may increase the risk of infection (Vielitz, 2016), but many outbreaks involve intensively housed laying hen or breeding flocks. In the USA and the United Kingdom the serovar is referred to as Pullorum (Hitchner, 2004), even though the strains are now considered to be the same serovar that is derived from *S*. Enteritidis by gene deletion events (Thomson *et al*., 2008). In this chapter the terms serovar Gallinarum or Pullorum will be used, as this more usefully distinguishes the two biovars that cause clearly distinct clinical syndromes and are therefore epidemiologically different. *Salmonella* Gallinarum recurred in some European countries in the first decade of the 21st century (Ivanics *et al.,* 2008). *Salmonella* Pullorum remains as a constant reservoir in wild and game birds (Barrow *et al*., 2012; Shivaprasad *et al*., 2013).

Salmonellosis caused by *Salmonella bongori* or subspecies of *Salmonella enterica* is covered in Chapter 3.10.7 *Salmonellosis*.

Clinical signs of fowl typhoid are typical of a septicaemic condition in poultry and include increased mortality and poor quality in chicks hatched from infected eggs. Older birds show signs of anaemia, depression, laboured breathing and diarrhoea causing adherence of faeces to the vent. The highest mortality in pullorum disease occurs in birds of 2–3 weeks of age. In older birds disease may be mild or inapparent. In breeding and laying flocks susceptibility is increased at the point of lay (Wigley *et al.,* 2005), but reduced egg production and hatchability may be the only signs of pullorum disease. Trans-ovarian infection resulting in infection of the egg and hatched chicks or poults is one of the most important transmission routes for both diseases (Haider *et al.,* 2014).

Post-mortem signs of pullorum disease in newly hatched chicks are those of peritonitis with generalised congestion of tissues and an inflamed unabsorbed yolk sac. Longer standing infections commonly lead to typhlitis with development of necrotic caecal casts and small necrotic foci in the liver, lungs and other viscera. Small lesions in the liver and spleen of Pullorum-infected birds may show a 'white spot' appearance that is not seen with Gallinarum; however, this lesion is not pathognomic. These *Salmonella* are very poor at colonisation and survival in the gastrointestinal tract is often indicative of later stages of clinical disease. Adult birds may develop misshapen, discoloured and/or shrunken ovaries with follicles attached by pedunculated fibrous stalks. Variant strains of *S*. Pullorum do not normally cause clinical disease or may result in mild, nonspecific signs but may lead to seroconversion.

In fowl typhoid, as well as generalised signs of septicaemia, the liver is usually enlarged, dark and friable with a distinctive coppery bronze sheen that may only develop after exposure to air. The bone marrow is also often dark brown. Although clinical signs and post-mortem findings of pullorum disease and fowl typhoid may be highly suggestive of the conditions, they are not sufficiently distinct from other causes of septicaemia to be pathognomic. It is therefore necessary to confirm disease by isolation of the organisms. Serological tests can be used to establish the presence of the disease in a flock.

1. Zoonotic risk and biosafety requirements

Salmonella Gallinarum and *S*. Pullorum are host adapted to avian species (Eswarappa *et al.,* 2009) and are considered to pose a minimal zoonotic risk (Shivaprasad, 2000). Although the genome is adapted to a non-intestinal environment and has lost flagella genes to help evade host immune responses (Lopes *et al*., 2016), it is continually evolving, which could theoretically widen the host range in future (Liu *et al*., 2002). Non-typhoidal *Salmonella* serovars should be handled with appropriate biosafety and containment procedures as determined by biological

risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of fowl typhoid and Pullorum disease and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Bacterial isolation	+++	+++	+++	+++	+++	–
Rapid alternative methods e.g. PCR	+	+	+	+	+	–
Detection of immune response						
WBT	++	–	+++	–	+	++
RSA	++	–	++	–	+	++
SAT	++	–	++	–	++	+++
ELISA	+	–	+	–	+	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction-based tests; WBT = whole blood agglutination test; RSA = rapid slide agglutination test;
SAT = serum agglutination test; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

1.1. Bacteriological culture methods

In the acute stages of disease, the agent of both diseases can be recovered from almost all organs, tissues and faeces. In older birds that have become carriers, *S.* Pullorum is most commonly recovered from the ova and oviduct; and it is recovered only occasionally from other organs and tissues, including the alimentary tract. In the acute phase of fowl typhoid the organism is also widely distributed, but in carrier birds, the organism is found most often in the liver, spleen and reproductive tract, and occasionally in the caecal tonsils.

Salmonella Pullorum and *S.* Gallinarum belong to the White-Kauffmann–Le Minor scheme serogroup D, along with *S.* Enteritidis, which is closely related (Grimont & Weill, 2007). The organisms are Gram-negative non-sporogenic rods 1.0–2.5 μm in length and 0.3–1.5 μm in width. They are considered to be non-motile under normal conditions but inducement of flagellar proteins and motility has been shown in some strains of *S.* Pullorum when grown in special media (Holt & Chaubal, 1997).

For optimal recovery of the organisms, the birds being sampled should not have been treated with antimicrobial drugs for approximately 2–3 weeks previously.

Samples may be obtained from live birds, preferably after identifying them as highly sero-positive birds. Fresh or freshly chilled carcasses, egg materials, fresh faeces, or any contaminated materials from housing, incubators or transport boxes may also be taken, but faecal and environmental samples often fail to reveal the presence of the organism because of inconsistent shedding and the poor sensitivity of bacteriological detection methods[2]. Swabs may be taken from the cloaca of sick live birds but post-mortem tissues are preferable. Samples from visibly abnormal tissues are preferred, but aseptically gathered samples can also be taken from the spleen, liver, gall-bladder, kidneys, lungs, heart, ova, testes, alimentary tract or joint lesions. The preferred tissues for routine investigation are liver, ileo–caecal junction and ovaries/oviduct. The surface is seared with a hot spatula and a sample is obtained by inserting a sterile cotton swab or sterile loop through the heat-sterilised surface. The demonstration of infection in serological reactor birds that are apparently normal may, in some cases, require the culture of large volumes of homogenised tissues as well as direct swabbing. Tissue pools may be made from tissues collected from a number of birds, and, for routine testing, five ileo-caecal junction samples may be pooled. Larger numbers of aseptically collected non-intestinal samples can be pooled, but for practical purposes, composite samples of liver, spleen and ovary from five birds are often tested.

When floor litter, faecal material or hatchery material such as hatcher basket liners is sampled, it should be remembered that *S.* Pullorum and *S.* Gallinarum in low numbers associated with subclinically infected carrier birds are more difficult to isolate from faecal and environmental samples than other salmonellae and it is always preferable to culture sick or recently deceased birds or 'dead in shell' embryos. Red mites associated with poultry that are infected with *S.* Gallinarum often contain the organism after feeding and can be cultured. These samples should be cultured by direct inoculation of a selective enrichment broth such as selenite cysteine or selenite F, followed by plating on selective media such as brilliant green agar (Parmar & Davies, 2007; Proux *et al.*, 2002).

Both *S.* Pullorum and *S.* Gallinarum grow well in pure culture on non-selective media, but selective and enrichment media have been described that contain substances to inhibit the growth of extraneous organisms. *Salmonella* Pullorum may grow slowly and produce very small colonies on selective media so incubation of plates for 48 hours is recommended. The efficiency of recovery of *Salmonella* varies according to circumstances, and experience in the use of a medium is an important but unquantifiable factor. Some complex media may have an inhibitory effect on these organisms, so that it is advisable to use both non-selective and selective media for isolation from tissues. Both solid media and broths can be employed. As the toxic properties of selective media may vary, it is preferable to monitor these by comparing growth of control cultures on both types of medium. The inhibitory media should grow at least 75% of the colonies of the corresponding non-inhibitory medium (Ellis *et al.*, 1976; Mallinsen & Snoeyenbos, 1989)

All the media mentioned below are examples of commonly used media, but there are many others found to be equally satisfactory and it is recommended that the most suitable products are validated locally in relation to strains that are circulating in a particular region.

Non-inhibitory media include nutrient agar and blood agar, on which colonies are seen to be smooth, translucent, slightly raised, and about 1–2 mm in diameter. *Salmonella* Gallinarum grows more rapidly than *S.* Pullorum and produces larger colonies with a distinctive smell resembling that of seminal fluid on most media. Broths include buffered peptone water and nutrient and meat infusion broths or universal pre-enrichment broth.

1.1.1. Selective media

i) MacConkey agar

MacConkey agar is inhibitory to non-enteric organisms; it differentiates lactose fermenters (pink colonies) from non-lactose fermenters (colourless colonies). NaCl is omitted to limit the spread of *Proteus* colonies. *Salmonella* colonies are smooth and colourless. *Salmonella* Pullorum produces smaller colonies than other salmonellae. MacConkey is the agar of choice for direct plating from tissues.

2 A combination of sample types and agent identification methods applied on the same clinical sample is sometimes recommended.

ii) Xylose lysine deoxycholate agar

This agar is inhibitory to non-enteric organisms. *Salmonella* Pullorum grows sparsely as small red translucent colonies. *S.* Gallinarum colonies are small, dome-shaped, and may have a central black spot due to H_2S production, but this reaction may be delayed or variable.

iii) Brilliant green agar

Brilliant green agar (BGA) is inhibitory to coliforms and most *Proteus* strains; useful for distinguishing enteric organism colonies. Salmonellae form low, convex, pale red, translucent colonies of 1–3 mm in diameter, similar to *Citrobacter*. *Proteus* forms pin-point colonies, *Pseudomonas aeruginosa* appears as small red colonies, and lactose fermenters are green. *Salmonella* Pullorum produces smaller more pale colonies than other salmonellae. BGA is the agar of choice following enrichment, but low numbers of *Salmonella* can be easily overgrown by competing organisms when BGA is used for faecal and environmental samples.

iv) Brilliant green sulphapyridine agar

This agar is inhibitory to coliforms and *Proteus* strains. The sulphapyridine is added to stabilise selectivity in the presence of egg materials. *Salmonella* Pullorum produces small colonies.

Salmonella Pullorum and Gallinarum grow poorly and do not produce typical colonies on some newer chromogenic agars such as Rambach agar, but more recently developed chromogenic media with a wider detection range could be evaluated for suitability.

1.1.2. Liquid enrichment and selective media

i) Selenite cysteine and F broths

These broths are inhibitory to coliforms but not *Proteus* and are improved by addition of brilliant green. Loss of activity after 24 hours limits its use. Selenite cysteine broth is more stable and less inhibitory than selenite F broth, so is normally preferable (e.g. for hatchery-based monitoring of hatcher basket liners or meconium) except in the case of fresh faecal samples from mature birds where highly competitive flora may be present. Although selenite-based broths are considered to be preferable for isolation of *S.* Pullorum and *S.* Gallinarum from faeces by direct enrichment (Shivaprasad *et al.*, 2013), if there are difficulties with issues of toxicity or shelf life in particular laboratories the other enrichment broths mentioned below may be used. Most of these other broths are however designed to be used following a non-selective enrichment stage and *S.* Gallinarum and *S.* Pullorum are readily overgrown by competitor organisms in non-selective faecal culture resulting in false-negative tests. Direct selective enrichment is therefore recommended for faeces and intestinal or environmental samples. Non-selective enrichment may give better results for tissues obtained by aseptic post-mortem where there should be no competing organisms (Mallinson & Snoeyenbos, 1989).

ii) Tetrathionate/brilliant green broth

This broth is inhibitory to coliforms and *Proteus*, but may also inhibit some strains of *S.* Pullorum/Gallinarum.

iii) Rappaport–Vassiliadis soya (RVS) peptone broth

This broth is normally only used for selective enrichment following pre-enrichment but is more stable than selenite broth; use 1 part inoculum to 100 parts medium. *Salmonella* Pullorum and Gallinarum are more likely to be overgrown by other organisms during pre-enrichment of faeces or intestinal contents than salmonellae that are not host-adapted so direct enrichment with RVS may also be attempted.

1.2. Recovery of salmonellae

The methods for recovering *S.* Pullorum and *S.* Gallinarum vary according to the origin of the samples. Although their isolation from cloacal swabs and faeces may be unrewarding, examination of tissues taken at post-mortem is usually more successful. The methods are described.

1.2.1. Cloacal swabs and fresh faeces from live birds

Swabs dipped in nutrient broth are suitable; small swabs being used for young chickens. The swabs are streaked on selective media, and placed in enrichment broth. The plates and the broth are incubated at 37°C. Higher temperatures may be used with some broths, e.g. 41.5°C for Rappaport–Vassiliadis (RVS). Subcultures are made on to selective media after 24 and 48 hours.

1.2.2. Gall-bladder contents

Swabs of gall-bladder contents are streaked on to non-selective and selective agars and placed in inhibitory and non-inhibitory broths, followed by incubation at 37°C and subculture on to selective agar after 24 and 48 hours.

1.2.3. Organs and tissues

Swabs or segments of tissues are taken in an aseptic manner from individual tissues and lesions and cultured on to non-selective and selective media, and into similar non-selective and selective broths. These are incubated at 37°C and subcultured on to selective agar after 24 and 48 hours. Parallel incubation at a higher temperature, e.g. 40°C, can also be used to enhance the overall isolation rate. Carrier birds: larger amounts of material may be required to identify the carrier birds. The ovary and oviduct are the tissues of choice for *S.* Pullorum, and the liver, gall-bladder and caecal tonsils as well as ovary and oviduct should be tested for *S.* Gallinarum. In practice it is usually best to pool samples from a variety of tissues including the spleen, but intestinal tissues should not be pooled with other tissues. Tissues are homogenised in a small volume of broth and directly plated out. Approximately 10 ml of homogenate is also added to 100 ml of non-selective enrichment broth (e.g. buffered peptone water) and selective enrichment broth (e.g. selenite cysteine broth or brilliant green broth), and incubated at 37°C. These broths are subcultured on to non-selective and selective agar after 24 hours.

1.2.4. Alimentary canal, including the caecal tonsils and intestinal contents

After grinding or homogenisation in a small volume of broth, 10 ml of the homogenate is incubated in 100 ml of selective enrichment broth at 37°C. In general, better isolation is achieved with selenite cysteine broth.

1.2.5. Eggshells

The broken eggshells are placed in a tenfold volume of enrichment broth (e.g. selenite cysteine broth). The broth is incubated at 37°C and subcultured on to selective agar after 24 and 48 hours.

1.2.6. Egg contents

Aseptically taken contents of fresh eggs are homogenised and mixed with 200 ml of buffered peptone water or nutrient broth, incubated at 37°C, and subcultured on to non-selective and selective agar after 24 and 48 hours. Incubated eggs, whether infertile or containing small embryos, can be similarly treated.

1.2.7. Embryos

Homogenised viscera and swabs from the yolk sacs of well-developed embryos may be streaked on to non-selective and selective agar, one swab being placed in 10 ml of both non-selective and enrichment broth (e.g. selenite cysteine broth or brilliant green broth). Incubation is carried out at 37°C, and subcultures are made on to non-selective and selective agars after 24 and 48 hours.

1.2.8. Environmental samples

These include hatcher fluff, debris and macerated egg/chick waste samples and chick box liners or floor faecal or litter samples; 25 g is mixed with 225 ml of enrichment broth (e.g. selenite cysteine broth, brilliant green broth), incubated at 37°C, and subcultured on to selective agar after 24 and 48 hours.

Polymerase chain reaction (PCR) based tests may also be used to confirm the serovar, or its vaccinal status, but have not been fully validated internationally (Batista *et al.,* 2016; Kang *et al.,* 2012; Soria *et al.,* 2012).

1.3. Confirmatory procedures

Typical *S.* Gallinarum colonies on non-selective media are round, translucent, glistening, domed, smooth, and 1–2 mm in diameter after 24–48 hours' incubation. *Salmonella* Pullorum colonies are slightly smaller and translucent. On selective media their appearance varies with the medium, but suspect colonies can be investigated serologically by testing for 'O'9 somatic antigens, observing for motility and testing biochemically.

After incubation for 20–24 hours, the plates should be examined carefully for the presence of typical *S.* Pullorum and *S.* Gallinarum colonies. The plates should be re-incubated for a further 24 hours and examined again. For biochemical and serological confirmation from each plate, five typical or suspect colonies should be chosen for further examination. If there are fewer than five typical or suspect colonies, all of them should be tested. Selected colonies should be inoculated onto the surface of nutrient agar, in a manner that allows the growth of separate colonies. For biochemical confirmation, only pure cultures taken from non-selective media should be used. The following media should be inoculated using an inoculating loop: triple sugar iron (TSI) agar; lysine iron agar (or l-lysine decarboxylation medium); urea agar according to Christensen; tryptone/tryptophan medium for indole reaction; glucose with an inverted Durham tube for acid and gas production; dulcitol, maltose, ornithine decarboxylation medium and semisolid agar, for motility. The reactions shown in Table 2 occur.

Identification kits are commercially available for Enterobacteriaceae. Molecular tests using ribotyping techniques and PCR have been developed in research laboratories (Kang *et al.,* 2012), and can be used for confirmation and differentiation between *S.* Gallinarum and *S.* Pullorum.

For serological confirmation to serogroup level, colonies from non-selective media (nutrient or blood agar) are used. The first stage is elimination of autoagglutinable strains. For this, material taken from a single colony of pure culture is transferred to a glass slide and mixed with a drop of sterile saline. The slide is rocked gently or the drop stirred with a loop for 30–60 seconds and observed for agglutination against a dark background, preferably with the aid of a magnifying glass or dissecting microscope. If the bacteria have clumped into more or less distinctive units, the strain is considered to be autoagglutinable and must not be submitted for the following tests. If the bacterial sample is recognised as non-autoagglutinable, it is tested with a polyvalent 'O' (A–G) antiserum. For this purpose, the material from a single colony is dispersed in the drop of polyvalent 'O' antiserum on the glass slide to obtain a homogenous and turbid suspension. After gently rocking for 30–60 seconds, the reaction is observed against a dark background for agglutination. Alternatively the slide agglutination test may be carried out with smaller volumes of suspension under a dissecting microscope. In this case a portion of the colony to be checked is added to a loopful of saline on the microscope slide to produce a light suspension to check for autoagglutination ('rough strains'). If no agglutination takes place, one or two loops of antisera are added, the drop stirred with a loop and observed for agglutination. *Salmonella* Pullorum and *S.* Gallinarum should agglutinate with polyvalent 'O' antisera but not with polyvalent flagella (poly 'H' phase 1 and phase 2) antisera. If the reaction is positive, the single colony is tested further in the same manner using group-specific sera for *S.* Pullorum and *S.* Gallinarum serovar ('O'9 antiserum). After serogrouping, isolates may be sent to a reference laboratory for serotyping.

Table 2. Biochemical investigation of Salmonella *Pullorum and* S. *Gallinarum*

	Salmonella Pullorum	*Salmonella* Gallinarum
TSI glucose (acid formation)	+	+
TSI glucose (gas formation)	v	–
TSI lactose	–	–
TSI saccharose	–	–
TSI hydrogen sulphide	v	v
Gas from glucose (medium with Durham tube)	+	–
Urea hydrolysis	–	–
Lysine decarboxylation	+	+
Ornithine decarboxylation	+	–
Maltose fermentation	– or late +	+
Dulcitol	–	+
Motility	–	–

+ = 90% or more positive reaction within 1 or 2 days; – = No reaction (90% or more); v = Variable reactions.

It is also possible to confirm and differentiate *S.* Gallinarum by specific PCR (Kang *et al.,* 2012).

1.4. Test procedure for culture of visceral, faecal, intestinal and environmental samples for *S.* Pullorum and *S.* Gallinarum

i) Where possible, begin laboratory procedures on the same day as samples are collected.

ii) Homogenise the material as much as possible by manual mixing, gentle macerating or stomaching with a small volume of sterile saline if the material is dry.

iii) Stir the mixture with a small rectal swab or loop and streak thickly on to one-quarter of a brilliant green agar plate. (Swabs from uncontaminated tissues sampled in an aseptic manner can also be streaked on to blood agar.)

iv) From this deposit of material on the plate, streak the rest of the plate to obtain individual colonies.

v) Add 5–25 g of the homogenised sample to freshly made selenite cysteine broth (see note on liquid enrichment and selective media above) to make a 1:10 sample to broth ratio. Shake or stir to disperse the sample in the broth.

vi) Incubate the brilliant green agar plates and selenite cysteine broth at 37°C for 24 hours.

vii) Examine the plate after 24 hours' culture. Carry out agglutination tests on up to five suspect colonies with polyvalent 'O' (A–G) antisera and polyvalent H (phase 1 and phase 2) antisera. If agglutination is unclear subculture suspect colonies on to nutrient agar or blood agar and repeat tests after 24 hours' incubation of those media.

viii) If poly 'O' is positive then check with 'O'9 antiserum. If 'O'9 is positive and poly 'H' is negative, this is indicative of the possible presence of *S.* Pullorum or *S.* Gallinarum.

ix) If there are no positive colonies on the brilliant green agar plate, streak out a 10 µl loop of incubated selenite cysteine broth onto brilliant green agar as in step iv above.

xi) Incubate the brilliant green agar plates at 37°C for 24 hours and re-incubate the previous (negative) brilliant green agar plates and the selenite cysteine broths for a further 24 hours.

xi) Repeat examination of plates as in step vii above.

xii) If plates are still negative, re-plate from selenite cysteine broth and incubate brilliant green agar plate, that was inoculated in step ix, for a further 24 hours and examine as in step vii above.

xiii) Confirm *S.* Pullorum and *S.* Gallinarum using biochemical tests as shown in Table 2. Isolates can be sent to a *Salmonella* reference laboratory for confirmation of serotype and for further molecular typing if required for epidemiological purposes.

1.5. Molecular epidemiology

Standard molecular 'fingerprinting' techniques used for *Salmonella*, such as plasmid profile analysis, pulsed field gel electrophoresis PCR-restriction fragment length polymorphism (RFLP) or ribotyping can be used for investigating outbreaks of *S.* Pullorum or *S.* Gallinarum. It is often necessary to use combinations of such methods and different restriction enzyme combinations to obtain maximum discrimination because of a high level of clonality. The most effective techniques may also vary by country because of the nature of circulating clones in that region. High throughput whole genome sequencing has also been applied to *S*. Gallinarum, but is not yet available or economically viable in all countries (De Carli *et al*., 2016).

2. Serological tests

Serological tests are best applied as a flock test as results for individual birds will vary according to the stage of infection. It is therefore necessary to take sufficient individual samples to determine infection in the flock. The number of samples will depend on the expected prevalence and level of confidence desired (see Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*). If the test is to be used for detecting individual infected birds for culling, it should be repeated at least twice and preferably until the whole flock has given at least two negative tests.

The tests that are most readily applied include rapid whole blood agglutination, rapid serum agglutination (RST), tube agglutination and micro-agglutination (USDA, 1996). Other invasive *Salmonella* such as *S.* Enteritidis and *S.* Typhimurium or use of vaccination may lead to false-positive results in serological tests for *S.* Pullorum.

Both *S.* Pullorum and *S.* Gallinarum possess 'O' antigens 9 and 12 and may also possess O antigen 1 (Brooks *et al.,* 2008). However, in the case of *S.* Pullorum, there is a variation in the ratio of 12_1, 12_2 and 12_3; the standard strain contains more 12_3 than 12_2, while the reverse is true of the variant form. Intermediate forms also exist. (There appears to be no such form variation in the case of *S.* Gallinarum.) As this variation occurs, it is necessary to use a polyvalent antigen in immunodiagnostic tests. The same antigen is used to detect both *S.* Pullorum and *S.* Gallinarum, but detection of the latter may be relatively poor (Proux *et al.,* 2002).

2.1. Rapid whole blood agglutination test

The rapid whole blood agglutination test can be used under field conditions for detecting both *S.* Pullorum and *S.* Gallinarum, and the reactors can be identified immediately. However, it is not reliable in turkeys or ducks as the test results in a significant proportion of false-positive results. Sera can be screened by rapid slide agglutination test and positive reactions confirmed by the more specific tube agglutination test. Chickens can be tested at any age, although some authorities specify a minimum age of 4 months (Shivaprasad *et al*., 2013; USDA, 1996) and positive results from chicks less than 4 weeks of age may be due to maternal antibodies.

2.1.1. Preparation of stained antigen for the rapid whole blood or rapid serum agglutination test

Incubate one standard form strain of *S.* Pullorum (antigenic structure 9, 12_1, 12_3) and one variant form (antigenic structure 9, 12_1, 12_2) at 37°C and harvest separately until final mixing for the complete antigen.

Sow strains on to separate agar slopes, incubate at 37°C for 24 hours, emulsify growth with sterile normal saline and spread an inoculum over an agar plate to produce easily selected discrete colonies. For this the plates are incubated for 48 hours, a number of colonies are marked out and each is tested for agglutination on a slide with 1/500 acriflavine in saline. Smooth-phase colonies do not produce agglutination. Pick off typical colonies that do not produce any agglutination, seed on to agar slopes, and incubate for 24 hours. Emulsify the growth in saline and evenly distribute 2 ml over the surface of the medium (200 ml) in a Roux or similar flask. Incubate the flasks for 60 hours.

For harvesting the bacterial growth, flood the surface of each flask with approximately 10 ml sterile buffered formol saline, pH 6.5 (8.5 g/litre sodium chloride, 10 ml/litre neutral formalin, 4 ml/litre 0.5 M sodium phosphate: made up to 1 litre with distilled water, pH adjusted to 6.5 using 1 M orthophosphoric acid or 1 M sodium hydroxide), to give dense cell suspensions. Add 12–15 sterile glass beads of 3–5 mm diameter and rock the flasks until all the culture is in even

suspension; leave in a vertical position for at least 15 minutes. Check the morphology and purity of the suspensions by preparing and examining Gram-stained films. Bulk the suspension from each flask containing the same strains. To each 100 ml of suspension, add 200 ml of absolute alcohol. Shake the mixture and allow to stand for 36 hours, or until precipitation is complete. Check the agglutinability of the standard and variant precipitate by first centrifuging a sample to separate the alcohol, which is removed, dilute with normal saline and test with a known positive and negative serum. If satisfactory, remove the clear supernatant alcohol (centrifugation at 2000 ***g*** for 10 minutes may be helpful for precipitation), and add sufficient phosphate buffered saline (PBS) containing 10% (v/v) glycerol to standardise the density to 75 × No. 1 Wellcome opacity tube (or 50 × tube No. 1.0 on the McFarland scale). Add equal volumes of standard and variant strains, and add 1% (v/v) of 3% (w/v) alcoholic crystal violet solution to the final mixture, and allow to stand for 48 hours at room temperature. Store in a tightly closed container at 0–4°C for up to 6 months. To assess safety, carry out a culture test on blood agar to confirm non-viability of the unwashed antigen before standardisation. Each bottle of antigen must be tested after alcoholic precipitation and before standardisation against standard titre antisera for *S.* Pullorum and *S.* Gallinarum, and against a negative serum. If possible, also test with known positive and negative serum and blood from positive and negative chickens.

Stained antigen products for the whole blood plate agglutination test are available commercially, and although there seem to be some slight differences in their sensitivity (Gast, 1997), it is unlikely that poultry flocks infected with the different variants of *S.* Pullorum would be missed.

2.1.2. Test procedure

i) Use a clean white tile marked into squares of about 3 × 3 cm. If a tile with 3 × 4 squares is used, up to 12 blood samples can be tested at the same time.

ii) Place 1 drop (about 0.02 ml) of crystal-violet-stained antigen in the centre of each square.

iii) Obtain a sample of fresh whole blood. This is conveniently done by making a stab of a wing vein using a needle with a triangular point.

iv) Place an equal size drop of fresh whole blood next to a drop of antigen.

v) Mix the drops of antigen and blood using a fine glass rod, which is wiped clean between samples.

vi) Use a gentle rocking motion to keep the drops agitated for up to 2 minutes. Several tests may be carried out simultaneously on the same tile, but the drops should not be allowed to dry out during this time. In very warm conditions, a smaller number of larger drops per plate may be required to avoid drying out.

vii) A positive reaction is indicated by easily visible clumping of the antigen within 2 minutes.

viii) A negative reaction is indicated by absence of clumping of the antigen within 2 minutes.

ix) Include known positive and negative control sera on each testing occasion, using them in the same way as the blood.

x) On completion of a set of tests, the tile is washed and dried, ready for further use.

In the absence of positive reactions, any inconclusive reactions can only be interpreted in the light of the previous *Salmonella* testing history of the flock. Where there are positive reactors, any doubtful reactor should be regarded as positive. Also, recently infected birds may not show a typical positive reaction until they are retested after 3–4 weeks.

2.2. Rapid serum agglutination test

The RST is performed in the same manner, except that serum is substituted for whole blood. For export test purposes an initial screening of sera by RST followed by confirmation of positives by the tube agglutination test is the optimal approach. Ideally serum samples tested by any method should be tested within 72 hours of collection as nonspecific reactions may increase in older samples. Fresh samples can be frozen for later testing if a delay is unavoidable.

2.3. Tube agglutination test

Fresh serum from chickens, turkeys or other birds is used at an initial dilution of 1/25, obtained by mixing 0.04 ml of serum with 1.0 ml of antigen[3]. Positive and negative control sera are included in each test. The antigen is prepared from unstained *S.* Pullorum or *S.* Gallinarum cultures diluted to a concentration of No. 1 on the McFarland scale (as described above). The mixture is incubated at 37 or 50°C for 18–24 hours before reading. A positive reaction consists of a granular white deposit with a clear supernatant fluid; a negative reaction shows uniform turbidity. Samples positive at a dilution of 1/25 are retested at a higher range of dilutions and a titre of 1/50 is usually considered to be positive, although this figure seems to vary in the literature. In many cases a single dilution of 1/50 is used but this may fail to detect some flock infections if only small numbers of samples are taken.

2.4. Micro-agglutination test

This resembles the tube agglutination test, but requires much smaller volumes of reagents. The test is performed in microtest plates. Sera are first diluted by adding 10 µl of serum to 90 µl of normal saline, and then adding 100 µl of previously standardised stained microtest antigen to give a final dilution of 1/20. By titrating the serum in doubling dilutions and adding an equal volume of standardised stained antigen, an end-point (titre) can be obtained. The plates are sealed and incubated at 37°C for 18–24 or 48 hours. A positive reaction consists of a fine diffuse precipitation, whereas a negative reaction shows a button-like precipitate. Titres of 1/40 are usually considered to be positive but this test is more liable to produce false-positive results with turkey sera.

Other serological tests include micro-antiglobulin (Coombs), immunodiffusion, haemagglutination and enzyme-linked immunosorbent assay (ELISA).

ELISA techniques have been described for detecting antibodies to *S.* Pullorum and *S.* Gallinarum (Oliviera *et al.,* 2004). The indirect ELISA using lipopolysaccharide antigen is likely to be the most sensitive and specific serological flock test for *Salmonella*, including *S.* Gallinarum and *S.* Pullorum. It is relatively easy to perform with serum or yolk, and can be used for quantifying the titre of antibody (Barrow, 1992; 1994). No commercial ELISA kits for *S.* Pullorum and *S.* Gallinarum are currently available, but an indication of likely infection can often be obtained using a lipopolysaccharide (LPS)-based commercial ELISA for *S.* Enteritidis; these tests have not been validated for this purpose.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Although both live and inactivated vaccines have been prepared for use against *S.* Gallinarum (Paiva *et al.,* 2009), the vaccine most widely used is made from the rough 9R strain (Harbourne *et al.*, 1963; Revolledo & Ferreira, 2012). It is normally only employed in chickens. The number of viable organisms per dose is important; these organisms can survive in vaccinated birds for many months and may be transmitted through the egg (and perhaps from bird to bird). Vaccination may reduce flock losses, but will not prevent infection with field strains. In addition, vaccination with 9R may sometimes precipitate high mortality in infected birds (Silva *et al.*, 1981), and may stimulate the production of transient antibodies. It is usual to vaccinate at 8 weeks and again at 16 weeks of age. Antimicrobials should be avoided before and after vaccination.

Currently available vaccines, however, have only a minor role to play in the control of fowl typhoid as they offer short-lived protection against clinical disease and limited or variable protection against infection. Autogenous or locally produced vaccines can also be used to control clinical disease, but care must be taken to avoid strain instability leading to reversion to virulence (Okamoto *et al.*, 2010; van Immerseel *et al.,* 2013). Control can best be achieved by biosecurity, hygiene, good management, monitoring and removal of infected flocks, although the routine vaccination against *S.* Enteritidis and *S.* Typhimurium that

3 For preparation of small volumes of somatic antigens see chapter 3.10.7.

is carried out in breeding and laying hen flocks in many countries may be partially protective against introduction of *S*. Gallinarum (Lee, 2015). Commercially available 9R vaccines have been used for reduction of *S.* Enteritidis in laying flocks in some countries but may be prohibited or are not commercially available in some countries where fowl typhoid is not present (Lee *et al*., 2005). Even in countries with fowl typhoid, use of vaccine may complicate control as it does not prevent infection, only reduce clinical disease and allow production to continue from infected flocks. It is therefore preferable to aim for eradication of the organism rather than acceptance of on-going disease, but this is often not economically viable in large multi-age holdings as eradication of red mites is necessary to ensure continued freedom from infection (Wales *et al.,* 2010).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Most vaccines are produced in highly industrial commercial processes and are regulated by national veterinary medicines licensing authorities. Smaller quantities of emergency herd vaccines or autogenous vaccines are produced by private laboratories, but each production has to be specifically licensed. It is recommended that a validated commercial vaccine is used unless there is no alternative because of the need to maintain quality and avoid risk associated with reversion to virulence. Live vaccines must also be bacteriologically distinguishable from field strains or surveillance and control programmes may be compromised. Observations from some countries suggest that it is not always straightforward to distinguish between *S*. Gallinarum vaccines and field strains (van Immerseel *et al.,* 2013). There will inevitably be some interference with serological monitoring for *S*. Gallinarum and potential interference with serological monitoring for *S*. Enteritidis, unless a stepwise approach is used in which a sensitive LPS-based ELISA is used to test for antibodies to O9 antigens and positive sera are further tested with a flagella antigen ELISA, which will give a negative reaction in cases of *S*. Gallinarum infection (Shivaprasad *et al.,* 2013). Recent work on the molecular mechanisms of infection should lead to the development of improved vaccines in future (Barrow *et al.,* 2012).

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

For killed or live vaccines, the bacterial strain should be an organism as closely related to currently circulating field strains as possible. It should be carefully chosen from cases of severe clinical disease, and virulence and antigen production should be assessed. It is best to evaluate a panel of potential strains in this way before testing the final selection. The final vaccinal strain should be identified by historical records and characterised by stable phenotypic and/or genetic markers, preferably using whole genome sequencing. Live vaccinal strains should be marked by stable characters allowing easy distinction from wild strains. Markers such as resistance to antimicrobials, for example rifampicin, or auxotrophism may be used. Attenuation of virulence should be stable and preferably obtained by two independent defined mutations. The stability of live vaccine strains can be verified by regular checks using whole genome sequencing.

Live fowl typhoid vaccine is a suspension of suitably attenuated living organisms of a rough strain of *S.* Gallinarum, e.g. 9R. The organisms in the vaccine give the biochemical reactions characteristic of *S.* Gallinarum. Colonies of a 24-hour culture prepared from the vaccine on nutrient agar plates are rough when examined by the acriflavine slide test. The culture should not produce any smooth colonies or contain the somatic antigens characteristic of the smooth forms of *S.* Gallinarum.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

i) Sterility and purity

The vaccine strain must be checked as follows:

a) Staining of a smear of bacterial suspension on a glass slide using Gram stain.

b) Homogeneity of culture on non-selective media.

c) Metabolic requirements as indicated by biochemical tests.

d) Detection of phenotypic and/or genetic markers.

e) Agglutination with specific antiserum.

f) The vaccine culture and any adjuvants, preservatives or other materials must be microbiologically sterile and non-toxic at the concentrations used.

ii) Safety

The LD_{50} (50% lethal dose) or ID_{50} (50% infectious dose) may be determined in chickens or, preferably, signs of more mild adverse reactions should be checked in the target species. Ten times the field dose of live vaccine or twice the dose for killed vaccines must be given to the target species at the recommended age and by the recommended route. The animals are observed for absence of adverse reactions. Stability and non-reversion to virulence after serial passages in susceptible species should be shown for live vaccines. It is also necessary to consider repeat vaccination. Live vaccine should be shown not to persist for long periods in vaccinated animals or be transmitted to meat or eggs that may be consumed, and the method of application should not present a hazard to operators. In the case of *S*. Gallinarum vaccine at least six healthy, susceptible (preferably specific pathogen free [SPF]) chickens, 8–16 weeks of age, are each injected subcutaneously with a ten-fold dose of vaccine, and are observed for at least 7 days; no local or systemic reaction should develop.

iii) Efficacy

Laboratory experiments and field trials should be used to show that the vaccine is effective. The laboratory experiments consist of vaccination–challenge tests in the target species at the recommended dose and age. The efficacy data can also be used as the basis for a batch potency test. Field trials are more difficult to undertake with respect to testing efficacy because of difficulties with standardising the challenge and providing appropriate controls. In the case of *S*. Gallinarum 9R vaccine or similar vaccines, at least fifteen healthy chickens, 8–16 weeks of age, of a brown layer hybrid breed, and taken from a stock that is free from *S*. Pullorum infection, are each injected subcutaneously with a quantity of vaccine corresponding to one field dose, i.e. 5×10^7 viable organisms. After an interval of 21–28 days, the vaccinated chickens and an equal number of similar unvaccinated chickens are deprived of food for approximately 18 hours. The chickens are then challenged by oral administration of 1 ml of a broth suspension containing 5×10^7 organisms of a virulent strain of *S*. Gallinarum mixed with 300 mg of a powder consisting of chalk (40%), light kaolin (43%) and magnesium trisilicate (17%). All the chickens are observed for 14–21 days. The vaccine passes the test if at the end of this period the number of surviving vaccinated chickens that show no macroscopic lesions of fowl typhoid at post-mortem exceeds by eight or more the number of similarly defined control chickens.

iv) Environmental aspects

Live vaccine strains should be tested for their ability to persist in the environment and infect non-target species such as rodents and wild birds that are likely to be exposed. Prolonged survival of some live vaccines in faeces, litter or dust may present an unacceptable environmental hazard when the material is removed from the animal houses. Live vaccines should not be used in commercial laying flocks during lay.

2.2. Method of manufacture

2.2.1. Procedure

The seed culture is propagated and maintained using suitable media for growth of *Salmonella*. The media used should not contain serum or animal tissues, unless permitted by national regulations. Culture may be on solid medium, in Roux flasks, or in liquid medium, in which case large-scale fermentation equipment may be used. Iron limitation or low temperature incubation on minimal media may enhance LPS antigen production by the vaccine strain. In the case of *S*. Gallinarum (9R), the vaccine may be prepared by inoculation of a suitable medium, such as peptone broth, with a fresh culture of *S*. Gallinarum (9R) and incubation at 37°C for 24 hours, with agitation. The organisms are harvested by sedimentation or centrifugation.

Alternatively the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In either case, the suspension is diluted in PBS solution, pH 7.0, and may be freeze-dried. The dose used per bird is between 5×10^6 and 5×10^7 organisms.

Vaccine must be produced in suitable clean rooms to which only approved personnel have access. Care must be taken to avoid cross-contamination between areas where live organisms are processed and other areas. Contamination from operators and/or the environment must be avoided and vaccine preparation should take place in a separate area from diagnostic culture work. Operators must not work with vaccine whilst ill and must not be subject to immunosuppressive conditions or medications. Personnel must be provided with protective clothing in production areas and in animal rooms.

Seed-lot cultures are prepared from the primary seed-lot, and the number of passages is dependent on the validation of the process. The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture and incubation on a shaker at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation. Alternatively, the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In the case of live vaccines, the suspension is diluted in PBS, pH 7.0, and may be freeze-dried.

The time of inactivation of dead vaccines should be at least 33% more than that taken to reduce the viable number to an undetectable level. The inactivation process must be applied to the whole volume of the vaccine cell harvest.

Preservatives, excipient for lyophilisation, stabiliser for multi-dose containers or other substances added to or combined with a vaccinal preparation must have no deleterious effect on the immunising potency of the product.

2.2.2. Requirements for substrates and media

All chemicals and growth media used should be guaranteed to be fit for purpose and checked by the use of suitable controls.

2.2.3. In-process controls

The following points require attention:

i) Visual control of the suspension, homogeneity by Gram stain, culture on non-selective medium.

ii) Slide agglutination with specific antisera.

iii) Titration of bacteria by turbidimetry and/or plate count.

iv) Test of effective inactivation (killed vaccine) by plating on non-selective medium or use of a medium that gives optimum chance of recovery, e.g. production medium with neutralisation of the inactivating compound.

v) Titration of viable bacteria (living vaccine) before and after lyophilisation.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use are found in chapter 1.1.9 of this *Terrestrial Manual*.

ii) Safety

A laboratory test that has previously shown a correlation with safety in the target species may be used to determine the absence of deleterious effects on vaccinated animals. Each batch should be tested in the target species at the recommended age and route, using at least twice the field dose for killed vaccines and ten times the dose for live vaccines.

Observations are made on any adverse effects on the demeanour and health of the vaccinated animals and an assessment may be made of tissue reactions at the injection site.

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

iii) Batch potency

Potency is tested using vaccination–challenge assay in chickens and/or other species, including (if practicable) any other target species and immunological response in target species.

2.3. Requirements for authorisation

2.3.1. Safety requirements

Certain killed vaccines may occasionally cause reactions in vaccinated animals because of their LPS content or the adjuvant used, and likewise live vaccines should be used with caution in animals that are not completely healthy at the time of vaccination. It is often necessary, however, to vaccinate flocks in the face of clinical fowl typhoid. Vaccines may also cause swelling at the site of injection, particularly if an oil-emulsion adjuvant is used.

i) Target and non-target animal safety

Killed vaccines are assessed in a double-dose test, and live vaccines are assessed in a test using ten times the dose, ideally in the target species. Live vaccines should be proven to be harmless in relevant non-target species that could be exposed to vaccine shed by vaccinated animals. As *S*. Gallinarum and *S*. Pullorum are host specific, non-target species are less of a concern.

ii) Reversion to virulence for attenuated/live vaccines

Live vaccines shall be shown in replication tests in target species to not revert to virulent strains during a suitably large number of replications. Mutations, especially undefined mutations, should be shown to be stable, and checks on stability can be made by whole genome sequencing. It is recommended that live vaccines that contain *Salmonella* serovars that are not endemic in a particular region should not be used for control of other serovars (van Immerseel *et al.,* 2013). Special care should also be taken to ensure that attenuated vaccines are not incompletely attenuated or contaminated with seed organisms.

iii) Environmental consideration

Live vaccines should not be able to replicate in the environment or persist for more than a short period.

2.3.2. Efficacy requirements

i) For animal production

The duration of immunity is likely to vary considerably between products, vaccination regimes and individual vaccinated animals. The vaccine should provide protection throughout the laying period, and this can be measured by potency (efficacy) tests at stages during lay. A booster dose during lay may be required, but live vaccines should not be used during lay in flocks providing eggs for human consumption.

Immunity to *Salmonella* is normally serovar or serogroup specific. Consultation among colleagues suggests that most killed vaccines will provide some protection for 6 months, while some live vaccines given by injection may elicit stronger immunity, which may persist for 1 year or more. Orally administered vaccines may produce more variable protection, particularly in the case of labile vaccines that are administered in drinking water. Vaccination of day old chicks by coarse spray may be beneficial where there is early challenge, and programmes that combine live and killed vaccines may provide superior protection. It

should be remembered however, that a strong challenge such as that associated with continually occupied farms or infected wild birds and mite populations may overwhelm vaccinal immunity and commercial live vaccines may be attenuated to reduce environmental survival in a way that reduces the immune response. There may also be problems with ensuring accuracy of injection with killed and live injectable vaccines. The *Salmonella* vaccines are intended to limit the extent of clinical disease in poultry, and also to reduce the risk of introduction of infection to flocks. If possible, the potency test should relate to the efficacy of the vaccine in the target species, and suitable criteria should be applied for passing batches. It may be possible to assess killed and injected vaccines by the O-H antibody response produced, although it should be remembered that serum antibodies are only part of the host's protective mechanism against *Salmonella*. Alternatively, the potency of the vaccine may be assessed by its effect on challenged vaccinated animals compared quantitatively and statistically with unvaccinated controls.

ii) For control and eradication

Vaccines for *Salmonella* are not capable of eradicating infection from flocks but can increase the threshold for infection, reduce the level of shedding of the organism and reduce vertical transmission in poultry that results in contamination of hatching or table eggs. Vaccination is therefore an aid to other eradication and control measures such as culling, all in-all out production, biosecurity and farm hygiene.

2.3.3. Stability

Information is lacking on the stability of killed vaccines. Stability is affected by storage conditions and by the presence of contaminating microorganisms growing in the product. Chemicals with antimicrobial activity, such as thiomersal, phenol or crystal violet, are often included as preservatives in killed bacterial vaccines. The stability is assessed by potency tests repeated at appropriate time intervals. The stability of live vaccines can be assessed by performing counts of the number of viable organisms repeated at appropriate time intervals, and genotyping tests to identify genetic changes during fermentation production.

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NB: At the time of publication (2018) there were no WOAH Reference Laboratories for fowl typhoid and pullorum disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.3.12.

INFECTIOUS BURSAL DISEASE (GUMBORO DISEASE)

SUMMARY

Description of the disease: *Infectious bursal disease (IBD) virus (IBDV, genus* Avibirnavirus, *family* Birnaviridae) *infects chickens, turkeys, ducks, guinea fowl and ostriches, but causes clinical disease solely in young chickens. Severe acute disease, usually in 3- to 6-week-old birds, is associated with high mortality, but less acute or subclinical infections are common earlier in life. IBDV causes lymphoid depletion in the bursa of Fabricius. Significant depression of the humoral antibody responses may result, thus promoting secondary infections. Two serotypes of IBDV, designated serotypes 1 and 2, are recognised. Clinical disease has been associated only with serotype 1, against which all commercial vaccines are prepared. Some antigenic variants of serotype 1 IBDV may require special vaccines for maximum protection. Very virulent strains of serotype 1 IBDV are common worldwide and cause serious disease.*

Clinical IBD, also known as Gumboro disease, can be diagnosed by a combination of characteristic signs and post-mortem lesions. Subclinical IBD can be confirmed in the laboratory by demonstrating a humoral immune response in unvaccinated chickens, or by detecting viral antigens or viral genome in tissues. In the absence of such tests, histological examination of bursae may be helpful.

Detection of the agent: *IBDV isolation is seldom carried out in routine diagnosis. Specific antibody-negative (SAN) chickens, embryonated eggs from SAN sources, or cell cultures, may be used. It may be difficult to adapt IBDV to the latter two systems. The identity of the isolated virus should be confirmed by virus neutralisation (VN).*

Viral antigens can be detected in the bursa of Fabricius before anti-IBDV antibodies are elicited; this can be useful for early diagnosis. In the agar gel immunodiffusion (AGID) test, a bursal homogenate is used as an antigen against a known positive antiserum. Antigen-capture enzyme-linked immunosorbent assays (AC-ELISAs) using plates coated with IBDV-specific antibodies can also detect IBDV antigens in bursal homogenates. IBDV antigens may be evidenced by immunostaining of infected tissues, using an IBDV-specific chicken antiserum.

The reverse-transcription polymerase chain reaction (RT-PCR) may be used to detect viral RNA.

Strain characterisation: *IBDV strains can be characterised by pathotyping in SAN chickens, by antigenic typing in cross VN assays or in tests based on monoclonal antibodies, or by nucleotide sequencing of RT-PCR amplification products derived from both segments of IBDV genome. Tests should be performed by specialised laboratories and should include reference control strains.*

Serological tests: *AGID, VN or ELISA may be carried out. IBDV infection usually spreads rapidly within a flock: only a small percentage of the flock needs to be tested for antibodies. If positive reactions are found in unvaccinated birds, then the whole flock must be regarded as infected.*

Requirements for vaccines: *Live attenuated vaccines, inactivated (killed) vaccines, live recombinant vaccines expressing the capsid (VP2) antigen of IBDV or Immune-complex (Icx) vaccines are available. Live attenuated, recombinant or Icx vaccines are used to actively immunise young chickens. A complementary approach is to provide young chickens with passive protection by vaccinating the parents using a combination of live and killed vaccines. Effective vaccination of breeding stock is therefore of great importance.*

Live attenuated IBDV vaccines should be stable, with no tendency to revert to virulence. Live vaccines are referred to as mild, intermediate, or 'intermediate plus' ('hot' or 'invasive'), based on their

increasing ability i) to replicate and cause lymphocytic depletion in the bursa and ii) to overcome residual maternally derived antibodies (MDA). Mild vaccines are rarely used in broilers, but are used widely to prime broiler parents prior to inoculation with inactivated vaccine. When MDA are present at 1 day of age, vaccination with live vaccines should be delayed until MDA in most of the flock has waned. The best schedule can be established by serological testing to determine when MDA has fallen to a low level. Live vaccines are usually administered by spray or in drinking water.

Recombinant and Icx vaccines allow for automated administration by injection, either in ovo *at 18 days of incubation, or at 1-day old, even in the presence of MDA.*

Killed vaccines need to have a high antigen content to be effective. They are mostly used to stimulate high and uniform levels of antibody in parent chickens, and as a consequence in their progeny, but they can occasionally be used in young valuable birds with MDA. The killed vaccines are manufactured in oil emulsion adjuvant and given by injection. They must be used in birds already sensitised by either live vaccine or field virus. This can be checked serologically. High levels of MDA can be obtained in breeder birds by giving, for example, live vaccine at approximately 8 weeks of age, followed by inactivated vaccine at approximately 18 weeks of age.

A. INTRODUCTION

Infectious bursal disease (IBD), also known as Gumboro disease, is caused by a virus that is a member of the genus *Avibirnavirus* (family *Birnaviridae*). Although turkeys, ducks, guinea fowl, pheasants and ostriches may be infected, clinical disease occurs solely in chickens. Only chickens younger than 10 weeks are usually clinically affected. Older chickens usually show no clinical signs.

Severe acute disease of 3- to 6-week-old birds is associated with high mortality, and signs including prostration, diarrhoea, and sudden death. Post-mortem examinations of acute IBD cases reveal a combination of muscular and proventricular haemorrhages, nephritis and bursal inflammation, with bursal oedema or haemorrhages in the first 4 days, followed by bursal atrophy later in the course of the disease (see Section B.1 *Identification of the agent* for details). Differential diagnosis of acute IBD should take into account other diseases that can induce sudden death in young chickens, with either haemorrhages or nephritis or bursal lesions. This certainly includes infectious diseases such as Newcastle disease (ND), chicken infectious anaemia (CIA), and infections by infectious bronchitis viruses (IBV) with nephropathogenic tendencies. Bursal lesions in the early stages of the disease are critical in the differential identification of acute IBD.

A less acute or subclinical disease is common in 0- to 3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and, especially if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. The only lesions associated with subclinical IBD may be bursal atrophy and lesions associated with secondary infections. The characterisation of histopathological changes associated with bursal atrophy will be of utmost importance in identifying subclinical IBD.

Two serotypes of infectious bursal disease virus (IBDV) are known to exist. Serotype 1 viruses replicate in the bursa of Fabricius and some serotype 1 viruses cause clinical disease in chickens. Antibodies or virus are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 viruses have been detected from the respiratory tract of turkeys, cloacal swabs of ducks or in the bursae of Fabricius of chickens. Antibodies against serotype 2 viruses are very widespread in turkeys and are sometimes found in chickens and ducks. There is no report of clinical disease caused by infection with serotype 2 virus (Eterradossi & Saif, 2013).

IBD has not been reported to have any zoonotic potential (Eterradossi & Saif, 2013).

B. DIAGNOSTIC TECHNIQUES

Isolation and identification of the agent provide the most certain diagnosis of IBD, but are not usually attempted for routine diagnostic purposes as the virus may prove difficult to isolate. In practice, laboratory diagnosis of IBD depends on detection of specific antibodies to the virus, or on detection of the virus in tissues, using immunological or molecular methods. Several methods are available for diagnosis depending on the objectives (Table 1).

Table 1. Test methods available for IBD diagnosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Pathology and virus[a]						
Histopathological examination of bursae	+[b]	–	–	+++	+[b]	+[c]
Virus isolation	+[b]	–[d]	–	+[e]	+[e]	–
Virus characterisation (pathotyping, antigenicity, nucleotide sequencing)	+[f]	–	–	+++	+[f]	+[c]
Virus detection in the bursa by immunoassays (AGID, AC-ELISA, immunostaining)	+[b]	–[d]	–	+++	+	–
Virus detection by RT-PCR	+[b]	–[d]	+[b]	+++	–[g]	+
Detection of immune response						
AGID for antibody detection	++[b]	++	++	–	–	+
ELISA for antibody detection	+++[b]	+++	+++	–	–	+++
Virus neutralisation	+[h]	++[h]	–	–	–	++[h]

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
AGID = agar gel immunodiffusion assay; AC-ELISA = antigen capture enzyme-linked immunosorbent assay.
RT-PCR = reverse transcription - polymerase chain reaction.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]If performed on a large scale and always negative in an area where no live vaccination is performed, or to check for subclinical IBD;
[c]Could be used post-vaccination to check replication of live vaccine in the bursa of Fabricius;
[d]Not suitable as could be negative if infection occurred several weeks before testing;
[e]Labour intensive and needs to be complemented with virus characterisation to differentiate between live vaccines and field isolates;
[f]Could be necessary if live vaccines are used in the investigated area;
[g]Not suitable as does not normally differentiate live vaccines from field isolates;
[h]Labour intensive, however reference method in non-poultry birds, or non-avian species, or when small number of chickens are investigated, or when it is critical to correlate the presence of detected antibody with protection.

1. Detection of the agent

Clinical IBD has clearly characteristic signs and post-mortem lesions. A flock will show very high morbidity with severe depression in most birds lasting for 5–7 days. Mortality rises sharply for 2 days then declines rapidly over the next 2–3 days. Usually between 5% and 10% of birds die, but mortality can reach 30–40% or more with very virulent IBDV (vvIBDV). The main clinical signs are watery diarrhoea, ruffled feathers, reluctance to move, anorexia,

trembling and prostration. Post-mortem lesions include dehydration of the muscles with numerous ecchymotic haemorrhages, enlargement and discoloration of the kidneys, with urates in the tubules. The bursa of Fabricius shows the main diagnostic lesions. In birds that die at the peak of the disease outbreak, the bursa is enlarged and turgid with a pale yellow discoloration. Intrafollicular haemorrhages may be present and, in some cases, the bursa may be completely haemorrhagic giving the appearance of a black cherry. Peribursal straw-coloured oedema will be present in many bursae. Confirmation of clinical disease or detection of subclinical disease is best done by using immunological methods as IBDV is difficult to isolate. For virus isolation, the methods described below should be followed. Differentiation between serotypes 1 and 2 or between serotype 1 subtypes or pathotypes should be undertaken by a specialised laboratory (e.g. the WOAH Reference Laboratories for infectious bursal disease[1]).

1.1. Sample preparation

Remove the bursae of Fabricius aseptically from approximately five affected chickens in the early stages of the disease. Chop the bursae using two scalpels, add a small amount of peptone broth containing penicillin and streptomycin (1000 µg/ml each), and homogenise in a tissue blender. Centrifuge the homogenate at 3000 ***g*** for 10 minutes. Harvest the supernatant fluid for use in the investigations described below. Filtration through a 0.22 µm filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titre.

1.2. Identification by the agar gel immunodiffusion test

A protocol for the agar gel immunodiffusion (AGID) test is described in Section B.2.1. For detection of antigen in the bursa of Fabricius by AGID, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in scissor movement, then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue, and the freeze–thaw exudate may be used to fill the wells.

1.3. Identification by immunofluorescence

Sections of bursa are prepared using a microtome cryostat, dried at room temperature and then fixed in cold acetone. Fluorescent-labelled IBDV-specific antisera are applied to the sections, which are then incubated at 37°C for 1 hour in a humid atmosphere. At the end of the incubation period, they are washed for 30 minutes using phosphate-buffered saline (PBS), pH 7.2, then rinsed in distilled water. The sections are mounted using buffered glycerol, pH 7.6, and examined by UV microscopy for IBDV-specific fluorescence (Meulemans *et al*., 1977).

1.4. Identification by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA)

Since the first protocol was described by Snyder *et al.* (1988) for the detection of serotype 1 IBDV using an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA), many other assays have been developed (Eterradossi & Saif, 2013). Briefly, ELISA plates are coated with IBDV-specific antibodies. Depending on the chosen AC-ELISA protocol, the capture antibody may be a mouse anti-IBDV monoclonal antibody (MAb), or a mix of such MAbs, or a chicken post-infectious anti-IBDV polyclonal serum. It has been suggested that AC-ELISAs using polyclonal antibodies may have a higher sensitivity. Samples of bursal homogenates (see above) diluted 1/10 to 1/25 (w/v) in a suitable dilution buffer are incubated in the coated wells. Unbound antigens are discarded at the end of the incubation period by washing with a suitable washing buffer (e.g. PBS, pH 7.2 + 0.2% Tween 20). The captured antigens are then revealed, as in an indirect ELISA, with a detection antibody (which must have been developed from a different animal species than the capture antibody), followed by an enzyme conjugate that binds to the detection antibody only (in some protocols the detection antibody may be directly conjugated to the enzyme), followed by the enzyme substrate. Finally, optical densities, which parallel the amount of captured IBDV antigens, are read with an ELISA reader.

AC-ELISA is based on the use of samples possibly containing live virus and should be performed only in suitable containment facilities such as a class II safety cabinet. All liquid (washing buffers) and solid

1 For details see the list on line at: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

wastes should be considered to be contaminated by IBDV and decontaminated accordingly before disposal.

Critical steps in the implementation or assessment of AC-ELISA are i) the need to perform extensive washings between each step of the reaction to keep background reactions low, ii) the requirement for known positive and negative samples to be included in each assay as controls, and iii) the need for both the capture and detection antibodies to positively react with all serotype 1 IBDV strains (i.e. neither capture nor detection should critically depend on IBDV antigenic variation that occurs among serotype 1 strains).

1.5. Identification by molecular techniques

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation. The most frequently used molecular method is the detection of IBDV genome by the reverse-transcription polymerase chain reaction (RT-PCR) (Lin *et al*., 1993; Wu *et al*., 1992). This method can detect the genome of viruses that do not replicate in cell culture, because it is not necessary to grow the virus before amplification.

RT-PCR is performed in three steps: extraction of nucleic acids from the studied sample, reverse transcription (RT) of IBDV RNA into cDNA, and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotidic primers that are short sequences complementary to the virus-specific nucleotidic sequence. Different areas of the genome will be amplified depending on the location from which the primers have been selected. The example below allows the amplification of the middle third of the gene encoding the outer capsid protein VP2 (Eterradossi *et al*., 1998) or the partial amplification of the 5' extremity of the VP1 gene in IBDV segment B (Le Nouen *et al*., 2006).

1.5.1. Extraction of nucleic acids

Unlike single-stranded RNA, the IBDV double-stranded RNA (dsRNA) genome resists degradation by RNases. However, infected cells also contain IBDV-derived positive-sense single-stranded RNA species that can be used as a template at the RT step and may contribute to improving the sensitivity of the assay. It is thus important that RNA extraction be performed using gloves and RNase-free reagents and labware.

IBDV RNA can be extracted from infected tissues using some kits available from commercial suppliers of molecular biology reagents. Alternatively, IBDV RNA can be extracted by adding 1% (weight/volume final concentration) sodium dodecyl sulphate and 1 mg/ml proteinase K to 700 µl of virus suspension (e.g. bursal homogenate). Incubate for 60 minutes at 37°C. Nucleic acids are obtained using a standard protocol for phenol/chloroform extraction (caution: phenol is toxic and should be handled and disposed accordingly). Nucleic acids are harvested from the final aqueous phase by ethanol precipitation and are resuspended in RNase-free distilled water or a suitable buffer. Water-diluted RNA should be kept frozen at a temperature below –20°C until use.

1.5.2. Reverse transcription

A variety of reverse transcriptases are commercially available. Follow the supplier's instructions to prepare the RT reaction mix. Use the 'lower' PCR primer (complementary to the positive strand of IBDV genome, see below) for reverse transcription, as this allows the synthesis of cDNA both from the positive strand of IBDV dsRNA genome and from IBDV-derived positive-sense single-stranded RNAs previously contained in infected cells. Alternatively, random primers (hexanucleotides) can be used to prime cDNA synthesis.

The IBDV RNA matrix must be denaturated before transfer to the RT reaction mix. Add one part (by volume) molecular biology grade dimethylsulfoxide to four parts the unfrozen solution of IBDV RNA. Heat for 3 minutes at 92°C and chill on ice; an alternative method is to heat for 5 minutes and immediately incubate the mixture in liquid nitrogen. Transfer the relevant volume of denaturated matrix to the reaction mix. Incubate according to the instructions of the enzyme supplier.

The cDNA solution obtained after the RT step should be kept frozen at a temperature below –20°C. Delaying the PCR step for several weeks after the cDNA synthesis may cause false-negative PCR results.

1.5.3 Polymerase chain reaction

A variety of DNA polymerases suitable for PCR are commercially available. Follow the manufacturer's instructions to prepare the PCR reaction mix. Protocols for the amplification and molecular typing of IBDV have been reviewed recently (Wu *et al*., 2007). As an example, the U3/L3 and +290/–861 pairs of PCR primers shown below can be suggested and have been found useful for amplifying the middle third of the VP2 gene in segment A of serotype 1 IBDV strains (Eterradossi *et al*., 1998), and a region at the 5' extremity of IBDV segment B (Le Nouen *et al*., 2006), respectively. Both regions have been shown to be suitable for molecular epidemiology studies (Le Nouen *et al*., 2006), and the amplified region in segment B encompasses the B-marker subsequently confirmed to reliably represent the phylogenetic information derived from full B-segment (Alfonso-Morales *et al.,* 2015). Although a significant number of IBDV strains have two nucleotide changes at position 35 (G–A) and 38 (T–C) of the U3 primer (including isolates from Japan [OKYM], Hong Kong [HK46], UK [UK661], Nigeria [N4]), it has been shown that the U3-L3 primer pair successfully amplifies some of these viruses that exhibit both mutations. This is probably because the 3' extremity of U3 is highly conserved. However, as with most PCR assays, IBDV strains may exist with nucleotide changes at the annealing positions of the primers, thus requiring the use of other primers for optimised RT-PCR detection.

The combination of segment A- and segment B-targeted RT-PCR protocols enhances the probability that, if present, serotype 1 IBDV will indeed be detected; it also allows a thorough genetic characterisation of the IBDV strains detected.

Nucleotide sequence of the U3 and L3 IBDV-specific PCR primers (specific for Segment A, VP2 gene):

Upper U3: 5'-**TGT-AAA-ACG-ACG-GCC-AGT**-GCA-TGC-*GGT-ATG-TGA-GGC-TTG-GTG-AC*-3'

Lower L3: 5'-**CAG-GAA-ACA-GCT-ATG-ACC**-GAA-TTC-*GAT-CCT-GTT-GCC-ACT-CTT-TC*-3'

Nucleotide sequence of the +226 and –793 IBDV-specific PCR primers (specific for Segment B, VP1 gene):

Upper +290: 5'-**TGT-AAA-ACG-ACG-GCC-AGT-**GAA-TTC-*AGA-TTC-TGC-AGC-CAC-GGT-CTC-T*-3'

Lower -861: 5'-**CAG-GAA-ACA-GCT-ATG-ACC**-CTG-CAG-*TTG-ATG-ACT-TGA-GGT-TGA-TTT-TG*-3'

The U3 and L3 primers are both 44 nucleotides long, whereas primers +290 and –861 are 46 and 47 nucleotides long, respectively. The four primers include an IBDV-specific 3' extremity (in italics in the sequence shown above) corresponding to nucleotide positions 657–676 and 1193–1212 of IBDV segment A in primers U3 and L3, respectively (numbering as in segment A of strain P2, Acc No X84034), and to nucleotide positions 290–311 and 861–883 of IBDV segment B in primers +290 and –861, respectively (numbering as in segment B of strain D6948, Acc No AF240687). The IBDV-specific extremity is coupled to a non-IBDV 5' extremity (bold type in the sequence above) corresponding to the M13 and RM13 universal primers in the upper and lower primers, respectively. The M13 and RM13 universal primers are commonly used as primers in DNA sequencing reactions, so that purified PCR products resulting from amplification with the U3/L3 and +290/–861 primer pairs can be easily sequenced in both directions. Finally, restriction sites (underlined in the above sequence) are included for the following restriction endonucleases: *Sph*I (in primer U3), *Eco*RI (in primers L3 and +290), and *Pst* I (in primer –861). These restriction sites are positioned so that the PCR products resulting from amplification with the U3/L3 or +290/–861 pairs can be cloned if required. The U3/L3 pair generates a 604 base pair (bp) product, 516 bp of which are specific of the amplified IBDV sequence and encompass the region encoding the hyper-variable region of the VP2 protein. The +290/–861 pair generates a 642 bp product, 549 bp of which are specific of the amplified IBDV sequence. Both products are derived from genomic regions that are suitable for phylogenetic analysis (Eterradossi *et al*., 1998; Le Nouen *et al*., 2006).

Perform an initial denaturation step as recommended by the DNA polymerase supplier, followed by 35 cycles, each including one denaturation, one annealing and one elongation step. In such cycles, denaturation at 95°C for 30 seconds and annealing at 64°C for 45 seconds may be used with both the U3/L3 and +290/–861 primer pairs (the annealing temperature should be adapted if other primers are used). The parameters for the elongation step should be set according to the supplier's recommendations.

Revelation may be performed by electrophoresis with the PCR products and DNA molecular weight markers in a 1% agarose gel stained with ethidium bromide (caution: ethidium bromide is toxic and carcinogenic. It should be handled and disposed accordingly).

Three PCR reactions should be performed for each cDNA sample (pure, 10- and 100-fold diluted cDNA) to avoid false-negative results due to PCR inhibition in mixes containing high amounts of the cDNA preparation.

Each PCR should include negative and positive control reactions. Protocols that include an internal control to test for the presence of PCR inhibitors have been developed (Smiley *et al*., 1999).

Delaying the PCR for several weeks after the RT step may cause false-negative PCR results.

One step RT-PCR may also be used for IBD diagnosis with both conventional and real-time methods.

1.6. Isolation of virus in cell culture

Inoculate 0.5 ml of sample into each of four freshly confluent chicken embryo fibroblast (CEF) cultures (from a specific pathogen free [SPF] source) in 25 cm^2 flasks. Adsorb at 37°C for 30–60 minutes, wash twice with Earle's balanced salt solution and add maintenance medium to each flask. Incubate the cultures at 37°C, observing daily for evidence of cytopathic effect (CPE). This is characterised by small round refractive cells. If no CPE is observed after 6 days, discard the medium, then freeze–thaw the cultures and inoculate the resulting lysate into fresh cultures. This procedure may need to be repeated at least three times. If CPE is observed, the virus should be tested against monospecific IBDV antiserum in a tissue culture virus neutralisation (VN) test (see Section B.2.2 *Virus neutralisation tests*). The more pathogenic IBDV strains usually cannot be adapted to grow in CEF unless the virus has first been submitted to extensive serial passage in embryos (see below).

1.7. Isolation of virus in embryos

Inoculate 0.2 ml of sample into the yolk sac of five 6- to 8-day-old specific antibody negative (SAN) chicken embryos and on to the chorioallantoic membrane (American Association of Avian Pathologists, 2008) of five 9- to 11-day-old SAN chicken embryos. SAN embryos are derived from flocks shown to be serologically negative to IBDV. Candle daily and discard dead embryos up to 48 hours post-inoculation. Embryos that die after this time are examined for lesions. Serotype 1 IBD produces dwarfing of the embryo, subcutaneous oedema, congestion and subcutaneous or intracranial haemorrhages. The liver is usually swollen, with patchy congestion producing a mottled effect. In later deaths, the liver may be swollen and greenish, with areas of necrosis. The spleen is enlarged and the kidneys are swollen and congested, with a mottled effect. If lesions are observed, the virus should then be tested against a monospecific anti-IBDV serum in an embryo-revealed virus neutralisation assay.

Serotype 1 IBDV usually causes death in at least some of the embryos on primary isolation.

Serotype 2 IBDV does not induce subcutaneous oedema or haemorrhages in the infected embryos, but embryos are of a smaller size with a pale yellowish discolouration.

For the preparation of embryo-propagated stock virus or for subsequent passaging, embryos with lesions or embryos suspected to be infected, respectively, are harvested aseptically. Their head and limbs are discarded and the main body is minced as described in Section B.1.1 *Sample preparation* for the preparation a virus suspension.

1.8. Isolation of virus in chickens

This method has been used in the past but is no longer recommended due to animal welfare concerns. Five susceptible and five IBD-immune chickens (3–7 weeks of age) are inoculated by the eye-drop route with 0.05 ml of sample. Humanely euthanise the chickens 72–80 hours after inoculation, and examine their bursae of Fabricius. The bursae of chickens infected with virulent serotype 1 IBDV appear yellowish (sometimes haemorrhagic) and turgid, with prominent striations. Peribursal oedema is sometimes present, and plugs of caseous material are occasionally found. The plicae are petechiated.

The presence of lesions in the bursae of susceptible chickens along with the absence of lesions in immune chickens is diagnostic of IBD. The bursae from both groups may be used as antigen in an AGID test against known positive IBD antiserum (see Section B.1.2 *Identification by the agar gel immunodiffusion test*).

The extent of bursal damage may vary considerably with the pathogenicity of the studied IBDV strain. However, as the samples submitted for virus isolation may vary in virus content, the extent of bursal damage observed in susceptible chickens at the isolation stage gives only a limited indication on strain pathogenicity.

The bursae of chickens infected with serotype 2 IBDV do not exhibit any gross lesions.

1.9. Strain differentiation

IBDV strains can be further identified by testing their pathogenicity in SAN chickens, by investigating their antigenic reactivity in cross VN tests or using MAbs, by determining the nucleotide sequence of RT-PCR amplification products derived from IBDV genome, or by studying the number and size of the restriction fragments obtained following digestion of such RT-PCR products with restriction endonucleases. Several protocols have been described for each of these approaches. Tests should be performed by specialised laboratories and should include a panel of reference strains as controls. Although the molecular basis for antigenic variation is now better understood, no validated virulence marker has been described yet.

1.9.1. Pathogenicity testing

Studies to compare the pathogenicity of IBDV strains must be carried out in secure biocontainment facilities to avoid the dissemination of the studied virus (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). SAN birds with a known microbial status (ideally SPF chickens) must be used to avoid interference by contaminating agents.

The main variables when comparing the results of pathogenicity trials are the breed, age and immune status of the challenged chickens, the dose and route of inoculation of the challenge virus, and the possible presence of contaminating agents in the inoculum. Light layer breeds have been reported to be more susceptible than heavy broilers (Van den Berg & Meulemans, 1991). Differences in susceptibility may also occur between different SPF chicken lines. The highest susceptibility to acute IBD occurs in chickens between 3 and 6 weeks of age (Eterradossi & Saif, 2013). (The influence of the immune status is described in Section C.) A high dose of challenge virus, such as that recommended in Section C.1.3 *Live recombinant vector vaccines: methods of use,* is necessary so that all inoculated chickens become infected at once without requiring bird-to-bird transmission of the inoculated virus. Finally, the presence in the inoculum of contaminating agents, such as adenovirus or chicken infectious anaemia virus, may modify the severity of IBD and signs observed after challenge (Rosenberger *et al*., 1975).

The terms 'variant', 'classical' and 'very virulent' have been used to describe IBDV strains that exhibit differences in pathogenicity. Based on the signs and lesions observed in two lines of White Leghorn SPF chickens during acute experimental IBD following a 10^5 50% embryo infective dose (EID_{50}) challenge, North American 'variant' IBDVs induce little if any clinical signs and no mortality but marked bursal lesions, 'classical' IBDVs induce approximately 10–50% mortality with typical signs and lesions whereas 'very virulent' IBDVs induce approximately 50–100% mortality with typical signs and lesions (Eterradossi *et al.,* personal observation).

1.9.2 Antigenicity testing

Antigenic relatedness among IBDV strains may be assayed in cross VN tests, which correlate best with cross protection. Such tests have to be performed in SAN embryonated eggs when the studied viruses do not grow in CEF (e.g. vvIBDV). Differences in cross VN results among serotype 1 IBDV strains have led to the definition of serotype 1 'subtypes', some of which include the antigenically 'variant' North American IBDV isolates (Jackwood & Saif, 1987).

Another approach to the study of genetic relatedness is the use of mouse MAbs that bind to IBDV neutralising epitopes. Several panels of MAbs exist world-wide for use in AC-ELISA (Eterradossi *et al*., 1999; Snyder *et al*., 1992). Some of the MAbs have been included in commercially available kits, but no unified MAb panel as yet been proposed. All neutralising epitopes of IBDV characterised to date have been mapped into a major immunogenic domain in the middle third (amino acid positions 200 to 340) of the VP2 capsid protein (Eterradossi *et al*., 1998; Schnitzler *et al*., 1993; Vakharia *et al*., 1994). This region is termed 'VP2 variable domain' because most amino acid changes observed among IBDV strains are clustered in it. Within vVP2, four amino acid stretches are of critical importance to antigenicity and are referred to as vVP2 hydrophilic peaks. These are amino acid positions 210 to 225 (major peak A), 249 to 252 (minor peak 1), 281 to 292 (minor peak 2) and 313 to 324 (major peak B) (Van den Berg *et al*., 1996). According to the crystal structure of the VP2 protein and IBDV particles, the amino acid stretches previously known as "VP2 hydrophilic peaks" correspond to the most exposed amino acid loops in the projection domain of the VP2 protein (Coulibaly *et al.,* 2005). Both North American 'variants' and 'very virulent' IBDV exhibit in these areas amino acid changes that correlate with epitope variation (Eterradossi *et al*., 1998; Vakharia *et al*., 1994). To date, no antigenic marker has been shown to correlate strictly with IBDV pathogenicity.

1.9.3. Molecular identification

Most efforts at molecular identification have focused on the characterisation of the larger segment of IBDV (segment A) and especially of the vVP2 encoding region. Efforts were made initially to characterise RT-PCR products using restriction endonucleases (Lin *et al.*, 1993). These approaches are known as RT-PCR/RE or RT-PCR-RFLP (restriction fragment length polymorphism). The usefulness of the information they provide depends on the identification of enzymes that cut in restriction sites that are phenotypically relevant. Several RE or RFLP protocols resulted in defining a high number of profiles, which may prove confusing to use in molecular epidemiology studies and difficult to correlate with antigenicity or pathogenicity. Nucleotide sequencing of RT-PCR products provides an approach to assessing more precisely the genetic relatedness among IBDV strains. Using a reverse genetics approach, it was demonstrated that cell culture adaptation of IBDV strains critically depends on VP2 amino acid pairs 279 N–284 T or 253 H–284 T (Mundt, 1999). In most very virulent viruses, four typical amino acids are present (222 A, 256 I, 294 I and 299 S) (Brown *et al*., 1994; Lin *et al*., 1993). Several recent studies indicated that although VP2 is an important virulence determinant, segment B also appears to be important (Boot *et al.*, 2000; Escaffre *et al.*, 2013; Jackwood *et al*., 2011). It has been reported that segment A and B of IBDV mostly co-evolve (i.e. most significant IBDV clusters, such as vvIBDV-related strains, may be identified by analysis of both genome segments). However, some potentially reassortant viruses have been identified. The pathogenicity of putative reassortant IBDV is often modified, as compared with what would have been expected from the characterisation of their segment A alone (Le Nouen *et al.*, 2006; Jackwood *et al.*, 2011; Wei *et al.*, 2008). Molecular identification of IBDV isolates based on the sequencing of both genome segments is therefore highly recommended.

2. Serological tests

Blood samples should be taken early in the course of the disease, and repeat samples should be taken 3 weeks later. As the virus spreads rapidly, only a small proportion of the flock needs to be sampled. Usually 20 blood samples are enough.

2.1. Agar gel immunodiffusion test

The AGID test is the most simple of the serological tests for the detection of specific antibodies in serum.

2.1.1. Preparation of positive control antigen

Inoculate 3- to 5-week-old susceptible chickens, by eye-drop, with a clarified 10% (w/v) bursal homogenate known to contain viable IBDV [2]. Humanely euthanise the birds 3 days post-inoculation, and harvest the bursae aseptically. Discard haemorrhagic bursae and pool the remainder, weigh and add an equivalent volume of cold distilled water (or of a suitable buffer such as PBS or tryptose phosphate broth) and an equivalent volume of undiluted methylene chloride. (Caution: methylene chloride is toxic and possibly carcinogenic. It should be handled and disposed accordingly. A possible alternative to avoid health hazards caused by methylene chloride is to use trichlorotrifluoroethane, which is however an environmental hazard and should be handled and disposed accordingly). Thoroughly homogenise the mixture in a tissue blender and centrifuge at 2000 ***g*** for 30 minutes. Harvest the supernatant fluid and dispense into aliquots for storage at –40°C. The antigen contains live virus and should be handled only in suitable containment facilities such as a class II safety cabinet. If required, the antigen can be inactivated prior to dispensing: add 0.3% (v/v) ß-propiolactone to the harvested supernatant, then further incubate at 37°C for 2 hours. It is important that incubation takes place on an orbital shaker or a mechanical rocker, so that any inner part of the vial that has been in contact with live virus indeed gets into contact with ß-propiolactone. Dispense and store as above. Check the efficacy of the inactivation process by attempting IBDV isolation from the inactivated antigen, with three serial passages on SAN embryonated eggs (see Section B.1.7 *Isolation of virus in embryos*).

2.1.2. Preparation of positive control antiserum

Inoculate 4–5-week-old susceptible chickens, by eye-drop, with 0.05 ml of a clarified 10% (w/v) bursal homogenate known to contain viable IBDV (see footnote 2). Exsanguinate 28 days post-inoculation. Pool and store serum in aliquots at –20°C.

2.1.3. Preparation of agar

Dissolve sodium chloride (80 g) and phenol (5 g) in distilled water (1 litre) (caution: phenol is toxic and should be handled and disposed of accordingly). Add agar (12.5 g) and steam until the agar has dissolved. To avoid the health and environmental hazards caused by the use of phenol, another suitable recipe for the preparation of agar is as follows: sodium chloride (80 g), kalium dihydrogenophosphate (0.45 g), sodium hydrogenophosphate dihydrate (1.19 g), agar (10 g) and distilled water to a final volume of 1 litre (final pH 7.1 at 20–25°C). This second recipe can be homogenised by heating up to 90°C under agitation. While the mixture is still very hot, filter it through a pad of cellulose wadding covered with a few layers of muslin and dispense the medium in 20 ml volumes into glass bottles. The medium without phenol can further be sterilised by autoclaving at (at most) 115°C for 15 minutes. Store the bottles at 4°C until required for use.

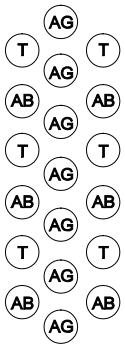
2.1.4. Test procedure

i) Prepare plates from 24 hours to 7 days before use. Dissolve the agar by placing in a steamer or boiling water bath. Take care to prevent water entering the bottles.

ii) Pour the contents of one bottle into each of the required number of 9 cm plastic Petri dishes laid on a level surface. (Some laboratories prefer to pour the gel on 25 × 75 mm glass slides, 3 mm deep.)

iii) Cover the plates and allow the agar to set, and then store the plates at 4°C. Poured plates may be stored for up to 7 days at 4°C. (If the plates are to be used the same day that they are poured, dry them by placing them opened but inverted at 37°C for from 30 minutes to 1 hour.)

2 A suitable classical strain of IBDV (serotype 1, classical pathotype) is strain 52/70, obtainable from one of the WOAH Reference Laboratories (https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).



AG = Positive antigen

AB = Positive antiserum

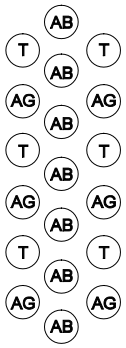


Fig. 1. Protocol for tests for antibody.

T = test sera

Fig. 2. Protocol for tests for antigen.

T = test tissues

Notes:

1. The linear pattern of wells is preferred although a hexagonal pattern may be used. Each test serum or test bursa (T in Figs 1 and 2 above) should be placed adjacent to a positive control antibody (AB) or antigen (AG), respectively.
2. Wells, 3 mm deep, 6 mm in diameter, and 3 mm apart (or wells of any other size previously shown to be effective), are used.

iv) Cut three vertical rows of wells 6 mm in diameter and 3 mm apart, using a template and tubular cutter.

v) Remove the agar from the wells by aspiration or remove using a pen and nib, taking care not to damage the walls of the wells.

vi) Using a pipette, dispense 50 µl of the test sera into the wells as shown in Figure 1.

Or, for the detection of IBDV antigens in bursae:

Dispense small pieces of finely minced test bursae by means of curved fine-pointed forceps into the wells, as shown in Figure 2, to just fill the wells. Alternatively, the freeze–thaw exudate of minced tissues can be used to fill the wells.

vii) Dispense 50 µl of the positive and negative control reagents into the relevant wells.

viii) Incubate the plates at between 22°C and 37°C for up to 48 hours in a humid chamber to avoid drying the agar.

ix) Examine the plates against a dark background with an oblique light source after 24 and 48 hours.

2.1.5. Quantitative agar gel immunodiffusion tests

The AGID test can also be used to measure antibody levels by using dilutions of serum in the test wells and taking the titre as the highest dilution to produce a precipitin line (Cullen & Wyeth, 1975). This can be useful for measuring maternal or vaccinal antibodies and for deciding on the best time for vaccination; however, this AGID quantitative determination has now been largely replaced by the ELISA.

2.2. Virus neutralisation tests

VN tests are carried out in cell culture. The test is more laborious and expensive than the AGID test, but is more sensitive for detecting antibody. This sensitivity is not required for routine diagnostic purposes, but may be useful for evaluating vaccine responses or for differentiating between IBDV 1 and 2 serotypes. The test uses either SPF chicken embryo fibroblast cells, or a suitable continuous cell line (such as QT-35, BGM-70, MA-104, Vero or DF1), in conjunction with an adapted strain of IBDV.

First, 0.05 ml of virus diluted in tissue culture medium to contain 100 $TCID_{50}$ (50% tissue culture infective doses) per 0.05 ml is placed in each well of a tissue-culture grade microtitre plate (See American Association of Avian Pathologists, 2008, for virus titration methods). The test sera are heat-inactivated at 56°C for 30 minutes. Serial doubling dilutions of the sera are made in the diluted virus. After 30 minutes

at room temperature, 0.2 ml of cell suspension, with a cell density allowing confluent layers to be obtained after 24 hours of incubation, is dispensed into each well. Plates are sealed and incubated at 37°C for 4–5 days, after which the monolayers are observed microscopically for typical CPE. The end-point (serum titre) is expressed as the reciprocal of the highest serum dilution that did not show CPE. To reduce test-to-test and operator-to-operator variation, a standard reference antiserum may be included with each batch of tests [3] and the titre of the virus suspension must be reassessed in each new experiment using a sufficient number of repeats (wells) per virus dilution.

2.3. Enzyme-linked immunosorbent assay

ELISAs are in use for the detection of antibodies to IBD. Coating the plates requires a purified, or at least semipurified, preparation of virus, necessitating special skills and techniques. Methods for preparation of reagents and application of the assay were described by Marquardt *et al.* (1980). Commercial kits are available.

The test sera are diluted according to the established protocol or kit instructions and each is dispensed into the requisite number of wells. After incubation under the appropriate conditions, the sera are discarded from the plates, and the wells are washed thoroughly. Anti-chicken immunoglobulins conjugated to an enzyme are dispensed into the wells, and the plates are again incubated as appropriate. The plates are emptied and rewashed before substrate containing a chromogen that gives a colour change in the presence of the enzyme used is added to the plate. After a final incubation step, the substrate/chromogen reaction is stopped by addition of a suitable stopping solution and the colour reactions are quantified by measuring the optical density of each well. The Sample to Positive (S/P) ratio for each test sample is calculated.

2.4. Interpretation of results

The AGID test is surprisingly sensitive, though not as sensitive as the VN test; the latter will often give a titre when the AGID test is negative. Positive reactions indicate infection in unvaccinated birds without maternal antibodies. As a guide, a positive AGID reaction in a vaccinated bird or young bird with maternal antibody indicates a protective level of antibody. ELISA gives more rapid results than VN or AGID and is less costly in terms of labour, although the reagents are more expensive. VN and AGID titres correlate well, but as VN is more sensitive, AGID titres are proportionally lower. Correlation between ELISA and VN and between ELISA and AGID is more variable depending on the source of the ELISA reagents, however it should be kept in mind that both VN and ELISAs are highly sensitive and subject to both intra- and inter-laboratory variations. It is therefore highly advisable that a positive sentinel serum with a known titre be introduced in every test in laboratories that perform IBDV ELISA or VN routinely (De Wit *et al.*, 2007; Kreider *et al.*, 1991). When testing for the decay of maternally derived antibodies (MDA), it is not uncommon to find residual VN antibodies at an age when ELISA results are already negative. Formulae have been devised that allow ELISA titres to be used to calculate the optimal age for vaccination, which will vary depending on the vaccine used (Block *et al.*, 2007). Nonspecific positive reactions may occur with most ELISAs because they are usually designed for monitoring vaccine responses, in which case sensitivity is regarded as more important than specificity. This should be taken into account when the ELISA is used for diagnosis. In commercial chicken flocks or experimentally infected chickens, a serotype 1 ELISA antigen also detects antibodies induced by serotype 2 IBDV (Ashraf *et al.*, 2006), however this cross reactivity has not yet been demonstrated to interfere with serological monitoring programmes of IBD based on the ELISA.

3 A suitable reference antiserum may be obtained from the WOAH Reference Laboratories (https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

C. REQUIREMENTS FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principle of veterinary vaccine production*. The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

IBDV vaccines have been reviewed recently (Muller *et al*., 2012). Four major types of vaccines are available for the control of IBD, these are: i) live attenuated vaccines; ii) immune-complex vaccines; iii) live recombinant vectored vaccines expressing IBDV antigens; and iv) inactivated oil-emulsion adjuvanted vaccines.

To date, IBD vaccines have been made with serotype 1 IBDV only, although a serotype 2 virus has been detected in poultry. The serotype 2 virus has not been associated with disease, but its presence will stimulate antibodies. Serotype 2 antibodies do not confer protection against serotype 1 infection, neither do they interfere with the response to type 1 vaccine. There have been numerous descriptions of antigenic variants of serotype 1 virus (Rosenberger & Cloud, 1986). Cross-protection studies have shown that inactivated vaccines prepared from 'classical' serotype 1 virus require a high antigenic content to provide good protection against some of these variants. IBD vaccines that contain both classical and variant IBD serotype 1 viruses have been authorised. vvIBDV strains with limited antigenic changes as compared with 'classical' serotype 1 viruses have emerged since 1986. Active immunisation with a 'classical' serotype 1 virus or vaccine provides a good protection against the vvIBDVs, however the latter viruses are less susceptible to neutralisation by MDA than 'classical' pathogenic viruses (Van den Berg & Meulemans, 1991).

1.1. Live vaccines: methods of use

Live IBD vaccines are produced from fully or partially attenuated strains of virus, known as 'mild', 'intermediate', or 'intermediate plus' ('hot'), respectively.

Mild or intermediate vaccines are used in parent chickens to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine. They are susceptible to the effect of MDA so should be administered only after all MDA has waned. Application is by means of intramuscular injection, spray or in the drinking water, usually at 8 weeks of age (Skeeles *et al*., 1979).

Intermediate or intermediate plus vaccines are used to elicit protection in broiler chickens and commercial layer replacements. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with virulent IBD. Although intermediate vaccines are susceptible to the presence of MDA, they are sometimes administered at 1-day old, as a coarse spray, to protect any chickens in the flock that may have no or only minimal levels of MDA. This also establishes a reservoir of vaccine virus within the flock that allows lateral transmission to other chickens when their MDA decay. Second and third applications are usually administered, especially when there is a high risk of exposure to virulent forms of the disease or when the vaccinated chicks exhibit uneven MDA levels. The timing of additional applications will depend on the antibody titres of the parent birds at the time the eggs were laid. As a guide, the second dose is usually given at 10–14 days of age when about 10% of the flock is susceptible to IBD, and the third dose 7–10 days later. The route of administration is by means of spray or in the drinking water. Intramuscular injection or eye-drop is used rarely. If the vaccine is given in the drinking water, clean water with a neutral pH must be used that is free from odour or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per litre. Care must be taken to ensure that all birds receive their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the vaccine is made available and care must be taken that no residual water remains in the pipes or in the drinkers. It is possible to divide the medicated water into two parts, giving the second part 30 minutes after the first.

Live IBD vaccines are generally regarded as compatible with other avian vaccines. However, it is possible that live IBD vaccines that cause bursal damage could interfere with the response to other vaccines. Only healthy birds should be vaccinated. The vials of vaccine should be kept at temperatures between 2°C and 8°C up to the time of use.

1.2. Immune complex vaccines: methods of use

To make an immune complex IBD vaccines a live infectious IBDV vaccine virus is blended with IBDV-specific antibodies. Such vaccines may be administered in the hatchery by *in-ovo* injection at 18 days of incubation. The eggs go on to hatch and the vaccine virus is supposedly released when the chicks are about 7–14 days of age. In this way, the problem of maternally derived IBD antibody is overcome and the chicks are effectively immunised (Haddad *et al*., 1997). The immune complex vaccine can also be injected subcutaneously at 1-day old in the hatchery (Ivan *et al*., 2005).

1.3. Live recombinant vector vaccines: methods of use

Live recombinant vaccines that use a viral vector (herpes virus of turkeys) to express the VP2 antigen of IBDV in chickens have been developed for *in-ovo* or day-old use and are currently authorised in many countries worldwide. Activity in the face of maternally derived IBD antibody, and compatibility with other Marek's disease vaccines have been documented (Le Gros *et al*., 2009, Lemiere *et al*., 2011). The anti-IBDV antibody response elicited by live recombinant IBDV vaccines expressing the VP2 protein will contain antibodies directed against VP2 only (as opposed to antibodies against all IBDV proteins, primarily VP2 and VP3, following infection by live IBDV). While neutralising antibodies against the VP2 protein will be readily detected in the standard VN test, detection of a VP2-specific antibody response in ELISA may require specific kits with an extended sensitivity. Antibodies against VP3 being absent in birds receiving the live recombinant IBDV vaccine, but present in birds infected with live IBDV, the combined use of ELISAs specific for anti-VP2 or anti-VP3 antibodies would allow implementation of a DIVA (detection of infection in vaccinated animals) strategy in birds vaccinated with such recombinant vaccines (Muller *et al*., 2012).

1.4 Inactivated vaccines: method of use

Inactivated IBD vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeding hens that have previously been primed by live vaccine or by natural exposure to field virus during rearing (Muller *et al*., 2012). The usual programme is to administer the live vaccine at about 8 weeks of age. This is followed by the inactivated vaccine at 16–20 weeks of age. Occasionally, inactivated vaccines may be used in programmes combining inactivated and live vaccines, in young valuable birds with high MDA levels reared in areas with high risk of exposure to virulent IBDV. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular into the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous route. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. Vaccine should be stored at between 2°C and 8°C. It should not be frozen or exposed to bright light or high temperature.

Only healthy birds, known to be sensitised by previous exposure to IBDV, should be vaccinated. Used in this way the vaccine should produce such a good antibody response that chickens hatched from those parents will have passive protection against IBD for up to about 30 days of age (Wyeth & Cullen, 1979). This covers the period of greatest susceptibility to the disease and prevents bursal damage at the time when this could cause immunosuppression. It has been shown that bursal damage occurring after about 15 days of age has little effect on immunocompetence as by that time the immunocompetent cells have migrated into the peripheral lymphoid tissues. However, if there is a threat of exposure to infection with very virulent IBDV, live vaccines should be applied as described above. The precise level and duration of immunity conferred by inactivated IBD vaccines will depend mainly on the concentration of antigen present per dose. The manufacturing objective should be to obtain a high antigen concentration and hence a highly potent vaccine.

Subunit vaccines, in which the inactivated whole IBDV antigen used in the inactivated vaccines is replaced by recombinant VP2 expressed either in the baculovirus system, or in *Escherichia coli*, or in the yeast *Pichia pastoris* (Pitcovski *et al.,* 2003), have been described. Similar to inactivated vaccines, they also require to be injected and result in a better immunisation when i) their antigen content is high and ii) they are administered as a booster in birds previously primed with a live vaccine (Muller *et al*., 2012).

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

See also Chapter 1.1.8 *Principles of veterinary vaccine production* and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

2.1.1. Biological characteristics of the master seed

i) Live vaccines

Virus strains used in live IBD vaccines are sometimes referred to as "mild", "intermediate" and "intermediate plus"/"invasive"/"hot" depending on their ability to replicate in the face of increasing amounts of residual maternally derived anti-IBDV antibodies. Consistently with the increasing replication ability of the least attenuated vaccine strains, these strains usually induce more severe vaccine-induced bursal lesions (microscopic lesions and reduced size) and may exhibit some levels of residual immunosuppressive properties (see Section C.2.1.3 *Validation as a vaccine strain*).

ii) Inactivated vaccines

Subtypes have been reported among serotype 1 IBDV, and it has been demonstrated that protection against a given subtype using an inactivated vaccine requires either an homologous antigen or a high antigenic content. As a result, information relating to the subtype of the strain used as an antigen in the inactivated vaccine may prove helpful.

2.1.2 Quality criteria

i) Purity

The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens. This includes freedom from contamination with other strains of IBDV.

ii) Lack of reversion to virulence of live vaccines

For vaccine strains that claim to be attenuated and with limited immunosuppressive properties, the seed virus must be shown to be stable, with no tendency to revert to virulence. This can be confirmed by carrying out sequential passage through five groups of SPF chickens, at 3- to 4-day intervals using bursal suspension as inoculum, in SPF chickens of the minimum age recommended for vaccination. It must be shown that the virus was transmitted: if the passage virus was not found at a passage level, the passage should be repeated by administration to a group of 10 chickens. A histological comparison is made to show that there is no difference between bursae from birds inoculated with the initial and the final passage material. Bursal scoring (Muskett *et al.,* 1979) and imaging techniques have been developed.

2.1.3. Validation as a vaccine strain

i) Live vaccine

Validation of an IBDV strain as a live vaccine requires the evaluation of its innocuity, immunosuppressive potential, lack of reverting potential and immunogenicity.

Innocuity may be tested in a number of ways. Some countries recommend vaccinating SPF chickens of the youngest recommended age for vaccination using a high dose (usually tenfold) of the vaccine at its least attenuated passage level, then checking the lack of signs and usually moderate and transient bursal lesions after this vaccination. There is no report documenting the innocuity of IBDV vaccines in non-target species.

The immunosuppressive potential is an important characteristic to assess, indeed the vaccine virus should not produce damage to the bursa of Fabricius such that it causes immunosuppression in susceptible birds. Live vaccines of the 'intermediate' or 'intermediate plus' type may be authorised even though they may be capable of causing immunosuppression. A possible protocol for the experimental assessment of

immunosuppression is the following: the IBD vaccine is administered by injection or eye-drop, one field dose per bird, to each of 10 SPF chickens, at 1-day old. Two further groups of 10 birds of the same age and source are housed separately as controls. At 2 weeks of age, each bird in both the IBDV-vaccinated group and in one of the control groups is given one field dose of live ND vaccine by eye-drop. Alternatively, the IBDV vaccine may be administered at the minimum age recommended for vaccination, and the ND vaccine at the time when bursal lesions induced by the IBDV vaccine are maximal. The haemagglutination inhibition (HI) response of each bird to ND vaccine is measured 2 weeks after the administration of the ND vaccine, and the protection is measured against challenge with $10^{5.0}$ to $10^{6.5}$ ELD_{50} (50% embryo lethal doses) Herts 33/56 strain (or similar) of ND virus (NDV) (the second control group, that was kept without IBDV- or NDV-vaccine, is used at this stage to validate the severity of the NDV challenge). The IBD vaccine fails the test if the HI response and protection afforded by ND vaccine is significantly less in the group given IBD vaccine than in the control group. In countries where NDV is exotic, an alternative is to use sheep erythrocytes or Brucella abortus-killed antigen as the test antigen, measuring the response using the haemagglutination or serum agglutination test, respectively. However, another live vaccine is a preferable test system because it also evaluates cell-mediated immunity.

Lack of reverting potential of the vaccine strain can be evaluated as described (see Section C.2.1.2.ii *Lack of reversion to virulence of live vaccines*).

a) Immunogenicity

The vaccine should be administered to birds in the way in which it will be used in the field. Live vaccine can be given to young birds and the response measured serologically and by resistance to experimental challenge: administer one vaccine dose of the minimum recommended titre to each of 20 SPF chickens of the minimum age of vaccination. Inoculate separate groups for each of the recommended routes of application. Leave 20 chickens from the same hatch as uninoculated controls. After 14 days, challenge each of the chickens by eye-drop with approximately 100 CID_{50} (50% chicken infective dose) of a virulent strain of IBDV as recommended by one of the WOAH Reference Laboratories for IBD[4]. Observe the chickens daily for 10 days. Register the number of birds that die or exhibit IBD signs. Perform a histological examination of the bursa in chickens that survive at day 10. The vaccine fails the test unless at least 90% of the vaccinated chickens survive without showing either clinical signs or severe lesions in the bursae of Fabricius at the end of the observation period. If more than half the controls do not show IBD signs, or one or more control chicken does not exhibit severe lesions of the bursa of Fabricius, or control or inoculated birds die from causes not attributable to the test, the test is invalid. Lesions are considered to be severe if at least 90% of follicles show greater than 75% depletion of lymphocytes, or if at least 51% of the bursal follicles exhibit a histopathological score of 3 or more according to the European Pharmacopoeia (2014).

ii) Inactivated vaccine

Validation of an IBDV inactivated vaccine requires the evaluation of its innocuity and immunogenicity.

Safety of the inactivated vaccine should be tested for all recommended administration routes and with a batch of vaccine whose activity is at least the maximal activity of future commercial batches. One dose, or a double dose to ensure maximal activity, of vaccine is administered to SAN or SPF chickens. Clinical signs in vaccinated chickens are checked daily and for 14 days. The vaccine passes the test if no signs are observed and no death can be attributed to the vaccine. The test is invalid if nonspecific death occurs.

Efficacy of IBD inactivated vaccines should be evaluated in older birds that go on to lay, using the recommended vaccination schedule, so that their progeny can be challenged to determine resistance due to MDA at the beginning and end of lay.

At least 20 unprimed SPF birds are given one dose of vaccine at the recommended age (near to point-of-lay) and by at least one of the recommended routes; an alternative

4 See footnote 1.

recommended procedure is to test one dose of vaccine in the recommended routes listed on the label, using 20 unprimed SPF birds for each route. The antibody response is measured between 4 and 6 weeks after vaccination by serum neutralisation with reference to a standard antiserum[5].

Eggs are collected for hatching 5–7 weeks after vaccination, and 25 progeny chickens are then challenged at 3 weeks of age by eye-drop with approximately 100 CID_{50} of a recognised virulent strain of IBDV. Ten control chickens of the same breed but from unvaccinated parents are also challenged. Protection is assessed 3–4 days after challenge by removing the bursa of Fabricius from each bird; each bursa is then subjected to histological examination or tested for the presence of IBD antigen by the agar gel precipitin test. Not more than three of the chickens from vaccinated parents should show evidence of IBD infection, whereas all those from unvaccinated parents should be affected.

These procedures may be repeated towards the end of the period of lay when the vaccinated birds are at least 60 weeks of age, but, on this occasion challenge of the progeny should be undertaken when they are 15 days old.

If the inactivated vaccine is intended to be used as a booster after a priming vaccination, the efficacy test should be repeated on primed birds vaccinated by the recommended schedule. The final dose of killed vaccine is given at the earliest recommended age. Chickens hatched from fertile eggs collected at the beginning and the end of lay are tested for protection against challenge as described above.

2.2. Methods of manufacture

2.2.1. Procedure

Seed virus may be propagated in various culture systems, such as SPF chicken embryo fibroblasts, or chicken embryos. In some cases, propagation in the bursa may be used. The bulk is distributed in aliquots and freeze-dried in sealed containers. There have been claims that bursal origin vaccines are better immunogens than tissue culture vaccines. In controlled studies, it was concluded that both types of virus, when included with a similar antigenic mass in inactivated vaccines, elicited similar immune responses; standardisation of the antigenic mass in inactivated vaccines would therefore appear to be desirable (Maas *et al.*, 2004).

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry.

Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. Live vaccines are made by growth in eggs or cell cultures. Inactivated IBD vaccines may be made using virulent virus grown in the bursae of young birds, or using attenuated, laboratory-adapted strains of IBDV grown in cell culture or embryonated eggs. A high virus concentration is required. Inactivated vaccines can be prepared as different types of emulsions. A typical water-in-oil formulation is to use 80% mineral oil to 20% suspension of bursal material in water, with suitable emulsifying agents, however vaccines prepared as double or micro-emulsions also exist.

2.2.2 Requirements for ingredients

i) Ingredients of animal origin

All ingredients of animal origin, including serum and cells, must be checked for the presence of viable bacteria, viruses, fungi or mycoplasma. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

SPF eggs must be used for all materials employed in propagation and testing of the vaccine.

5 See footnote 1.

ii) Preservatives

A preservative may be required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its efficacy until the end of the shelf life should be checked. A suitable preservative already established for such purposes should be used.

2.2.3. In-process control

i) Antigen content

Having grown the virus to high concentration, its titre should be assayed by use of cell cultures, embryos or chickens as appropriate to the strain of virus being used. The antigen content required to produce satisfactory batches of vaccine should be based on determinations made on test vaccine that has been shown to be safe and effective in laboratory and field trials.

ii) Inactivation of inactivated vaccines

This is often done with either ß-propiolactone or formalin. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine virus and any potential contaminants, e.g. bacteria, that may arise from the starting materials.

Prior to inactivation, care should be taken to ensure a homogeneous suspension is free from particles that may not be penetrated by the inactivating agent. A test for inactivation of the vaccine should be carried out on each batch of both the bulk harvest after inactivation and the final product. An alternative approach is to test inactivation of the final or bulk harvest, but not both. The test selected should be appropriate to the vaccine virus being used and should consist of at least two passages in susceptible cell cultures, embryos or chickens, with ten replicates per passage. No evidence of the presence of any live virus or microorganism should be observed.

iii) Sterility of inactivated vaccines

Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the European Pharmacopoeia (2014) or in title 9, *Code of Federal Regulations* (*9-CFR*), *part 113.26*.

2.2.4. Final product batch test

i) Identity

The identity of a live IBD vaccine can be confirmed at batch level by incubating an appropriate dilution of the vaccine with a monospecific anti-IBDV antiserum neutralising serotype 1 IBDV, then inoculating the mix to susceptible SAN or SPF eggs or susceptible cell cultures. The neutralised vaccine should not exhibit any infectivity.

The identity of inactivated IBD vaccine can be confirmed at batch level by administrating the vaccine to SAN or SPF chickens, and demonstrating that the vaccine does induce antibodies that neutralise serotype 1 IBDV. In some instances, this test can be combined with the potency test in order to reduce the number of animals used in the experiments.

ii) Sterility and absence of extraneous agents

Tests for sterility and freedom from contamination of biological materials by bacteria, fungi, mycoplasma and extraneous agents are described in Chapter 2.3.4 *Minimum requirements for the production and quality control of vaccines*.

iii) Safety

a) Live vaccine safety test

Ten field doses of vaccine are administered by eye-drop to each of 15 SPF chickens of the minimum age recommended for vaccination and not older than 2 weeks. The chickens are observed for 21 days. If more than two chickens die due to causes not related to the vaccine, the test must be repeated. The vaccine fails the test if any chickens die or show signs of

disease attributable to the vaccine. This test is performed on each batch of final vaccine, unless controls at earlier production stages complemented by implementation of GMP advocate for the safety of the overall process. Alternative safety tests may be used as described in *9-CFR* 113.212(d)(1) and 113.331(d)(2).

b) Extraneous agents in inactivated vaccines

Ten to 21 SPF birds, 14–28 days of age, are inoculated by the recommended routes with the recommended dose or twice the field dose. The birds are observed for 3 weeks. No abnormal local or systemic reaction should develop. No antibodies against any avian pathogen but the vaccine antigen should develop. The test is performed on each batch of final vaccine, unless controls at earlier production stages complemented by implementation of good manufacturing practices advocate for the safety of the overall process.

iv) Residual live vaccine in inactivated vaccines

The process described in Section C.2.2.3 *In process controls* is may be performed on each batch of final product.

v) Potency

a) Live vaccine potency test

A potency test (virus titration) in eggs or cell cultures must be carried out on each serial (batch) of vaccine produced.

In addition, the method described in Section C.2.1.3.i.a *Immunogenicity* must be used and yield satisfactory results on one batch representative of all the batches prepared from the same seed lot.

b) Inactivated vaccine potency test

Ten SPF chickens, approximately 4 weeks of age, are each vaccinated with one dose of vaccine given by the recommended route. An additional ten control birds of the same source and age are housed together with the vaccinates. The antibody response of each bird is determined 4–6 weeks after vaccination in a VN test with reference to a standard antiserum. The mean antibody level of the vaccinated birds should not be significantly less than the level recorded in the test for protection (see Section C.2.1.3.ii.a *Immunogenicity*). No antibody should be detected in the control birds. This test must be carried out on each batch of final vaccine. Alternatively a vaccination-challenge potency test may be used (*9-CFR* 113.212(d)(2)).

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning the manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and C.2.2 *Methods of manufacture*) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Live attenuated IBD vaccines with the highest replication ability and the potential to induce lymphoid depletion in the bursa are usually authorised for use in animals with high titres of maternally derived anti-IBDV antibodies and in premises characterised by a high infectious pressure of highly pathogenic viruses. This information should be indicated when relevant in the instructions for use of the vaccine.

No interaction of live IBD vaccines with non-target avian species has been documented so far. Any information regarding a negative effect in a non-target animal species should be provided in the vaccine instructions for use.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

It is critical that the potential of live attenuated IBD vaccines to revert to virulence is assessed prior to regulatory approval (See Section C.2.1.2.ii above).

Environmental considerations to be taken into account in the regulatory approval process include the knowledge of the IBDV strains that circulate in the area where the approved vaccine will be used, as this knowledge may help i) in selecting the vaccines suitable to control these strains and ii) in deciding whether it is justified or not to introduce a live attenuated IBDV vaccine strain possibly significantly different from the local IBDV strains.

iii) Precautions (hazards)

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident, the person should go at once to a hospital, taking the vaccine package with them. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injury. Such wounds should be treated by the casualty doctor as a 'grease gun injury'.

2.3.3. Efficacy requirements

The tests, challenge models and criteria used to assess the efficacy of IBD vaccines are described under Sections C.2.1.3.i *Live vaccine* and C.2.1.3.ii *Inactivated vaccine*. When assessing efficacy in an IBDV challenge model, it is advisable that the selected challenge virus be representative of contemporary IBDV strains that circulate in the area where the authorised vaccine will be used.

2.3.4. Vaccines permitting a DIVA strategy

Among the currently commercially available vaccines, live recombinant vectored vaccines expressing the VP2 protein of IBDV, and subunit vaccines containing the VP2 protein as the sole IBDV antigen, have the potential to be used in a DIVA strategy. Indeed, chickens vaccinated with such vaccines will develop anti-VP2 antibodies only, whereas birds infected by IBDV will present a broader antibody response directed at all IBDV antigens, including the VP3 protein (IBDV ribonucleoprotein). Based on the presence of anti-VP2 antibodies only, or of both anti-VP2 and anti-VP3 antibodies, it would therefore be theoretically possible to differentiate the birds that received only such vaccines, from the infected birds. However, implementation of the DIVA strategy would require ELISAs allowing the differential study of these two types of antibody responses. Although commercial ELISAs may exhibit different sensitivity to these different types of antibodies, the validation of the commercial assays for such a purpose has not been reported in the scientific literature.

2.3.5 Duration of immunity

As explained above (see Section C.2.1.3.ii *Inactivated vaccine*), repeating the evaluation of the efficacy of inactivated vaccines in breeder birds, both early after point of lay and later-on at the end of the laying period, may help in assessing whether the prolonged protection of progeny requires the implementation of a booster vaccination during the laying period.

2.3.6. Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at the requested shelf life or as an alternative at 3 months beyond.

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NB: There are WOAH Reference Laboratories for Infectious bursal disease (Gumboro disease) (see WOAH Web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for infectious bursal disease (Gumboro disease)

NB: FIRST ADOPTED IN 1990; MOST RECENT UPDATES ADOPTED IN 2016.

CHAPTER 3.3.13.

MAREK'S DISEASE

SUMMARY

Description and importance of disease: *Marek's disease* (*MD*) *is a lymphomatous and neuropathic disease of gallinaceous birds caused by an alphaherpesvirus, designated Marek's disease virus* (*MDV*).

Diagnosis is made on clinical signs and gross or microscopic lesions. Definitive diagnosis must be made by diagnosing the disease (*tumour*)*, not the infection. Chickens may become persistently infected with MDV without developing clinical disease.*

In chickens, classical MD can occur at any time, beginning at 3–4 weeks of age or older, sometimes even well after the onset of egg production. Clinical signs observed are paralysis of the legs and wings, with enlargement of peripheral nerves, although nerve involvement is sometimes not seen, especially in adult birds. MDV strains of higher virulence may also cause increased mortality in younger birds of 1–2 weeks of age, especially if they lack maternal antibodies. Depending on the strain of MDV, lymphomatous lesions can occur in multiple organs such as the gonads, liver, spleen, kidneys, lungs, heart, proventriculus and skin. Tumours produced by MDV may also resemble those induced by retroviral pathogens such as avian leukosis virus and reticuloendotheliosis virus and their differentiation is important. Compared with the uniform cell populations observed in lymphoid leukosis, MD lymphomas consist of pleomorphic lymphoid cells of various types.

Detection and identification of the agent: *Infection by MDV is detected by virus isolation and the demonstration of viral nucleic acid, antigen or antibodies. Under field conditions, most chickens become infected with MDV during the first few weeks of life and then carry the infection throughout their lives, often without developing overt disease. Virus isolation is performed by co-cultivating live buffy coat cells on monolayer cultures of chicken kidney cells or chicken/duck embryo fibroblasts, in which characteristic viral plaques develop within a few days. MDV belongs to the genus* Mardivirus *that includes three species* (*serotypes*) *designated as Gallid alphaherpesvirus 2* (*serotype 1*), *Gallid alphaherpesvirus 3* (*serotype 2*) and *Meleagrid alphaherpesvirus 1 or herpesvirus of turkeys* (*HVT*) (*serotype 3*). *Serotype 1 includes all the virulent strains and some attenuated vaccine strains. Serotype 2 includes the naturally avirulent strains, some of which are used as vaccines. Antigenically related HVT is also used as vaccine against MD, and, more recently, as a recombinant viral vaccine vector. MDV genomic DNA and viral antigens can be detected in the feather tips of infected birds using polymerase chain reaction* (*PCR*) *and the radial immunoprecipitation test, respectively. These molecular diagnostic tests can be used for differentiating pathogenic and vaccine strains.*

Serological tests: *Antibodies to MDV develop within 1–2 weeks of infection and are commonly recognised by the agar gel immunodiffusion test, or the indirect fluorescent antibody test.*

Requirements for vaccines: *MD is prevented by vaccinating chickens* in ovo *at 18–19 days of incubation, or at day of hatch. Attenuated variants of serotype 1 strains of MDV are the most effective vaccines. Serotype 2 strains may also be used, particularly in bivalent vaccines, together with HVT. Serotype 1 and 2 vaccines are only available in the cell-associated form. Live HVT vaccines are widely used and are available both as cell-free* (*lyophilised*) *and cell-associated* ('*wet*') *forms. Bivalent vaccines consisting of serotypes 1 and 3 or trivalent vaccines consisting of serotypes 1, 2, and 3 are also used. The bivalent and trivalent vaccines have been introduced to combat the very virulent strains of MDV that are not well controlled by the monovalent vaccines.*

Vaccination greatly reduces clinical disease, but does not prevent persistent infection and shedding of MDV. The vaccine viruses may also be carried throughout the life of the fowl.

A. INTRODUCTION

1. Description and impact of the disease

Marek's disease (MD) (Davison & Nair, 2004; Nair *et al.,* 2020) is a lymphoproliferative disease of gallinaceous birds caused by Marek's disease virus (MDV). Birds are infected by inhalation of contaminated dust from the poultry houses, and, following complex pathogenic pathways, the virus is shed from the feather follicle of infected birds. MD can occur at any time, beginning at 3–4 weeks of age or older, sometimes even well after the onset of egg production. During the early phases of the disease, atrophy of the bursa of Fabricius and the thymus are detected which often remains unnoticed. MD is associated with several distinct clinicopathological syndromes. In the classical neurological form of the disease, characterised mainly by the involvement of nerves, mortality rarely exceeds 10–15% and can occur over a few weeks or many months. In the lymphoproliferative form, which is usually characterised by visceral lymphomas in multiple organs, disease incidence of 10–30% in the flock is not uncommon and outbreaks involving up to 70% incidence can occur. Mortality may increase rapidly over a few weeks and then cease, or can continue at a steady or slowly falling rate for several months. In the lymphoproliferative form, birds are dull, lose condition and show signs of immunosuppression. Another syndrome is characterised by vasogenic oedema of the brain resulting in transient paralysis is increasingly recognised with MD induced by the more virulent strains of the virus.

In its classical neurological form, the most common clinical sign of MD is partial or complete paralysis of the legs and wings. The characteristic finding is enlargement of one or more peripheral nerves. Those most commonly affected and easily seen at post-mortem examination are the sciatic, brachial and sometimes vagal nerves. However, other nerves can also be affected. Affected nerves are often two or three times their normal thickness, the normal cross-striated and glistening appearance is absent, and the nerve may appear greyish or yellowish, and sometimes oedematous. Microscopic examination of the nerves reveals lymphoid infiltration which can vary from mild inflammatory lesions to marked lymphomatous infiltration. In certain neurological forms, similar lesions can be detected in the brain and the eye resulting in typical clinical signs. Tumours typical of other forms of MD are sometimes present in this form of MD, most frequently as small, soft, grey tumours in the gonads, liver, kidney, heart and other tissues.

In the lymphoproliferative form, the typical finding is a multicentric lymphoma with involvement of the liver, gonads, spleen, kidneys, lungs, proventriculus and heart. Grossly, the lymphomatous lesions can appear as distinct white masses or as marked ill-defined enlargement of the affected organ, mostly the liver, spleen and proventriculus. Histopathology reveals typical lymphoid proliferation. Lymphomas can also arise in the skin around the feather follicles and in the skeletal muscles. The eye can be similarly affected usually presenting with lymphomatous infiltration of the iris. Upon microscopic examination, lymphoid infiltrations can also be detected in other parts of the eye.

2. Nature and classification of the pathogen

MDV is a highly cell-associated alphaherpesvirus of the genus *Mardivirus*. The genus includes three species (serotypes) designated as Gallid alphaherpesvirus 2 (serotype 1), Gallid alphaherpesvirus 3 (serotype 2) and Meleagrid alphaherpesvirus 1 or herpesvirus of turkeys (HVT) (serotype 3). Serotype 1 includes all the virulent strains, which are further divided into pathotypes that include mild (m)MDV, virulent MDV (vMDV), very virulent MDV (vvMDV) and very virulent plus MDV (vv+MDV). Some attenuated vaccine strains also belong to serotype 1. Serotype 2 includes the naturally avirulent strains, some of which are used as vaccines. Antigenically related HVT is also used as vaccine against MD, and, more recently, as a recombinant viral vaccine vector.

MDV is a double-stranded DNA virus of approximately 160–180 kb in length. The structure is similar to other alphaherpesviruses with unique short and long sequences that are both flanked by inverted repeat sequences. Whole genome sequences are available for a number of MDV strains that have been used for both diagnostic and research purposes.

3. Zoonotic potential and biosafety and biosecurity requirements

MDV is not considered to be a zoonotic pathogen, as nearly all experimental data indicate that mammalian cells or animals cannot be infected with MDV (reviewed by Schat & Erb, 2014). Biosecurity, however, is an important component for prevention of MD in poultry, in combination with vaccination and improvements in host genetic

resistance. Limiting early MDV exposure of a newly hatched flock is crucial for maximum efficacy of vaccination programmes, and the failure to limit early exposure is likely to be the leading cause of vaccination failures. Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

4. Differential diagnosis

MD diagnosis requires differentiation from other avian tumour virus diseases, such as lymphoid leukosis and reticuloendotheliosis, as well as non-virus-induced tumours, and some non-tumour lesions. The heterogeneous population of lymphoid cells in MD lymphomas, as seen in haematoxylin-and-eosin-stained sections, or in impression smears of lymphomas stained by May–Grünwald–Giemsa, is an important feature in differentiating the disease from lymphoid leukosis, in which the lymphomatous infiltrations are composed of uniform lymphoblasts. Another important difference is that, in lymphoid leukosis, gross lymphomas occur in the bursa of Fabricius, and the tumour has an intrafollicular origin and pattern of proliferation. In MD, although the bursa is sometimes involved in the lymphoproliferation, the tumour is less apparent, diffuse and interfollicular in location. Peripheral nerve lesions are not a feature of lymphoid leukosis as they are in MD. The greatest difficulty comes in distinguishing between lymphoid leukosis and forms of MD sometimes seen in adult birds in which the tumour is lymphoblastic with marked liver enlargement and absence of nerve lesions. If post-mortems are conducted on several affected birds, a diagnosis can usually be made based on gross lesions and histopathology. However there are other specialised techniques described. The expression of the Meq biochemical marker has been used to differentiate between MD tumours, latent MDV infections and retrovirus-induced tumours (Schat & Nair, 2013). The procedure may require specialised reagents and equipment and it may not be possible to carry out these tests in laboratories without these facilities. Development of a number of polymerase chain reaction (PCR)-based diagnostic tests has allowed rapid detection of pathogenic MDV strains (Schat & Nair, 2013). Other techniques, such as detection by immuno-fluorescence of activated T cell antigens present on the surface of MD tumour cells (MD tumour-associated surface antigen or MATSA), or of B-cell antigens or IgM on the tumour cells of lymphoid leukosis can give a presumptive diagnosis, but these are not specific to MD tumour cells.

Nerve lesions and lymphomatous proliferations induced by certain strains of reticuloendotheliosis virus (REV) are similar, both grossly and microscopically, to those present in MD. Although REV is not common in the majority of chicken flocks, it should be considered as a possible cause of lymphoid tumours; its recognition depends on virological and serological tests on the flock. REV can also cause neoplastic disease in turkeys, ducks, quail and other species. Another retrovirus, designated lymphoproliferative disease virus (LPDV), also causes lymphoproliferative disease in turkeys. Although chicken flocks may be seropositive for REV, neoplastic disease is rare. The main features in the differential diagnosis of MD, lymphoid leukosis and reticuloendotheliosis are shown in Table 1. Peripheral neuropathy is a syndrome that can easily be confused with the neurological lesions caused by MDV. This is not very common but its incidence may be increasing in some European flocks.

Table 1. Features useful in differentiating Marek's disease, lymphoid leukosis and reticuloendotheliosis

Feature	Marek's disease	Lymphoid leukosis	Reticuloendotheliosis*
Age	Any age. Usually 6 weeks or older	Not under 16 weeks	Not under 16 weeks
Signs	Frequently paralysis	Non-specific	Non-specific
Incidence	Frequently above 5% in unvaccinated flocks. Rare in vaccinated flocks	Rarely above 5%	Rare
Macroscopic lesions			
Neural involvement	Frequent	Absent	Infrequent
Bursa of Fabricius	Diffuse enlargement or atrophy	Nodular tumours	Nodular tumours
Tumours in skin, muscle and proventriculus, 'grey eye'	May be present	Usually absent	Usually absent

Feature	Marek's disease	Lymphoid leukosis	Reticuloendotheliosis*
Microscopic lesions			
Peripheral nerves	Yes	No	Infrequent
Liver tumours	Often perivascular	Focal or diffuse	Focal
Spleen	Focal/multifocal in layers or diffuse in broiler breeders	Often focal	Focal or diffuse
Bursa of Fabricius	Interfollicular tumour and/or atrophy of follicles	Intrafollicular tumour	Intrafollicular tumour
Central nervous system	Yes	No	No
Lymphoid proliferation in skin and feather follicles	Yes	No	No
Cytology of tumours	Pleomorphic lymphoid cells, including lymphoblasts, small, medium and large lymphocytes and reticulum cells. Rarely can be only lymphoblasts	Lymphoblasts	Lymphoblasts
Category of neoplastic lymphoid cell	T cell	B cell	B cell

*Reticuloendotheliosis virus may cause several different syndromes. The bursal lymphoma syndrome is most likely to occur in the field and is described here.

B. DIAGNOSTIC TECHNIQUES

Table 2. Test methods available for the diagnosis of Marek's disease and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[(a)]						
Histopathology	–	–	–	+++	+	–
Virus isolation	–	–	–	+	–	–
Antigen detection	–	–	–	+	–	–
PCR	–	–	–	++	+	–
Real-time PCR	–	–	–	+++	+	–
LAMP	–	–	–	++	+	–
Detection of immune response						
AGID	–	–	–	–	+	+
IFA	–	–	–	–	+	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; LAMP = Loop-mediated isothermal amplification;
AGID = agar gel immunodiffusion; IFA = indirect fluorescent antibody.
[(a)]A combination of agent detection methods applied on the same clinical sample is recommended.

1. Detection and identification of the agent

1.1. Cell culture for virus isolation

Infection by MDV in a flock may be detected by isolating the virus from the tissues of infected chickens. However, the ubiquitous nature of MDV must be taken into consideration and the diagnosis of MD should be based on a combination of MDV isolation or detection of the genome and the occurrence of clinical disease. Commonly used sources are buffy coat cells from heparinised blood samples, or suspensions of lymphoma cells or spleen cells. When these samples are collected in the field, it is suggested that they be transported to the laboratory under chilled conditions but not frozen. As MDV is highly cell-associated, it is essential that these cell suspensions contain viable cells. The cell suspensions are inoculated into monolayer cultures of chicken kidney cells or duck embryo fibroblasts (chicken embryo fibroblasts (CEF) are less sensitive for primary virus isolation). Serotype 2 and 3 viruses (see Section C.2.1 *Characteristics of the seed*) are more easily isolated in CEF than in chicken kidney cells. Usually, a 0.2 ml suspension containing from 10^6 to 10^7 live cells is inoculated into duplicate monolayers grown in plastic cell culture dishes (60 mm in diameter). Inoculated and uninoculated control cultures are incubated at 37°C in a humid incubator containing 5% CO_2. Alternatively, closed culture vessels may be used. Culture medium is replaced at 2-day intervals. Areas of cytopathic effects, termed plaques, appear within 3–5 days and can be enumerated at about 7–10 days.

Another, less commonly used source of MDV for diagnostic purposes is feather tips. While this is more commonly used for PCR-based diagnosis, feather tips can also be useful for preparing cell-free MDV. Tips about 5 mm long, or minced tracts of skin containing feather tips, are suspended in an SPGA/EDTA (sucrose, phosphate, glutamate and albumin/ ethylenediamine tetra-acetic acid) buffer for extraction and titration of cell-free MDV (Calnek *et al.,* 1970). The buffer is made as follows: 0.2180 M sucrose (7.462 g); 0.0038 M monopotassium phosphate (0.052 g); 0.0072 M dipotassium phosphate (0.125 g); 0.0049 M L-monosodium glutamate (0.083 g); 1.0% bovine albumin powder (1.000 g); 0.2% EDTA (0.200 g); and distilled water (100 ml). The buffer is sterilised by filtration and should be at approximately pH 6.5.

This suspension is sonicated and then filtered through a 0.45 μm membrane filter for inoculation on to 24-hour-old chicken kidney cell monolayers from which the medium has been drained. After absorption for 40 minutes, the medium is added, and cultures are incubated as above for 7–10 days.

Using these methods, MDV of serotypes 1 and 2 may be isolated, together with HVT (serotype 3), if it is present as a result of vaccination. With experience, cytopathic effects and plaques caused by the different virus serotypes can be differentiated fairly accurately on the basis of time of appearance, rate of development, and plaque morphology. HVT plaques appear earlier and are larger than serotype 1 plaques, whereas serotype 2 plaques appear later and are smaller than serotype 1 plaques.

1.2. Antigen detection – immunolabelling techniques

A variation of the agar gel immunodiffusion (AGID) test used for serology (see below) may be used to detect MDV antigen in feather tips as an indication of infection by MDV. Glass slides are prepared with a coating of 0.7% agarose (e.g. A37) in 8% sodium chloride, containing MDV antiserum. Tips of small feathers (ideally blood feathers) are taken from the birds to be examined and are inserted vertically into the agar, and the slides are maintained as described below. The development of radial zones of precipitation around the feather tips denotes the presence in the feather of MDV antigen and hence of infection in the bird.

Antigen detection can be especially helpful in differentiating a MD tumour from other lymphoid tumours. In addition to confirming the tumour as T-cell lymphoma, immunolabelling with monoclonal or polyclonal antibodies can be used to confirm expression of Meq, which is the primary MDV oncogene (Ahmed *et al.*, 2018; Gimeno *et al.,* 2014). MDV and HVT cell culture plaques may also be identified as such using specific antibodies raised in chickens. Monoclonal antibodies may be used to differentiate serotypes (Lee *et al.,* 1983).

1.3. Molecular methods – detection of nucleic acids

The genomes of all three serotypes have been completely sequenced (Afonso *et al.,* 2001; Lee *et al.,* 2000). PCR tests have been developed for the diagnosis of MD using specific primer sets that amplify

specific MDV genes (Table 3). Real-time PCR to quantify MDV genome copies has also been described (Baigent *et al.,* 2005; Gimeno *et al*., 2005; Islam *et al.,* 2004). MD lymphomas have relatively high levels of MDV DNA compared with latently infected tissues and can be differentiated using defined cycle threshold ratio cut-off levels (Gimeno *et al.*, 2005). In addition, PCR tests that enable differentiation of oncogenic and non-oncogenic strains of serotype 1 MDV, and of MDV vaccine strains of serotypes 2 and 3 (Becker *et al.,* 1992; Bumstead *et al.,* 1997; Zhu *et al.,* 1992) have been described. Different studies have also described the differentiation of oncogenic and vaccine strains (Baigent *et al.,* 2016; Gimeno *et al.,* 2014; Renz *et al*., 2006) by real-time PCR using specific primer and probe sets (Table 4). Real-time PCR tests to distinguish virulent MDV-1 strains from CVI-988 (Rispens) vaccine are based on a single nucleotide polymorphism in the pp38 gene. Because of this constraint, there can be low-level cross reactivity between the virulent MDV-specific probe and CVI-988 (Rispens) DNA. This can be overcome by inclusion of CVI-988 (Rispens) DNA as a negative control in the PCR: in this case the reaction threshold must be set above the amplification signal of this CVI-988 (Rispens) negative control. Consideration must also be given to the possibility of the emergence of virulent MDV strains with a similar mutation but one that cannot be distinguished from CVI-988 (Rispens). For the emerging MDV gene-deleted vaccines (Zhang *et al.,* 2017), the tumorigenic strain can be differentiated from the vaccine strain by PCR for the specific gene deletion fragment. PCR may also be used to quantitate virus load in tissues (Baigent *et al.,* 2005; Bumstead *et al.,* 1997; Burgess & Davison, 1999) or differentially detect MDV and HVT in the blood or feather tips (Baigent *et al.,* 2005; Davidson & Borenshtain, 2002). A modification of the PCR test, designated LAMP (loop-mediated isothermal amplification), has also been used for the detection and differentiation of MDV serotypes (Wozniakowski *et al*., 2013).

Table 3. Example PCR primer sets used for identification of MDV

MDV specificity	Primer set (5' → 3')	Product size	Reference
pp38	Fwd: GTG-ATG-GGA-AGG-CGA-TAG-AA Rev: TCC-GCA-TAT-GTT-CCT-CCT-TC	225 bp	Cao *et al*., 2013
gB	Fwd: CGG-TGG-CTT-TTC-TAG-GTT-CG Rev: CCA-GTG-GGT-TCA-ACC-GTG-A	66 bp	Gimeno *et al*., 2005
Meq	Fwd: GAG-CCA-ACA-AAT-CCC-CTG-AC Rev: CTT-TCG-GGT-CTG-TGG-GTG-T	1.41 kb	Dunn *et al*., 2010

Table 4. Example PCR primer and probe sets used for differentiating virulent and vaccine strains using real-time PCR

Real-time PCR Target	Sequence (5' → 3'): 5' reporter and 3' quencher for probes	Product size	Reference
Virulent MDV-specific pp38	SNP FP: GAG-CTA-ACC-GGA-GAG-GGA-GA SNP RP: CGC-ATA-CCG-ACT-TTC-GTC-AA Probe CCC-ACT-GTG-ACA-GCC (5' FAM, 3' BHQ1)	99 bp	Baigent *et al*., 2016
CVI-988 (Rispens)-specific pp38	SNP FP: GAG-CTA-ACC-GGA-GAG-GGA-GA SNP RP: CGC-ATA-CCG-ACT-TTC-GTC-AA Probe CCC-ACC-GTG-ACA-GCC (5' FAM, 3' BHQ1)	99 bp	Baigent *et al*., 2016
MDV-2 DNA Pol gene	Pol FP: AGC-ATG-CGG-GAA-GAA-AAG-AG Pol RP: GAA-AGG-TTT-TCC-GCT-CCC-ATA Probe CGC-CCG-TAA-TGC-ACC-CGT-GAC-T (5' FAM, 3' BHQ1)	100 bp	Renz *et al*., 2006
HVT sORF-1 gene	sORF1 FP: GGC-AGA-CAC-CGC-GTT-GTA-T sORF1 RP: TGT-CCA-CGC-TCG-AGA-CTA-TCC Probe AAC-CCG-GGC-TTG-TGG-ACG-TCT-TC (5' FAM, 3' BHQ1)	77 bp	Renz *et al*., 2006

2. Serological tests

The presence of antibodies to MDV in non-vaccinated chickens from about 4 weeks of age is an indication of infection. Before that age, such antibodies may represent maternal transmission of antibody via the yolk and are not evidence of active infection.

Viruses, antigens and antisera can be obtained from commercial suppliers or from the WOAH Reference Laboratory for Marek's Disease[1], but international standard reagents have not yet been produced.

2.1. Agar gel immunodiffusion

There is no test suitable for certifying individual animals prior to movement, but the AGID test is employed commonly to detect antibody. The test is conducted using glass slides coated with 1% agar in phosphate buffered saline (PBS) containing 8% sodium chloride. Adjacent wells are filled with antigen or serum and these are incubated in a humid atmosphere at 37°C for 24 hours for diffusion to take place; positive sera show reactions of identity with known positive serum and antigen. The antigen used in this test is either disrupted MDV-infected tissue culture cells or an extract of feather tips, or skin containing feather tracts obtained from MDV-infected chickens. The cell culture antigen is prepared by propagating MDV in chicken kidney cells or CEF. When cytopathic effect is confluent, the cells are detached from the culture vessel and suspended in culture medium or phosphate buffered saline without tryptose phosphate broth (presence of tryptose phosphate broth may produce non-specific precipitin lines) at a concentration of about 1×10^7 cells/ml. This suspension is then freeze–thawed three times and used as antigen.

2.1.1. Test procedure

i) Make a 1% solution of agar in 8% sodium chloride by standing the mixture in a boiling water bath.

ii) Pour the agar to a thickness of 2–3 mm on either a microscope slide or a Petri dish.

iii) Cut holes in the agar using a template with a centre well and 6 wells spaced at equal distance around the centre well. The diameter of wells should be approximately 5.3 mm, and the wells should be about 2.4 mm apart. A template with cutters is commercially available.

iv) Add the antigen in the centre well and the standard antiserum in alternate exterior wells. Add the serum samples to be tested to the remaining three wells so that a continuous line of identity is formed between an unknown sample that is positive and the known positive control serum.

v) Incubate the slide for 24 hours at 37°C in a humid container and read the results over a lamp in a darkened room.

2.2. Indirect immunofluorescence test

The indirect immunofluorescence test demonstrates the ability of a test serum to stain MDV plaques in cell cultures (Silva *et al.*, 1997; Spencer & Calnek, 1970). These tests are group specific and more sensitive than the AGID test.

2.2.1. Test procedure

i) Prepare MDV antigen in cell culture dishes or 96-well microtitre plates.

ii) Fix cells with acetone–alcohol mixture for 10 minutes then air dry. Plates may be held in refrigerator until ready to be stained or frozen for longer periods of time.

iii) Wet surface of plate with PBS, discard PBS, then add serum at multiple dilutions (1:5, 1:10, 1:20, 1:40). Incubate in water bath or incubator at 37°C for 30–60 minutes.

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

iv) Discard serum, wash plates three times with distilled water followed by three washes with PBS, blot.

v) Add fluorescein-labelled affinity purified antibody to chicken IgG. Incubate in water bath or incubator at 37°C for 30–60 minutes.

vi) Repeat washing, then read plates immediately using fluorescent microscope

2.3. Other tests

A virus neutralisation test for the ability of a serum to neutralise the plaque-forming property of cell-free MDV can also be employed. However, this test is more suitable for research purposes than for routine diagnostic use. Enzyme-linked immunosorbent assays (ELISA) for detecting MDV antibodies are available (Cheng *et al*., 1984; Zelnik *et al.,* 2004).

C. REQUIREMENTS FOR VACCINES

1. Background

Control of MD is essentially achieved by the widespread use of live attenuated vaccines (Nair, 2004, Schat & Nair, 2013). Commercial biological products mainly used in the control of MD are the cell-associated live virus vaccines. Lyophilised cell-free vaccines are rarely used. Marek's disease vaccines are injected subcutaneously into day-old chicks after hatch or *in ovo* at 18–19 days of embryonation (Sharma, 1999).

2. Outline of production and minimum requirements for conventional vaccines

The requirements for producing vaccines are outlined below, and in Chapter 1.1.8 *Principles of veterinary vaccine production*, but other sources should be consulted for further information on the procedures (Code of Federal Regulations title 9 [9CFR], 2016; European Pharmacopoeia, 1997a and 1997b; Ministry of Agriculture, Fisheries and Food, UK, 1990; Thornton, 1985). Protocols are given in the British Pharmacopoeia Monograph 589, and 9CFR, Part 113 (9CFR, 2016). The guidelines in this *Terrestrial Manual* are intended to be general in nature and may be supplemented with national and regional requirements.

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Viruses of the MDV group are classified under three serotypes – 1, 2, and 3 – on the basis of their antigenic relatedness.

i) Serotype 1

This includes all the pathogenic strains of the virus, ranging from strains that are very virulent plus (e.g. 648A), very virulent (e.g. Md/5, Md/11, Ala-8, RB-1B), virulent (e.g. HPRS-16, JM GA), mildly virulent (e.g. HPRS-B14, Conn A) and finally to weakly virulent (e.g. CU-2, CVI-988). These strains may be attenuated by passage in tissue culture, with loss of pathogenic properties but retention of immunogenicity, to provide strains that have been used as vaccines. Those that have been used commercially include attenuated HPRS-16 and CVI-988 (Rispens) strains. Serotype 1 vaccines are prepared in a cell-associated ('wet') form that must be stored in liquid nitrogen.

ii) Serotype 2

This includes naturally avirulent strains of MDV (e.g. SB-1, HPRS-24, 301B/1, HN-1), and several of these have been shown to provide protection against virulent strains. The SB-1 and 301B/1 strains have been developed commercially and used, particularly with HVT, in bivalent vaccines for protection against the very virulent strains. Serotype 2 vaccines exist only in the cell-associated form.

iii) Serotype 3

This contains the strains of naturally avirulent HVT (e.g. FC126, PB1), which are widely used as a monovalent vaccine, and also in combination with serotype 1 and 2 strains in bivalent or trivalent vaccines against the very virulent strains of MDV. HVT may be prepared in a cell-free form as a freeze-dried (lyophilised) vaccine or in a cell-associated ('wet') form.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The substrates used for commercial vaccine production are primary CEF derived from specific pathogen free (SPF) flocks or duck embryo fibroblasts. CEF from SPF flocks are preferred to duck cells because more is known about chicken-embryo-transmitted pathogens and methods for their detection.

Methods for testing SPF flocks for freedom from infection are available (Ministry of Agriculture, Fisheries and Food, UK, 1990; Thornton, 1985; Veterinary Services Memorandum 800.65[2]). SPF chicken flocks should be free from avian adenoviruses, including egg-drop syndrome 76 virus, avian encephalomyelitis virus, avian leukosis virus (subgroups A, B and J), avian nephritis virus, avian reoviruses, avian rotaviruses, fowl pox virus, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, influenza type A virus, MDV, *Mycoplasma gallisepticum, M. synoviae,* Newcastle disease virus, reticuloendotheliosis virus, *Salmonella* spp., and turkey rhinotracheitis virus. Requirement for freedom from chicken anaemia virus varies between different countries. Freedom from other infections may also be required as they become recognised.

SPF duck flocks should be free from avian adenoviruses, avian reoviruses, *Chlamydia*, duck virus enteritis, duck virus hepatitis types I and II, influenza type A virus, Newcastle disease virus, *Pasteurella* (now *Riemerella*) *anatipestifer,* REV, and *Salmonella* infections. Some countries, including the USA, do not have an official definition of SPF duck flock. Freedom from other infections may also be required as they become recognised.

Seed virus must be free from the agents listed for SPF flocks and from other contaminants. A vaccine strain derived from turkeys must also be free from LPDV and haemorrhagic enteritis virus.

The ability of the master seed virus – and derived virus at the limit of the passage range used to produce vaccinal virus (usually not more than five tissue culture passages) – to protect against MD must be determined. Standardised protection tests are published but requirements may vary according to the relevant regulatory authority. They involve vaccination of MD-susceptible SPF chickens at 1 day of age (or by *in ovo* route for an *in ovo* label claim) and challenge with sufficient virulent MDV 5–8 days later to cause at least a 70–80% incidence of MD in unvaccinated chickens. Two types of tests are used. In the protection index test, a single field dose (1000 PFU) (plaque-forming units) of vaccine is given and the incidence of MD in vaccinated birds is compared with that in unvaccinated birds. Protective indices should be greater than 80, i.e. vaccinated birds should show at least 80% reduction in the incidence of gross MD, compared with unvaccinated controls.

A PD_{50} (50% protective dose) test may also be used, involving the inoculation of five four-fold serial dilutions of vaccine virus selected to provide protection above and below the 50% level, followed by challenge 8 days later to determine the PD_{50} value. The assays are conducted using a standard reference vaccine for comparison. The PD_{50} may be as low as 4 PFU, but higher values can be obtained depending on the vaccine strain, whether cell-free or cell-associated and the presence or absence of maternal antibodies in the test chickens. On the basis of the PD_{50} test, it has been suggested that the minimum vaccine field dose should be the greater of two values: 10^3 PFU or 100 PD_{50}.

2.1.3. Validation as a vaccine strain

The vaccine strain shall be non-pathogenic for chickens as shown by the inoculation of 50 MD susceptible SPF egg/chickens with a 10× field dose by the route of vaccination(s) planned. At

2 https://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_65.pdf

120 days of age, the birds are evaluated for MD lesions. If lesions are found, the seed is considered unsatisfactory.

The master seeds are tested for purity, identity, and extraneous agents and are approved for use in vaccine production following satisfactory testing.

The serotype 2 MDVs used in vaccines function in a synergistic fashion with HVT protecting against the milder challenges and the bivalent/trivalent combinations with serotypes 1 and 2 protecting against more virulent challenges.

An effective titre of each serotype of MDV is established in a vaccination-challenge study.

2.2. Method of manufacture

2.2.1. Procedure

Vaccines against MD are prepared from live attenuated strains belonging to the 3 serotypes using CEF (derived from SPF embryonated eggs) as the substrates.

2.2.2. Requirements for ingredients

Substrate cells are seeded into flat-bottomed vessels for stationary incubation, or into cylindrical vessels for rolled incubation. Media commonly used are Eagle's minimal essential medium, or 199 medium, buffered with sodium bicarbonate and supplemented with 5% calf serum. Incubation is at 37°C for 48 hours.

For cell-associated vaccine, cultures are infected with production HVT or MDV seed-virus stock, in cell-associated form, which is usually two passages beyond the master seed stock. Cultures are incubated for 48 hours (depending on the vaccine strain) then the infected cells are harvested by treating the washed cell sheet with an EDTA/trypsin solution to allow the cells to begin to detach. The flasks are then returned to the incubator (37°C) to allow complete detachment. The cells are subjected to low-speed centrifugation, and then resuspended in the freezing mixture consisting of cell growth medium containing 7.5–15% dimethylsulphoxide (DMSO), and held at 4°C or dispensed immediately into the final vaccine containers, usually glass ampoules, which are flame sealed and frozen in liquid nitrogen.

Cell-free lyophilised vaccine may be prepared from HVT, but not from MDV strains. For the production of this form of vaccine, HVT-infected cultures are incubated for 72 hours, infected cells are detached from the vessel as described above, or scraped from the walls of the vessel. The cells are suspended in a small volume of growth medium, centrifuged, and resuspended in a buffered stabiliser solution containing 8% sucrose, but free from protein to prevent frothing. The cell suspension is sonicated to release virus, the cell debris is removed, the suspension is diluted with a complete stabiliser – such as SPGA – filled into the final containers, and lyophilised.

The dilution rate for both cell-associated and cell-free vaccines is based on previous experience, as is the number of doses required per container, because the virus content of the harvested material cannot be assayed prior to filling the final containers. The virus content of the finished product can subsequently be added to the label.

2.2.3. In-process controls

For optimal results in preparing cell-associated vaccine, a slow rate of freezing (1–5°C per minute) and rapid thawing are essential. The infectivity titre of the infected cells, and hence the number of doses per ampoule, are determined after filling the ampoules. Similarly for cell-free vaccine, the virus content of the final suspension, and hence the number of doses per container, is determined after filling.

2.2.4. Final product batch tests

Using immunofluorescence assay (IFA) with monospecific serum, checks should be carried out to show that the product is of the same specificity as the seed virus. This is best done using

monoclonal antibodies. When serotypes are combined for a product, the product is titred in serotype-specific fashion to confirm a sufficient quantity of each serotype.

i) Sterility/purity

Extensive testing is required of the materials used to produce the vaccine, and of the final product. Substrate cells should come from an SPF flock, in particular, free from vertically transmitted agents. Substances of animal origin used in the preparation of vaccines such as serum, trypsin, and bovine serum albumin, must be free from extraneous agents.

Batches of the final vaccine produced should be tested for freedom from contaminating bacteria, fungi, mycoplasma and the viruses listed for SPF flocks; tests for purity of the diluent should also be conducted. Suitable tests for the detection of extraneous agents at all stages of vaccine production are recommended by several official bodies (9CFR, 2016; Ministry of Agriculture, Fisheries and Food, UK, 1990; Thornton, 1985) and in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

When firms use different MDV serotypes and HVT constructs in manufacturing, serials may be screened for the presence of other contaminating serotypes and constructs.

ii) Safety

Ten doses of vaccine or a quantity of diluent equivalent to 10 doses of vaccine should be inoculated into separate groups of 10–25 1-day-old SPF chickens. No adverse reactions should occur during a 21-day observation period.

With cell-associated vaccine, care is necessary to avoid injury from ampoules that may explode when they are removed from liquid nitrogen. Eye protection must be worn.

iii) Batch potency

The standard dose of each type of vaccine is 1000 PFU per chicken or egg but may be higher based on the titre used in the efficacy study. Virus content assays are conducted on batches of vaccine to ensure that the correct dose of each serotype per bird will be achieved.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and Section C.2.*2 Method of manufacture*) should be submitted to the relevant authorities. The manufacturing information should be consistent with the production of the serial used in the efficacy study. Testing information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume. This testing may be confirmed by a regulatory authority laboratory.

In-process controls are part of the manufacturing process in the form of cell substrate testing and working seed testing to ensure the purity and identity of the seeds and cells.

2.3.2. Safety requirements

i) Target and non-target animal safety

The master seed virus should be shown to be non-pathogenic for chickens by inoculating ten times the field dose into 1-day-old SPF chickens (or embryonated eggs when using the *in-ovo* route) of a strain susceptible to MD, to ensure that it does not cause gross lesions or significant microscopic lesions of MD by 120 days of age. It should be noted that some vaccine strains of MDV and HVT may produce minor and transient microscopic nerve lesions.

Three serials are evaluated in a field safety study to evaluate the product prior to approval for use under field conditions. Pharmacovigilance evaluates the product post-licensure.

Recombinant seeds should be tested in non-target animals to ensure no unexpected changes in virulence had resulted from the gene insertion.

ii) Reversion-to-virulence for attenuated/live vaccines

No increase in virulence should occur during at least four serial passages of the vaccine strain in 1-day-old SPF MD-susceptible chickens. Ten times the field dose of vaccine is inoculated initially and then passaged by inoculation of heparinised blood at 5–7-day intervals, and tests for viraemia are run to check that virus is transferred at each passage. The birds receiving the final passage are kept for 120 days and should be free from MD lesions. However, some strains such as CVI-988 (Rispens), may cause some mild MD lesions. The important observation is that the virulence should not change. This is a difficult test because the genetic resistance of the chickens fundamentally affects the apparent virulence of the virus, so does the type of inoculum. After successful completion of laboratory safety tests, the safety of the strain should be confirmed in extensive field trials. The CVI-988 (Rispens) strain must be field safety tested alone or it must be marketed in combination with the other Marek's serotypes as the other serotypes may attenuate the CVI-988 (Rispens).

2.3.3. Efficacy requirements

One group of SPF eggs or chickens receives the vaccine at a minimum dose at a minimum age. Other chickens or eggs from the same hatch serve as controls. The non-vaccinated challenged group serve as challenge controls. Another group is neither vaccinated nor challenged to serve as test controls. If a bivalent or trivalent vaccine is being evaluated, a group is vaccinated with HVT. This group should not be well protected to validate the virulence of the challenge. Challenge occurs at 5–8 days of age and the challenge culture must be carefully handled to preserve virulence. The birds are observed until 7 weeks of age. Birds lost during this time or birds evaluated at the end of the study, are evaluated for grossly observable Marek's disease lesions. For a satisfactory test, at least 80% of the vaccinates must remain free of grossly observable lesions.

A titre is associated with the efficacy study. From this titre, a titre for serial release and a throughout dating titre are assigned based on test variation and titre loss over time. For bivalent or trivalent products, two or three titres are assigned to the efficacy.

A test for duration of immunity may be carried out on the product. Such immunity is apparently lifelong for Marek's disease vaccines but must be supported by data to be included on the labelling. Preservatives may be included in the vaccine or diluent. During use, reconstituted vaccine must be kept cool and cell-associated vaccine should be agitated to keep cells in suspension. If duration of immunity data are not created, the labelling must specify that the duration of immunity is not known.

2.3.4. Duration of immunity

As part of the regulatory approval procedure, the manufacturer may demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test at the end of the claimed period of protection. In the case of Marek's disease vaccines it is a vaccination–challenge study design. The level of immunity at the short-term study described above and at the claimed duration should be similar. If it is demonstrated/approved, it may be included on the labelling as opposed to a statement that the duration is not known.

2.3.5. Stability

Tests for stability are carried out on representative batches of vaccine to show that titre is maintained during the stated shelf life of the vaccine – a real-time stability study. These tests should be conducted under the conditions of storage of the vaccine. The lyophilised product should have a shelf life of 12 months when stored at 2–8°C. Manufacturers increase the virus content of the vaccine to compensate for some loss of titre during storage but must maintain a titre above the throughout dating titre. The throughout dating titre is set based on the titre of the efficacy serial. Appropriate diluting fluids are provided for use with cell-associated and freeze-

dried vaccines. The stability of reconstituted vaccine over a 2-hour period is tested as part of the serial or batch release testing. The diluted virus is held on ice for 2 hours to mimic field use.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Genetically engineered recombinant vaccines (Reddy *et al.*, 1996) based on the existing live MD vaccines can offer simultaneous protection against other avian diseases, depending on the protective antigens engineered into the recombinant vaccine. They can also offer the *in ovo* route for other antigens. A number of recombinant vaccines based on HVT vectors that induce protection against avian diseases such as avian influenza, infectious bursal disease, Newcastle disease and infectious laryngotracheitis are commercially available.

3.2. Special requirements for recombinant vaccines

For HVT constructs expressing other antigens, the potency test is a two-part assay. First, the HVT backbone is titred. Second, the protein expression from the resulting plaques is assayed. The foreign gene expression should be equal to or greater than the HVT titre. Combining two HVT constructs in a single vaccine is not acceptable due to interference. However, the combination of more than one antigen in a single HVT construct is allowed. For recombinants, specific studies to support no changes to the tissue tropism, non-target animal safety, environmental stability, and shed and spread are conducted to support environmental release of the vaccine. Additionally, for duration of immunity claims, the duration of the Marek's disease protection and the protection provided by the inserted gene must be measured for the claim.

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NB: There is a WOAH Reference Laboratory for Marek's disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Marek's disease

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.3.14.

NEWCASTLE DISEASE (INFECTION WITH NEWCASTLE DISEASE VIRUS)

SUMMARY

Description of the disease: *Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus type 1 (APMV-1), also known as Newcastle disease virus (NDV), of the genus* Orthoavulavirus *belonging to the family* Paramyxoviridae*. At present, there are 21 serotypes of avian paramyxoviruses designated APMV-1 to APMV-21. Each virus belongs to a virus species that are dispersed amongst three genera termed: avian metaavulavirus, avian orthoavulavirus and avian paraavulavirus.*

APMV-1 has been shown to be able to infect over 200 species of birds, but the severity of disease produced varies with both host and strain of virus. Even APMV-1 strains of low virulence may induce severe respiratory and enteric disease when exacerbated by the presence of other organisms or by adverse environmental conditions. The preferred method of diagnosis is validated reverse transcription polymerase chain reaction (RT-PCR) and sequencing, but virus isolation still remains an important laboratory tool.

Detection of the agent: *Suspensions in an antibiotic solution prepared from tracheal or oropharyngeal and cloacal swabs (or faeces) obtained from live birds, or of faeces and pooled organ samples taken from dead birds, are inoculated into the allantoic cavity of 9- to11-day-old embryonated chicken eggs. The eggs are incubated at 37°C for 2–7 days. The allantoic fluid of any egg containing dead or dying embryos, as they arise, and all eggs at the end of the incubation period are tested for haemagglutinating activity and/or by use of validated specific molecular methods to detect viral genome.*

Any haemagglutinating agents should be tested for specific inhibition with a monospecific antiserum to APMV-1. APMV-1 may show some antigenic cross-relationship with some of the other avian paramyxovirus serotypes, particularly APMV-3, APMV-7 and APMV-12. Real-time RT-PCR on the positive allantoic fluids could also be used as an alternative method for the initial APMV-1 identification.

Virulence of any newly isolated APMV-1 can be evaluated using molecular techniques, i.e. reverse-transcription polymerase chain reaction and sequencing. Alternatively, the intracerebral pathogenicity index (ICPI) can be used to determine virulence. ND is subject to official control in most countries and the virus has a high risk of spread from the laboratory; consequently, appropriate laboratory biosafety and biosecurity must be maintained; a risk assessment should be carried out to determine the level needed. Any APMV-1 with an ICPI ≥ 0.7, or with multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 of its F protein, is considered virulent.

Genetic-based tests are most commonly applied to routine diagnosis and offer rapid, sensitive, cost effective alternatives to conventional methods. Real-time RT-PCR targeting a highly conserved gene overcomes wide heterogeneity in the fusion (F) or haemagglutinin-neuraminidase (HN) genes. They can be applied directly to clinical samples once treated to extract viral RNA and can be used to test large sample numbers. Confirmation of detection using highly sensitive and inclusive screening real-time RT-PCR should then be followed by F gene sequencing to determine the virus virulence (the F gene proteolytic cleavage site) and virus genotype. It is important that tests selected have been shown to be able to sensitively detect viruses known to be circulating or an emerging threat to the region in which they are applied.

A screening test by real-time RT-PCR to identify both virulent and avirulent APMV-1 is recommended. For any positive tests, additional testing targeted to the fusion cleavage site can be used to identify viruses with a cleavage site compatible with NDV. Due to variability of the fusion cleavage site, it may require more than one test if using real-time RT-PCR to identify all virulent genotypes circulating in a country or region hence gene sequencing is the preferred approach. For outbreaks that have birds with signs compatible with Newcastle disease virus, which are positive by the real-time RT-PCR screening test, and are negative for the virulent fusion real-time RT-PCR tests, direct sequence of the fusion cleavage site or virus isolation and classical analysis may be required.

Serological tests: *The haemagglutination inhibition (HI) test is used most widely in ND serology, its usefulness in diagnosis depends on the vaccinal immune status of the birds to be tested and on prevailing disease conditions. Enzyme-linked immunosorbent assays also have a place and a number of commercial test kits are available.*

Requirements for vaccines: *Live viruses of low virulence (lentogenic) or of moderate virulence (mesogenic) are used for the vaccination of poultry depending on the disease situation and national requirements. Inactivated vaccines are also used.*

Live vaccines may be administered to poultry by various routes. They are usually produced by harvesting the infective allantoic/amniotic fluids from inoculated embryonated chickens' eggs; some are prepared from infective cell cultures. The final product should be derived from the expansion of master and working seeds.

Inactivated vaccines are given intramuscularly or subcutaneously. They are usually produced by the addition of formaldehyde to infective virus preparations, or by treatment with beta-propiolactone. Most inactivated vaccines are prepared for use by emulsification with a mineral or vegetable oil.

Recombinant Newcastle disease vaccines using viral vectors such as turkey herpesvirus or fowl poxvirus in which the HN gene, F gene or both are expressed have recently been developed and approved. If virulent forms of APMV-1 are used in the production of vaccines or in challenge studies, the facility should meet the requirements for an appropriate biosafety and containment level determined by biorisk analysis. Regulatory oversight may be required in some countries.

A. INTRODUCTION

Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) of the genus *Orthoavulavirus* belonging to the subfamily *Avulavirinae*, family *Paramyxoviridae*. The paramyxoviruses isolated from avian species have been classified by serological testing and phylogenetic analysis into 21 serotypes of avian paramyxoviruses designated APMV-1 to APMV-21. Each virus belongs to a virus species that are dispersed amongst three genera: metaavulaulavirus, orthoavulavirus and paraavulavirus (Amarasinghe *et al.*, 2019; ICTV 2019).

Since its recognition in 1926, ND is regarded as being endemic in many countries. Prophylactic vaccination is practised in all but a few of the countries that produce poultry on a commercial scale.

One of the most characteristic properties of different strains of APMV-1 has been their great variation in pathogenicity for chickens. Strains of APMV-1 have been grouped into five pathotypes on the basis of the clinical signs observed in infected chickens (Alexander & Senne, 2008b). These are:

1. Viscerotropic velogenic: a highly pathogenic form in which haemorrhagic intestinal lesions are frequently seen;
2. Neurotropic velogenic: a form that presents with high mortality, usually following respiratory and nervous signs;
3. Mesogenic: a form that presents with respiratory signs, occasional nervous signs, but low mortality;
4. Lentogenic or respiratory: a form that presents with mild or subclinical respiratory infection;
5. Subclinical: a form that usually consists of a subclinical enteric infection.

Pathotype groupings are rarely clear-cut and even in infections of specific pathogen free (SPF) birds, considerable overlapping may be seen. In addition, exacerbation of the clinical signs induced by the milder strains may occur

when infections by other organisms are superimposed or when adverse environmental conditions are present. As signs of clinical disease in chickens vary widely and diagnosis may be complicated further by the different responses to infection by different hosts, clinical signs alone do not present a reliable basis for diagnosis of ND. However, the characteristic signs and lesions associated with the virulent pathotypes will give rise to strong suspicion of the disease.

APMV-1 may infect humans where the most common sign of infection is conjunctivitis that develops within 24 hours of virulent APMV-1 exposure to the eye (Swayne & King, 2003). Reported infections have been non-life threatening and usually not debilitating for more than a day or two. The most frequently reported and best-substantiated clinical signs in human infections have been eye infections, usually consisting of unilateral or bilateral reddening, excessive lachrymation, oedema of the eyelids, conjunctivitis and sub-conjunctival haemorrhage. Although the effect on the eye may be quite severe, infections are usually transient and the cornea is not affected. There is no evidence of human-to-human spread. There is one report of the isolation of the pigeon variant of APMV-1 (PPMV-1) from immunocompromised patients who died of pneumonia.

ND, as characterised in Section B.1.6 of this chapter, is subject to official control in most countries and the virus has a high risk of spread from the laboratory. All laboratory manipulations with live virus or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis, as outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.* Countries lacking access to appropriate laboratory facilities should send specimens to a WOAH Reference Laboratory for the disease.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Virus isolation	–	+++	+	+++	+	–
Conventional PCR and sequencing	+	++	+	+++	+	–
Real-time RT-PCR	++	+++[1]	++	+++[(a)]	+	–
Detection of immune response						
ELISA	+	+	++	–	++	++
HI	–	–	+	–	++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
HI = haemagglutination inhibition.
[(a)]providing specified inclusive fit for purpose methods are used

1. Detection of the agent

Identification of APMV-1 viruses as the cause of infections and disease in poultry and other birds requires a thorough diagnostic investigation to differentiation from similar diseases caused by other viral agents especially influenza A

viruses. Individual APMV-1 and influenza A viruses isolates vary greatly in virulence, causing various syndromes evident as subclinical infections, drops in egg production, respiratory disease, and severe and high mortality disease. The latter clinical syndrome can be caused by either Newcastle disease (virulent APMV-1) or high pathogenicity avian influenza (HPAI) APMV viruses. It is judicious to have a single sampling procedure and simultaneously run specific differentiating diagnostic tests for both category of agents on field samples to obtain an accurate aetiological diagnosis for a single agent or, on occasion, confirmation of dual infection by APMV-1 and influenza A viruses.

1.1. Samples for virus isolation

Virus isolation is the reference method but is laborious and time intensive, used primarily for diagnosis of a first clinical case in an outbreak and to obtain virus isolates for further laboratory analysis.

For investigations of severe disease and high mortality in poultry flocks, it is usual to attempt virus isolation from recently dead birds or moribund birds that have been killed humanely. Samples from dead birds should include intestinal contents (faeces) or cloacal swabs and oropharyngeal or tracheal swabs. Samples from lungs, air sacs, intestine, spleen, kidneys, caecal tonsils, brain, liver and heart should also be collected and processed either separately or as a pool. When pooling samples the brain should be collected and processed first (to avoid cross contamination with other tissue types) and kept separate as presence of virus in the brain may be an indicator of NDV or HPAI. Further pools should be made consistent with known virus tropisms between NDV and non-NDV APMV-1 viruses, i.e. grouped at the level of respiratory, systemic and gastrointestinal.

Samples from live birds should include both tracheal or oropharyngeal and cloacal swabs, the latter should be visibly coated with faecal material. To avoid harming them, swabbing of small delicate birds should be done with the use of especially small swabs that are usually commercially available and intended for use in human paediatrics or the collection of fresh faeces may serve as an adequate alternative (caution that some influenza A viruses and type 1 avulaviruses in birds can have a strong respiratory tropism). Similar swab samples can be pooled from the same anatomical site (i.e. cloacal swabs with cloacal swabs, oropharyngeal swabs with oropharyngeal swabs), and most commonly pooling of five or occasionally more, if appropriately validated not to reduce sensitivity of detection, but specific swab types should be used (Spackman *et al.,* 2013). Further the type of swabs used may affect test sensitivity or validity with thin wire or plastic shafted swabs preferred.

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0–7.4 with antibiotics or a solution containing protein and antibiotics. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml); streptomycin (2 mg/ml); gentamycin (50 μg/ml); and mycostatin (1000 units/ml) for tissues and oropharyngeal or tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the concentrated stock solution to pH 7.0–7.4 following the addition of the antibiotics. It is recommended that a solution for transport of the swabs should contain protein to stabilise the virus (e.g. brain–heart infusion, up to 5% [v/v] cattle serum, 0.5% [w/v] bovine albumen or similar commercially available transport media). If control of *Chlamydophila* is desired, 0.05–0.1 mg/ml oxytetracycline should be included. Faeces and finely homogenised tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to 4 days. For prolonged storage, diagnostic samples and isolates should be kept at –80°C but for transport on dry ice (≤–50°C) is widely used. Repeated freezing and thawing should be avoided.

1.2. Virus isolation

The preferred method of growing APMV-1 viruses is by the inoculation of specific pathogen free (SPF) embryonated chicken eggs, or specific antibody negative (SAN) eggs. The supernatant fluids of faeces, swabs or tissue suspensions obtained through clarification by centrifugation at 1000 ***g*** for about 10 minutes at a temperature not exceeding 25°C, are inoculated in 0.2 ml volumes into the allantoic cavity of each of three to five embryonated SPF or SAN chickens' eggs of 9–11 days incubation. After inoculation, these are incubated at 35–37°C for 2–7 days. To accelerate the final isolation, it is possible to carry out up to two passages at 1- to 3-day intervals, obtaining results comparable to two passages at 2–7-day intervals (Alexander & Senne, 2008a). Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C for a minimum of 2 hours or

overnight (and checked for embryo death before proceeding) and the allantoic fluids tested for haemagglutination (HA) activity. Fluids that give a negative reaction should be passed into at least one further batch of eggs. Routine checks for contamination should be conducted by streaking samples in Luria Broth agar plates and reading these at 24 and 48 hours of incubation against a light source. BHI agar and blood agar plates may also be used. For larger numbers of sample initial culture could be in tryptose phosphate broth. Contaminated samples can be treated by incubation with increased antibiotic concentrations for 2–4 hours (gentamicin, penicillin g, and amphotericin b solutions at final concentrations to a maximum of 1 mg/ml, 10,000 U/ml, and 20 μg/ml, respectively). Samples heavily contaminated by bacteria that cannot be removed by centrifugation or controlled by antibiotics can be filtrated through 0.45 and 0.2 micron sterile filters. Filtration should be used only when other methods fail because aggregation may significantly reduce virus titre.

Suspensions of homogenised organs, faeces or swabs prepared as for isolation in eggs may also be used for attempted isolation in cell cultures. APMV-1 strains can replicate in a variety of cell cultures of avian and non-avian origin, among which the most widely used are: chicken embryo liver (CEL) cells, chicken embryo kidney (CEK) cells, chicken embryo fibroblasts (CEF), African green monkey kidney (Vero) cells, avian myogenic (QM5) and chicken-embryo-related (CER) cells (Terregino & Capua, 2009). Primary cell cultures of avian origin are the most susceptible. To optimise the chances of viral recovery for isolates of low virulence, trypsin should be added to the culture medium. The concentration of trypsin will vary depending on the type of trypsin and the type of cells used. One example is to add 0.5 μg/ml of porcine trypsin to CEFs. Viral growth is usually accompanied by cytopathic effects typically represented by disruption of the monolayer and formation of syncytia.

The optimal culture system for the virus is to some extent strain-dependent. Some strains of APMV-1 grow poorly in cell culture and replicate to higher titre in embryonated eggs, whereas some strains of Pigeon APMV-1 (PPMV-1) and of APMV-1, such as the avirulent Ulster strain, can be isolated in chicken liver or chicken kidney cells but not in embryonated eggs. If possible, mainly when dealing with samples suspected of being infected with PPMV-1, virus isolation should be attempted using both substrates (embryonated eggs and primary chicken embryo cells). As the viral titre obtained in cell culture is usually very low, additional replication steps in embryonated eggs should be performed prior to characterisation of the isolate by HI or other phenotypic methods. Comparing virus isolation rates for APMV-1 *in ovo* with real-time reverse-transcription polymerase chain reaction (RT-PCR) using positive wild bird surveillance samples, it was demonstrated that there were no significant differences in isolation frequency when using embryonated chickens' eggs, embryonated duck eggs or embryonated turkey eggs. In contrast, largely significant rates of virus isolation were reported with embryonated bird eggs compared with either Madin–Darby canine kidney or Vero cell cultures for APMV-1 real-time RT-PCR positive samples (Moresco *et al.,* 2012). For animal welfare reasons, the number of embryonated eggs should be kept to a minimum, applying the principles of the 3Rs.

1.3. Virus identification

HA activity detected in bacteriologically sterile fluids harvested from inoculated chicken eggs may be due to the presence of any subtype of APMV (including APMV-1), 16 haemagglutinin subtypes of influenza A viruses from birds, haemagglutinating adenoviruses or bacterial HA. APMV-1 can be confirmed by the use of specific antiserum in a haemagglutination inhibition (HI) test. Usually chicken antiserum that has been prepared against one of the strains of APMV-1 is used.

APMV-1 isolated from some wild bird species, e.g. cormorants, does not always demonstrate the ability to haemagglutinate RBCs. Replacement of chicken RBCs with turkey RBCs may be beneficial when testing viral-infected allantoic/amniotic fluid isolated from such species. Even when turkey RBCs are used, the HA activity of the virus remains low making the HA test unreliable for evaluating strains of APMV -1 from some wild bird species (Hines & Miller, 2012).

In the HI test, some level of cross-reactivity may be observed among the various avian paramyxovirus serotypes. Cross-reactivity can be observed between APMV-1 and APMV-3 viruses (particularly with the psittacine variant of APMV-3 , commonly isolated from pet or exotic birds), APMV-7 or APMV-12 . The risk of mistyping an isolate can be greatly reduced by using a panel of reference sera or monoclonal antibodies (MAbs) specific for APMV-1, APMV-3, APMV-7 and APMV-12. See also Section B.1.7.

At present, RT-PCR-based techniques for the detection and typing (pathotyping and genotyping) of APMV-1 RNA in allantoic fluid of inoculated fowl eggs is the common standard in diagnostic laboratories. However, the genetic variability of APMV -1 isolates should be considered carefully as potential cause for false negative results when using genetic-based laboratory tests if not shown to have inclusivity. See Sections B.1.5, B.1.8 and B.1.9 of the present chapter.

1.4. Pathogenicity index

The extreme variation in virulence of different APMV-1 isolates and the widespread use of live vaccines means that the identification of an isolate as APMV-1 from birds showing clinical signs does not confirm a diagnosis of ND, so that an assessment of the virulence of the isolate is also required (see Section B.1.6). In the past, tests including the mean death time in eggs, the intravenous pathogenicity test and variations of these tests have been used (Alexander & Senne, 2008b), but by international agreement, a definitive assessment of virus virulence is based on the intracerebral pathogenicity test (ICPI). The current WOAH definition (*Terrestrial Animal Health Code*, Chapter 10.9, *Infection with Newcastle disease virus*) also recognises the advances made in understanding the molecular basis of pathogenicity and allows confirmation of virus virulence, but not lack of virulence, by *in-vitro* tests that determine the amino acid sequence at the F0 protein cleavage site. Because of the severity of the procedure, ICPI should only be used where there is strong justification based on the epidemiological circumstances, for example in the first isolate from an outbreak (index case). It would not be appropriate to use ICPI for isolates detected in the course of routine surveillance of unvaccinated healthy birds. It is recommended to use only when other methods such as gene sequencing and clinical data provide anomalous results.

The *in-vivo* pathogenicity tests on strains isolated from species other than chickens (pigeons or doves for instance) can cause some problems and may not produce accurate readings until passaged in chickens or embryonated chickens' eggs as has frequently been reported for PPMV-1. A more accurate indication of the true pathogenicity of ND viruses for a susceptible species could come from experimental infection of a statistically significant number (≥10) of young and adult birds with a viral standard dose (e.g. $10^5 EID_{50}$) administered via natural routes (e.g. oro-nasal route).

1.4.1. Intracerebral pathogenicity index

i) Fresh infective allantoic fluid, free from influenza A and other extraneous agents, with a HA titre $>2^4$ (>1/16) is diluted 1/10 in sterile isotonic saline with no additives, such as antibiotics.

ii) 0.05 ml of the diluted virus is injected intracerebrally into each of ten chicks hatched from eggs from an SPF flock. These chicks must be over 24-hours and under 40-hours old at the time of inoculation.

iii) The birds are examined every 24 hours for 8 days.

iv) At each observation, the birds are scored: 0 if normal, 1 if sick, and 2 if dead. (Birds that are alive but unable to eat or drink should be killed humanely and scored as dead at the next observation. Dead individuals must be scored as 2 at each of the remaining daily observations after death.)

v) The intracerebral pathogenicity index (ICPI) is the mean score per bird per observation over the 8-day period.

The most virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic and asymptomatic enteric strains will give values close to 0.0. Any score of ≥ 0.7 is considered virulent.

1.5. Molecular basis for pathogenicity

During replication, APMV-1 particles are produced with a precursor glycoprotein, F0, which has to be cleaved to F1 and F2 for the virus particles to be infectious. This post-translational cleavage is mediated by host-cell proteases. Trypsin is capable of cleaving F0 for all NDV strains.

It would appear that the F0 molecules of viruses virulent for chickens can be cleaved by a host protease or proteases found in a wide range of cells and tissues, and thus allow the virus to spread throughout the host damaging vital organs, but F0 molecules in viruses of low virulence are restricted in their cleavability

to certain host proteases resulting in restriction of these viruses to growth only in certain host-cell types, such as epithelial cells of the respiratory and gastrointestinal tracts.

Most APMV-1 viruses that are pathogenic for chickens have the sequence ^{112}R/K-R-Q/K/R-K/R-R^{116} (Choi *et al.*, 2010) at the C-terminus of the F2 protein and F (phenylalanine) at residue 117, the N-terminus of the F1 protein, whereas the viruses of low virulence have sequences in the same region of ^{112}G/E-K/R-Q-G/E-R^{116} and L (leucine) at residue 117. Some of the pigeon variant viruses (PPMV-1) examined have the sequence ^{112}G-R-Q/K-K-R-F^{117}, but give high ICPI values (Meulemans *et al.*, 2002). Thus there appears to be the requirement of at least one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at 113 if the virus is to show virulence for chickens. However, some PPMV-1 may have virulent cleavage sites with variable ICPI values (Heiden *et al.,* 2014). This phenomenon has been associated not only with the fusion protein, but also with the replication complex consisting of the nucleoprotein, phosphoprotein and polymerase (Dortmans *et al.*, 2011; Heiden *et al.,* 2014).

Several studies have been undertaken using molecular techniques to determine the F0 cleavage site sequence by reverse-transcription polymerase chain reaction (RT-PCR), either on the isolated virus or on tissues and faeces from infected birds, followed by analysis of the product by nucleotide sequencing with a view to establishing a routine *in-vitro* test for virulence (Miller *et al.*, 2010). Determination of the F0 cleavage sequence may give a clear indication of the virulence of the virus, and this has been incorporated into the definition of ND (see Section B.1.6).

In the diagnosis of ND it is important to understand that the demonstration of the presence of virus with multiple basic amino acids at the F0 cleavage site confirms the presence of virulent or potentially virulent virus, but that failure to detect virus or detection of APMV-1 without multiple basic amino acids at the F0 cleavage site using molecular techniques does not confirm the absence of virulent virus. Primer mismatch, or the possibility of a mixed population of virulent and avirulent viruses, means that virus isolation and an *in-vivo* assessment of virulence, such as an ICPI, will still be required. Determination of the cleavage site by sequencing or other methods has become the method of choice for initial assessment of the pathogenicity of these viruses and has been incorporated into agreed definitions. This has reduced the number of *in-vivo* tests, although the initial Sanger sequencing result of a F0 cleavage site for APMV-1 should be confirmed by either inoculation of birds or deep sequencing using high throughput sequencing with a minimum of 1000 reads to confirm no virus subpopulations of virulent virus.

Analyses of viruses isolated in Ireland in 1990 and during the outbreaks of ND in Australia since 1998 have given strong evidence that virulent viruses may arise from progenitor viruses of low virulence. Virulent virus has also been generated experimentally from low virulence virus by passage in chickens (Dortmans *et al.,* 2011).

1.6. Description of Newcastle disease

The vast majority of bird species appear to be susceptible to infection with APMV-1 of both high and low virulence for chickens, although the clinical signs seen in infected birds vary widely and are dependent on factors such as: the virus, host species, age of host, infection with other organisms, environmental stress and immune status. In some circumstances infection with the extremely virulent viruses may result in sudden high mortality with comparatively few clinical signs. Less acute signs include depression, diarrhoea, oedema of the head and neurological signs including torticollis but with very high levels of mortality. Egg shells may be soft, or birds may stop laying altogether. Moderately virulent (mesogenic) strains typically produce respiratory signs with neurological sequelae and levels of mortality <50%. Some strains, such as those from pigeons, may induce diarrhoea with neurological signs in fowl together with greatly reduced egg production. Low virulence strains produce mild respiratory disease or none at all. Exacerbating circumstances including co-infection with other pathogens, and poor husbandry may cause apparent increases in virulence. Thus, the clinical signs are variable and influenced by other factors so that none can be regarded as pathognomonic. Furthermore, the clinical spectrum of signs cannot easily be distinguished from those of high pathogenicity avian influenza.

Even for susceptible hosts, ND viruses produce a considerable range of clinical signs. Generally, variation consists of clusters around the two extremes in the ICPI test, but, for a variety of reasons, some viruses

may show intermediate virulence. The broad variation in virulence and clinical signs necessitates the careful definition of what constitutes ND for the purposes of trade, control measures and policies.

The definition of Newcastle disease for the purpose of disease notification and control measures is given in the *Terrestrial Animal Health Code.*

1.7. Monoclonal antibodies

Mouse monoclonal antibodies (MAbs) directed against strains of NDV have been used in HI tests to allow rapid identification of NDV without the possible cross-reactions with other APMV serotypes that may occur with polyclonal sera. Many MAbs have been produced that give reactions in HI tests that are specific for particular strains or variant NDV isolates (Alexander *et al.*, 1997).

Panels of MAbs have been used to establish antigenic profiles of NDV isolates based on whether or not they react with the viruses. Typical patterns of reactivity of PPMV-1 strains to MAbs can be used to differentiate these from other APMV-1 rapidly and inexpensively.

1.8. Phylogenetic studies

Development of improved techniques for nucleotide sequencing, the availability of sequence data for an increasing number of APMV-1 viruses in online databases and the demonstration that even relatively short sequence lengths could give meaningful results in phylogenetic analyses, have led to such approaches being widely used. Considerable genetic diversity has been detected, but viruses sharing temporal, geographical, antigenic or epidemiological parameters tend to fall into specific genetic lineages or genotypes and this has proven valuable in assessing both the global epidemiology and local spread of ND (Diel *et al.,* 2012; Dimitrov *et al.,* 2019).

The greater availability and increased speed of production of sequence data using sophisticated, commercially available kits for RT-PCR and automatic sequencing hardware now means that phylogenetic studies are within the capabilities of an increasing number of diagnostic laboratories and can give meaningful results that are contemporaneous rather than retrospective (Miller *et al.*, 2010). To classify new APMV-1 viruses, Diel *et al.* (2012) designed a unified nomenclature and a classification system based on the phylogenetic analyses of complete F gene sequences and on objective criteria to separate APMV-1 into genotypes and sub-genotypes. This work confirmed the existence of two main distinct phylogroups termed class I and II (Miller *et al.,* 2010). Class I largely includes low virulence strains recovered from live bird markets and wild waterfowl worldwide, while class II comprises the vast majority of viruses of high and low virulence isolated from poultry and wild birds. Within classes, a single genotype was revealed for Class I viruses whereas class II NDV isolates have been divided in 18 genotypes. Further revisions to the system of Diel *et al.* have been adopted based on clearly defined metrics for assigning viruses to genotypes and subgenotypes. This system analysed 1956 viruses and defined class II into 22 genotypes and class I into two genotypes (Dimitrov *et al.,* 2019). Through the use of the reference sequence datasets provided, these nomenclatures can be applied to phylogenetic investigations, with any new virus being assigned to a specific class (Class I or II) and to an established or new genotype. However, any virus classification system in use must be reviewed regularly, to ensure that any new or emerging viruses that fall outside the current classification system can be incorporated. Overall this analysis enables rapid epidemiological assessment of the origins and spread of viruses responsible for outbreaks. Furthermore, it enables clear separation of APMV-1 from vaccine viruses that may be detected as part of routine surveillance in poultry and facilitate precise mapping of such viruses including likely vaccine in the case of the latter.

1.9. Molecular techniques in diagnosis

The use of molecular techniques to detect APMV-1 in clinical samples have become the methods of choice in many diagnostic laboratories. In addition, RT-PCR and sequencing is widely used for the determination of the virulence of APMV-1 viruses (see Section B.1.5) or for phylogenetic studies. Care should be taken in the selection of clinical samples as some studies have demonstrated lack of sensitivity in detecting virus in some organs and particularly in faeces (Creelan *et al.*, 2002). Tracheal or oropharyngeal swabs are often used as the specimens of choice, because they are easy to process and usually contain little extraneous organic material that can interfere with RNA recovery and amplification by PCR. However, tissue and organ samples and even faeces have been used with success as they may

contain higher viral loads of APMV-1. Strains of APMV-1 can have differential tropisms so it is recommended to collect and test both oropharyngeal and cloacal swabs when using this sample type.

The system used for RNA extraction will also affects the success of the RT-PCR on clinical samples and even with commercial kits care should be taken in selecting the most appropriate or validated system for the processing of samples to be analysed. Increasingly, given the difficulties in shipping biological materials containing infectious substances, commercially available chemically treated cards designed for the shipment of specimens for DNA and RNA analysis have been used (Perozo *et al.,* 2006), particularly in situations where the virus is already known to be endemic in a country.

Due to the large genetic diversity among APMV-1 viruses (Diel *et al.,* 2012; Dimitrov *et al.,* 2019), a laboratory testing algorithm should ideally be developed to take account of the purpose for which the test is being applied. Furthermore, this should consider the relevance of the test for the detection of local contemporaneously circulating strains in particular (but also consider risk for introduction of new viruses) and should be appropriately validated for the purpose for which it is used. This could include testing through inter laboratory ring trials. A molecular testing algorithm for NDV should ideally comprise a screening assay of high sensitivity and inclusivity targeting a conserved gene (i.e. matrix, L protein or polymerase genes) followed by a pathotyping assay. More usually applied to positive samples in screening assays is further examination using methodology directed to the F gene sequence determination enabling classification of virus as virulent (NDV) or avirulent APMV-1.

The main challenge with the use of conventional RT-PCR in diagnosis is the necessity for post-amplification processing because of the high potential for contamination of the laboratory and cross contamination of samples. Extreme precautions and strict regiments for handling samples are therefore necessary to prevent this (see Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases*). However, one of the strategies used to circumvent this issue is to apply real-time RT-PCR techniques. The advantages are that real-time RT-PCR assays are based on the fluorogenic hydrolysis probes or fluorescent dyes which eliminates the post-amplification processing step and can provide results in a few hours. These types of assay have been successfully used during ND outbreaks when the laboratory may be required to test large numbers of samples. It may be that during an outbreak a bespoke assay with a perfect match and high sensitivity to the circulating strain may be applied. In principle, when confirming a new event, it is appropriate to use a combination of tests (i.e. at least two distinct independent laboratory tests for antigen or nucleic acid detection) and is certainly recommended for the confirmation of an index case.

This could be a sensitive real-time RT-PCR screening assay using a highly conserved gene followed by gene sequencing to determine virulence.

It is possible that bespoke assays that amplify a specific portion of the genome might provide added value, for example by amplifying part of the F gene that contains the F0 cleavage site so the product can additionally be used for simultaneous pathotyping (Fuller *et al.,* 2009; Steyer *et al.,* 2010) or sequence analysis. However due to the genetic diversity described in Section B.1.8 *Phylogenetic studies* universal F gene specific RT-PCR tests to detect and pathotype virulence remains a challenge. Furthermore, due to the variability in the F0 region encoding the cleavage site, currently available tests are of limited use and may fail to detect variants.

Most of the viruses affecting poultry reside in APMV-1 class II, but within this class there is extensive genetic diversity and even highly conserved genes such as the matrix or L protein contain some heterogeneity that might lead to false negatives during investigations. Furthermore, given the extensive use of live vaccine strains, such assays will not reliably discriminate between lentogenic, mesogenic and velogenic viruses. A detection system based on the L gene has been extensively validated with a wide range of genotypes (Sutton *et al.,* 2019) and provides a sensitive and reliable assay for the detection of both class I 1 and class II strains. Furthermore, it has been proven to have high utility during inter-laboratory trials producing reliable and reproducible results. Other assays have been developed to include both class I and class II viruses (Kim *at al.,* 2008). There is a wide range of other assays that have been reported and used, but they generally lack consistency on a broad spectrum of analyses including sensitivity, so should be carefully selected according to the local environment and strains circulating.

Some assays use more than one gene for detection and pathotyping APMV-1, for example both the F-gene and the M-gene in a single-tube reaction. In addition, duplex real-time assays have been developed

that can simultaneously detect and differentiate NDV and avian influenza virus (AIV) (Nguyen *et al.,* 2013). However, more recent developments include the application of analyser-based multiplex PCR technologies, for the simultaneous detection and differentiation of an even wider range of avian pathogens (Xie *et al.*, 2014). Rapid high-throughput methods have been also developed, which combine one-tube multiplex RT-PCR with bead-based hybridisation and detection technology, to simultaneously identify a number of avian respiratory viruses in a single or mixed infection (Laamiri *et al.,* 2016). At present, it should be noted that multiplexing RT-PCR or real-time RT-PCR assays aiming at broadening the range of virus detection frequently result in reduced sensitivity of the test compared with single target assays (Fuller *et al.*, 2010).

1.10. Gene sequencing

Currently real-time RT-PCR is the preferred method of virus surveillance because the test provides rapid sensitive diagnostics for NDV and is available in high throughputs. However, greater use of sequencing technologies, particularly as unit costs reduce with improvement in technology, offer powerful opportunities to simultaneously detect and sequence from clinical samples in a laboratory or field setting, for example applying nanopore technology (Butt *et al.,* 2018).

Sanger sequencing methodology has been widely used for decades and enables the rapid determination of typically a single (F) target gene in 24–36 hours to define virus virulence and still has widespread utility. However, as genomic data can be rapidly determined using high throughput sequencing technology, it enables a broader analysis using a range of bioinformatics tools. For example, with the advent of greater access to sequencing methodology either through specialised laboratories or commercial providers it is now possible to determine the genomic sequences of NDV from birds to provide a level of characterisation important in rapid pathogen identification and outbreak intervention. Conventionally nucleotide sequences have been used in outbreak epidemiology to infer virus origin and precise relationships between different viruses associated within the same event (by phylogeny) to support outbreak management. Virus gene sequences of F or HN neuraminidase can rapidly be compared with known sequences in gene databases and used to reveal closest match thereby identifying the virus. This often avoids the need to culture the virus for rapid identification although reliability and quality of data reduces with increasing cycle threshold values in samples from real-time RT-PCR testing. Occasionally such analyses have been applied at the whole genome level and can offer greater analytical specificity to the analyses when dealing with groups of very closely related ND viruses.

2. Serological tests

NDV may be employed as an antigen in a wide range of serological tests, enabling neutralisation or enzyme-linked immunosorbent assays (ELISA) and HI to be used for assessing antibody levels in birds. At present, the HI test is most widely used for detecting antibodies to APMV-1 in birds while the use of commercial ELISA kits to assess post-vaccination antibody levels is common. In general, virus neutralisation or HI titres and ELISA-derived titres correlate at the flock level rather than at the level of individual birds. Serological assays are also used in diagnostic laboratories to assess antibody response following vaccination, but have limited value in surveillance and diagnosis of ND because of the almost universal use of vaccines in domestic poultry. Furthermore, reference antigens produced with historic strains may reduce the sensitivity of HI assay when used for the detection of antibodies against ND viruses currently circulating. For this reason it is important to investigate the antigenic relationships between the antigen used in the laboratory and current circulating viruses.

2.1. Haemagglutination and haemagglutination inhibition tests

Chicken sera rarely give nonspecific positive reactions in the HI test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken red blood cells (RBCs), so this property should first be determined and then removed by adsorption of the serum with chicken RBCs. This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, shaking gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 ***g*** for 2–5 minutes and the adsorbed sera are decanted.

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply in the use of V-bottomed (U-bottomed can be used but care in reading is required as the clarity is less defined) microwell plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic PBS (0.01 M), pH 7.0–7.2, and RBC taken

from a minimum of three SPF chickens and pooled in an equal volume of Alsever's solution (if SPF chickens are not available, blood may be taken from unvaccinated birds monitored regularly and shown to be free from antibodies to NDV). Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

2.1.1. Haemagglutination test

i) 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.

ii) 0.025 ml of the virus suspension (i.e. infective or inactivated allantoic fluid) is placed in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/5, 1/7, etc.

iii) Twofold dilutions of 0.025 ml volumes of the virus suspension are made across the plate.

iv) A further 0.025 ml of PBS is dispensed to each well.

v) 0.025 ml of 1% (v/v) chicken RBCs is dispensed to each well.

vi) The solution is mixed by tapping the plate gently. The RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, when control RBCs should be settled to a distinct button.

vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

2.1.2. Haemagglutination inhibition test

i) 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.

ii) 0.025 ml of serum is placed into the first well of the plate.

iii) Twofold dilutions of 0.025 ml volumes of the serum are made across the plate.

iv) 4 HAU virus/antigen in 0.025 ml is added to each well and the plate is left for a minimum of 30 minutes at room temperature, i.e. about 20°C, or 60 minutes at 4°C.

v) 0.025 ml of 1% (v/v) chicken RBCs is added to each well and, after gentle mixing, the RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for about 60 minutes at 4°C if ambient temperatures are high, when control RBCs should be settled to a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (positive serum, virus/antigen and PBS controls) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre >1/4 (>2^2 or >$\log_2$ 2 when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

The value of serology in diagnosis is clearly related to the expected immune status of the affected birds. HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2^4 or $\log_2$ 4 when expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (2^3 or $\log_2$ 3) or more. Back titration of antigen should be included in all tests to verify the number of HAU used.

In vaccinated flocks that are being monitored serologically, it may be possible to identify anamnestic responses as the result of a challenge infection with field virus, but great care should be exercised as variations may occur from other causes. For example, it has been demonstrated that APMV-3 virus infections of ND-virus-vaccinated turkeys will result in substantially increased titres to NDV (Alexander *et al.*, 1983).

2.2. Enzyme-linked immunosorbent assay

There is a variety of commercial ELISA kits available including those formally registered with WOAH[1] and these are based on several different strategies for the detection of NDV antibodies, including indirect, sandwich and blocking or competitive ELISAs using MAbs. At least one kit uses a subunit antigen. Usually such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully and their performance verified within the laboratory. The HI test and ELISA may measure antibodies to different antigens; depending on the system used ELISAs may detect antibodies to more than one antigen while the HI test is probably restricted to those directed against the HN protein. However, comparative studies have demonstrated that the ELISAs are reproducible and have high sensitivity and specificity; they have been found to correlate well with the HI test (Brown *et al*., 1990). Conventional ELISAs have the disadvantage that it is necessary to validate the test for each species of bird for which they are used. Competitive ELISAs may not recognise all strains of APMV-1 if they use MAb known for their specificity for single epitopes.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

A detailed account of all aspects of NDV vaccines, including their production and use, has been published (Brown *et al.,* 2019) and should be referred to for details of the procedures outlined here. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements. If virulent forms of APMV-1 (NDV) are used in the production of vaccines or in challenge studies, the facility should meet the requirements for an appropriate biosafety and containment level determined by biorisk analysis as described in chapter 1.1.4.

In this section, conventional live and inactivated vaccines will be considered, as these are still used universally. However, it should be remembered that there has been much recent work on the application of molecular biology techniques to the production of new vaccines, and success has been reported in obtaining protective immunity with recombinant fowlpox virus, vaccinia virus, pigeonpox virus, turkey herpesvirus and avian cells in which the HN gene, the F gene, or both, of NDV are expressed. Several of these recombinant viruses have been approved for use in certain countries.

NDV strains used in conventional commercial live virus vaccines fall into two groups: lentogenic vaccines, such as Hitchner-B_1, LaSota, V4, NDW, I2 and mesogenic vaccines, such as Roakin, Mukteswar and Komarov. Strains from both these groups have been subjected to selection and cloning to fulfil different criteria in their production and application. The mesogenic vaccine viruses all have two pairs of basic amino acids at the F0 cleavage site and ICPI values of around 1.4. This means that infections of birds with these viruses would fall within the intended definition of ND (Section B.1.6), but as these vaccines are used primarily in countries where ND is endemic this may not necessarily preclude their use. In the USA, the 9CFR 121.3b.818 states that NDV strains with ICPI values equal to or greater than 0.7 are virulent and reportable, leaving APMV-1 isolates of low virulence to be used as vaccines. The European Union stated in their Commission Decision 93/152/EEC (European Commission, 1993) that for routine ND vaccination programs the viruses used as live NDV vaccines are to be tested under specific conditions and have an ICPI of less than 0.4 or 0.5, depending on the dose of vaccine given. WOAH Biological Standards Commission similarly recommended in 2000 that in principle vaccines should have an ICPI <0.7. However, in order to account for interassay and interlaboratory variability a safety margin should be allowed so that vaccine master seed virus strains should not have an ICPI exceeding 0.4.

Live virus vaccines may be administered to birds by incorporation in the drinking water, delivered as a coarse spray (aerosol), or by intranasal or conjunctival instillation. A live vaccine formulated from a APMV-1 of low virulence for use *in ovo* has been approved for use in the USA. Some mesogenic strains

1 https://www.woah.org/en/scientific-expertise/registration-of-diagnostic-kits/background-information/

are given by wing-web intradermal inoculation. Vaccines have been constructed to give optimum results through application by specific routes.

Inactivated vaccines are considerably more expensive than live vaccines, and their use entails handling and injecting individual birds. They are prepared from allantoic fluid that has had its infectivity inactivated by the addition of formaldehyde or beta-propiolactone (BPL). This is incorporated into an emulsion with mineral oil or vegetable oil, and is administered intramuscularly or subcutaneously. Individual birds thus receive a standard dose. There is no subsequent spread of virus or adverse respiratory reactions. Both virulent and avirulent strains are used as seed virus although, from the aspect of safety control, the use of the latter appears more suitable. As no virus multiplication takes place after administration, a greater amount of antigen is required for immunisation than for live virus vaccination.

The duration of immunity depends on the vaccination programme chosen. One of the most important considerations affecting vaccination programmes is the level of maternal immunity in young chickens, which may vary considerably from farm to farm, batch to batch, and among individual chickens. For this reason, one of several strategies is employed. Either the birds are not vaccinated until 2–4 weeks of age when most of them will be susceptible, or 1-day-old birds are vaccinated by conjunctival instillation or by the application of a coarse spray. This will establish active infection in some birds that will persist until maternal immunity has waned. Revaccination is then carried out 2–4 weeks later. Vaccination of fully susceptible 1-day-old birds, even with live vaccines of the lowest virulence, may result in respiratory disease, especially if common pathogenic bacteria are present in significant numbers.

Re-vaccination of layers should be performed at sufficiently frequent intervals to maintain an adequate immunity. Vaccination programmes often employ slightly more pathogenic live virus vaccines to boost immunity than those used initially. These more pathogenic live vaccines may also be used following initial vaccination with oil emulsion inactivated vaccines. Layers that have high serological titres for NDV are protected against drop in egg production and poor egg quality (shell-less, soft shelled eggs, off-coloured eggs) (Brown *et al.,* 2019). The level of homology between the vaccine strain and the field virus can influence the degree of protection against reduced egg production (Cho *et al.,* 2008).

When devising a vaccination programme, consideration should be given to the type of vaccine used, the immune and disease status of the birds to be vaccinated, and the level of protection required in relation to any possibility of infection with field virus under local conditions (Brown *et al.,* 2019). Two examples of vaccination programmes that may be used in different disease circumstances are listed here. For the first example, when the disease is mild and sporadic, it is suggested that the following order of vaccination be adopted: live Hitchner-B_1 by conjunctival or spray administration at 1 day of age; live Hitchner-B_1 or LaSota at 18–21 days of age in the drinking water; live LaSota in the drinking water at 10 weeks of age, and an inactivated oil emulsion vaccine at point of lay. For the second example, when the disease is severe and more widespread, the same protocol as above is adopted up to 21 days of age, and this is followed by revaccination at 35–42 days of age with live LaSota in the drinking water or as an aerosol; this revaccination is repeated at 10 weeks of age with an inactivated vaccine (or a mesogenic live vaccine) and again repeated at point of lay (Brown *et al.,* 2019). The first protocol is generally applicable to countries were virulent NDV is not endemic and is intended to minimise productivity losses by using a milder vaccine during the initial vaccination. Considering possible constraints of ND vaccination, particularly applying to live vaccines, proper immunisation should be validated by serological testing of vaccinated flocks. Regardless of which test system would be applied, i.e. ELISA or HI, humoral immune response should be demonstrated at the flock level.

When HI is used to evaluate the immune response after vaccination, it should be taken into account that HI titres are greatly influenced by the quality of vaccine, the route and method of administration, environmental and individual factors, but also depend on the species (e.g. generally the HI response of some species, such as turkey and pigeon, is lower than that of chicken). It is also recommended to inactivate nonspecific haemagglutinating agents often present in the serum of some species such as game birds (pheasant, partridge, etc.), quails, ostriches and guinea fowl, by heat treatment in a water bath at 56°C for 30 minutes.

Single vaccinations with live lentogenic virus may produce a response in susceptible birds of about 4–6 $\log_2$, but HI titres as high as 11 $\log_2$ or more may be obtained following a vaccination programme involving oil-emulsion vaccines. The actual titres obtained and their relationship to the type of protection and duration of immunity for a given flock and programme are difficult to predict. Variation in HI titres

may occur for nonspecific factors, for instance due to the antigenic correlations, infection with other APMVs (e.g. APMV-3) may result in significant increased titres to NDV. The HI titre is also influenced by the characteristics of antigen used. For instance, the use of the homologous La Sota antigen in the HI assay after vaccination with this virus resulted in significantly higher titres than when heterologous Ulster virus was used (Maas *et al.,* 1998). Furthermore, reference antigens produced with historic strains may reduce the sensitivity of HI assay when used for the detection of antibodies against ND viruses currently circulating. For this reason, it is important to investigate the antigenic relationships between the antigen used in the laboratory and current circulating viruses, and between vaccine strains and reference HA antigens, to avoid misjudgements in estimating serum antibody titres.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The first principle to consider when selecting a strain for a live NDV vaccine is whether it is to be used as a primary or a secondary vaccine, the main consideration being its pathogenicity. The methods of application and frequency of use are valid considerations. In general, the more immunogenic live vaccines are more virulent, and are therefore more likely to cause adverse side effects. For example, vaccination with the LaSota strain will cause considerably greater problems in young susceptible birds than the Hitchner-B_1 strain, the Ulster based vaccines, or specific LaSota clones, although in general the regular LaSota vaccine induces a stronger immune response. There is detectable variation in the antigenicity of different circulating strains, which may indicate a need to tailor vaccines more carefully to relate antigenically to any prevalent field virus (Miller *et al.*, 2007).

Live vaccines using either of two avirulent Australian NDV strains selected for their heat stability, V4 or I-2, have been used with animal feed acting as carriers to combat the specific problems associated with village chicken rearing in developing countries with variable success. The intention is that this vaccine could be coated on food easily fed to roaming chickens while being slightly more resistant to inactivated by high ambient temperatures. Vaccines with both viral strains have been formulated that produce sufficient HI antibody titres (Olabode *et al.*, 2010) and in some instances prevent mortality after virulent challenge (Wambura, 2011).

The most important consideration in selecting a seed for the preparation of inactivated vaccine is the amount of antigen produced when grown in embryonated eggs; it is rarely cost-effective to concentrate virus. Both virulent and lentogenic strains have been used as inactivated vaccines, but the former offer an unnecessary risk because the manipulation of large quantities of virulent virus is involved, as well as the dangers of inadequate inactivation and possible subsequent contamination. Some lentogenic strains grow to very high titres in eggs.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The master seed should be checked after preparation for sterility, safety, potency and extraneous agents. The master seed should be free of bacterial (including Salmonella), fungal, and mycoplasma contamination, and should be free of extraneous viruses. In addition to laboratory tests for the detection of avian lymphoid leukosis, cytopathic and hemadsorbing agents, chicken anaemia virus and reticuloendotheliosis virus, the master seed used in live vaccines should be evaluated for pathogens by inoculation into embryonated chickens' eggs as well as by inoculation into healthy chickens that have not been vaccinated against ND.

2.2. Method of manufacture

2.2.1. Procedure

The vaccine production facility should operate under the appropriate bio-security procedures and practices. If ND, as defined in Section B.1.6 of this chapter, is used for vaccine production or for vaccine–challenge studies, that part of the facility where this work is done should meet the requirements for biosafety and biosecurity as outlined in chapter 1.1.4 of this *Terrestrial Manual.*

A master seed is established, and from this a working seed. If the strain has been cloned through a limiting dilution or plaque selection, the establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised aliquots (0.5 ml). Seed viruses of unknown pedigree should be passed through SPF eggs and cloned before producing the master seed. Some passage through SPF chickens may also be desirable (Allan *et al.*, 1978).

For vaccine production, a working seed, from which batches of vaccine are produced, is first established by expansion of an aliquot of master seed to a sufficient volume to allow vaccine production for 12–18 months. It is best to store the working seed in liquid form at –60°C or lower as lyophilised virus does not always multiply to high titre on subsequent first passage (Allan *et al.*, 1978).

Most ND vaccines are produced in embryonated chickens' eggs, and live virus vaccines should be produced in SPF eggs. The method of production is large-scale aseptic propagation of the virus with all procedures performed under sterile conditions. It is usual to dilute the working seed in sterile PBS, pH 7.2, so that roughly 10^3–10^6 EID_{50}/0.1–0.2 ml is inoculated into the allantoic cavity of 9- or 10-day-old embryonated SPF chickens' eggs. These are then incubated at 37°C. Eggs containing embryos that die within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure maximum yield with the minimum number of embryo deaths.

The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the allantoic fluids aspirated after depression of the embryo. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination before large pools are made for lyophilisation or inactivation. Live vaccines are usually lyophilised. The methodology depends on the machinery used and the expertise of the manufacturers, but this is a very important step as inadequate lyophilisation results in both loss of titre and a reduced shelf life.

In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000) or BPL (a typical final concentration is 1/2000–1/4000). The time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are not concentrated; the inactivated allantoic fluid is usually emulsified with mineral or vegetable oil. The exact formulations are generally commercial secrets.

Generally, oil-based inactivated vaccines are prepared as primary emulsions of water-in-oil. The oil phase usually consists of nine volumes of highly refined mineral oil, such as Marcol 52, Drakeol 6VR or BayolF, plus one volume of emulsifying agent, such as Arlacel A, Montanide 80 or Montanide 888. The aqueous phase is the inactivated virus to which a non-ionic emulsifier such as Tween 80 has been added. The oil phase to aqueous phase ratio is usually 1:1 to 1:4. Manufacturers strive to reach a balance between adjuvant effect, viscosity and stability. If the viscosity is too high viscosity and the vaccine is difficult to inject; too low viscosity and the vaccine is unstable.

2.2.2. Requirements for substrates and media

Most live virus vaccines are grown in the allantoic cavity of embryonated chickens' eggs but some, notably some mesogenic strains, have been adapted to a variety of tissue culture systems. In the USA, both live and killed ND vaccines are prepared in SPF eggs.

2.2.3. In-process controls

For those produced in eggs, the most important process control is testing for bacterial and fungal contamination. This is necessary because of the occasional occurrence of putrefying eggs, which may remain undetected at the time of harvest. In the USA, passage is not required unless the results are inconclusive.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. In the USA, several purity tests are conducted on each serial of a live vaccine. Most of these may be omitted for killed products if the inactivating agent renders the test results meaningless.

ii) Safety

Some countries also require back passage studies for live NDV vaccine to ensure that the pathogenicity is not increased by cycling through birds (Code of Federal Regulations [CFR], 2019).

iii) Batch potency

Each batch of live vaccine virus should be tested for viability and potency. For inactivated vaccines, the efficacy of the process of inactivation should be tested in embryonated eggs, taking 25 aliquots (0.2 ml) from each batch and passing each three times through SPF embryos (Allan *et al.*, 1978).

Most countries have published specifications for the control of production and testing of NDV vaccines, which include the definition of the obligatory tests on vaccines during and after manufacture. In Europe, the European Pharmacopoeia states that it is not necessary to repeat the potency test on each batch if it has been shown that a representative batch of the final product from the master seed has passed the potency test.

In the USA, each serial batch of inactivated ND vaccine is tested for potency by vaccination-challenge (CFR, 2019). At least ten vaccinates and ten control birds, 2–6 weeks of age, must be used. At least 90% of the control birds must show typical signs of Newcastle disease or die, and at least 90% of the vaccinates must remain normal during the 14 day post-challenge period. In the USA, each serial batch and each subserial of live ND vaccine must have a virus titer that is at least $10^{0.7}$ EID_{50} greater than the titer of the virus used in the immunogenicity study described above (CFR, 2019). The minimum titre shall not be less than $10^{5.5}$ EID_{50}.

The infectivity of live virus vaccines is tested by titrating the virus in embryonated chickens' eggs to calculate the EID_{50}. This involves making tenfold dilutions of virus; 0.1 ml of each dilution is inoculated into five 9 to 10-day-old embryonated chickens' eggs. After 5–7 days of incubation at 37°C, the eggs are chilled and tested for the presence of haemagglutinin activity, which is an indication of the presence of live virus. The EID_{50} end-point is calculated using a standard formula such as Spearman–Kärber or Reed Muench (Thayer & Beard, 2008).

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Live NDV vaccines may represent a hazard to humans. ND viruses, both virulent and of low virulence for chickens have been reported to have infected humans, usually causing acute conjunctivitis following direct introduction to the eye. Infections are usually transient and the cornea is not involved.

Mineral oil emulsion vaccines represent a serious hazard to the vaccinator. Accidental injection of humans should be treated promptly by washing of the site with removal of the material, including incision of tissues, as for a 'grease-gun' injury.

ii) Reversion-to-virulence for attenuated/live vaccines

The 9CFR 113.329.768 states that in the USA the use of chickens for the testing of NDV vaccines involves the inoculation of twenty-five SPF birds, five days of age or younger. Ten

doses of live vaccine are administered supraconjunctivally to each bird and the birds are then observed for 21 days. No chicken should show serious clinical signs and none should die from causes attributable to the vaccine. An alternative is to use the prechallenge part of the potency test, described below, as a safety test and if unfavourable reactions that are attributable to the product occur, the test is declared inconclusive and the safety test is repeated. If not repeated satisfactorily, the batch is declared unsatisfactory (CFR, 2019). In the USA the safety test is done with a single dose, administered to chickens 2–6 weeks old (CFR, 2019); the prechallenge part of the potency test can serve as the safety test.

In view of the finding that virulent NDV can emerge by mutation from virus of low virulence, the introduction of wholly new strains of ND in live vaccines should be considered carefully and the vaccines subjected to evaluation before use. Recombinant strains that are used in live vaccines in the USA are subject to additional safety requirements. The genetic stability of the virus should be demonstrated at the highest passage level to be used in production. The phenotypic effect of any genetic modification(s) should be thoroughly assessed to ensure that the genetic modifications have not resulted in any unexpected effects *in vivo*. Studies should be performed in chickens to evaluate possible alterations in tissue tropism, as well as to evaluate whether the vaccine virus is shed. Recombinant strains that are shed into the environment must be evaluated for safety in non-target avian species as well as in mammalian species, and the ability to persist in the environment under field conditions should be addressed.

iii) Environmental consideration

None.

2.3.2. Efficacy requirements

i) For animal production

Various methods for the testing of NDV vaccines for potency have been proposed. The importance of using a suitable challenge strain for assessment has been stressed (Allan *et al.*, 1978). Challenge strains used in Europe and the USA are Herts 33 or GB Texas, respectively. For live vaccines, the method recommended involves the vaccination of 10 or more SPF or other fully susceptible birds, some countries specify 20 birds, at the minimum recommended age by the suggested route using the minimum recommended dose. After 14–28 days, each vaccinated bird and ten control birds are challenged intramuscularly with at least 10^4 EID (50% egg infectious dose) or 10^5 LD_{50} (50% lethal dose) of ND challenge virus. Challenged birds are observed for 14 days; at least 90% of the control birds must develop clinical signs and die within 6 days of Newcastle disease. If at least 90–95% of the vaccinates do not remain free of clinical signs, the master seed is unsatisfactory.

For inactivated vaccines, in Europe 21- to 28-day-old SPF or susceptible chickens are used. Three groups of 20 birds each are injected intramuscularly with volumes of vaccine equivalent to 1/25, 1/50 and 1/100 of a dose. A group of ten chickens is kept as controls. All the birds are challenged by intramuscular injection of 10^6 LD_{50} of ND challenge virus, 17–21 days later. Chickens are observed for 21 days. The PD_{50} (50% protective dose) is calculated by standard statistical methods. The test is only valid if challenged control birds all die within 6 days. The vaccine complies with the test if the PD_{50} is not less than 50 per dose and if the lower confidence limit is not less than 35 PD_{50} per dose. Some control authorities accept a test at 1/50 only, for animal welfare reasons. It is not necessary to repeat the potency test on each batch if it has been shown that a representative batch of the final product from the master seed has passed the test.

The recommended efficacy test for inactivated vaccines in the USA is a vaccination–challenge study (CFR, 2009). At least ten SPF chickens, 2–6 weeks old, are vaccinated with the minimum recommended dose. The 9CFR 113.205.727 states that after 14 days post-vaccination, the vaccinates and at least ten unvaccinated controls are challenged with the GB Texas strain of Newcastle disease virus and the vaccinates are observed for 14 days. At least 90% of the control birds must develop clinical signs of Newcastle disease during the observation period. If at least 90% of the vaccinates do not remain free of clinical signs, the master seed is unsatisfactory.

ii) For control and eradication

The level of immunity reached with any single dose or regimen of ND vaccination will vary enormously with both vaccine and host species. The level of immunity required in a given host (i.e. to protect against death, disease, meat or egg production losses) is extremely complex and difficult to evaluate. Generally, some assessment of the longevity of serum antibodies should be made and vaccine regimens adopted to maintain these above an acceptable level (Allan *et al.*, 1978). Most commercial vaccines have been designed to control clinical signs however they do not prevent viral replication and are not suitable for eradication.

Transmission of the ND virus in an area might be interrupted only if a very high percentage of the resident susceptible population (> 80%) is sufficiently immunised showing an Ab titre ≥ 1:8 (Brown *et al.,* 2019).

2.3.3. Stability

When stored under the recommended conditions the final vaccine product should maintain its potency for at least the designated shelf life of the product. Accelerated stability tests such as reduction of infectivity following incubation at 37°C for 7 days (Lensing, 1974) may be used as a guide to the storage capabilities of a batch of live vaccine. Oil emulsion vaccines should also be subjected to accelerated ageing by storing at 37°C, for a minimum of 1 month, without separation of the aqueous and oil phases. The USA requires real-time stability to be demonstrated on at least three sequential serials of NDV vaccine (CFR, 2019). Each serial should be evaluated at multiple intervals until the expiration date has been reached in order to develop a degradation profile for the product.

Live virus vaccines must be used immediately after reconstitution. Inactivated vaccines must not be frozen. In most countries, preservatives must not be included in the freeze-dried live product, but antimicrobial preservatives may be incorporated in the diluent used to reconstitute the vaccine. An alternative used in the USA is to allow the use of certain preservatives, but they must be indicated on the labelling.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

The advent of recombinant DNA technology has resulted in the development of novel NDV vaccines. One class consists of vector vaccines, which consist of a suitable carrier virus that expresses one or more immunogenic NDV proteins (usually F and/or HN), thereby inducing an immune response against both NDV and the vector virus itself. Examples of such vector vaccines are recombinants based on Vaccinia virus (Meulemans, 1988), Fowlpox virus (Boursnell *et al.*, 1990; Olabode *et al.*, 2010), Pigeonpox virus (Letellier *et al.*, 1991), Herpesvirus of turkeys (Heckert *et al.*, 1996), Marek's disease virus (Sakaguchi *et al.*, 1998) and avian adeno-associated virus (Perozo *et al.*, 2008).

Other approaches include the development of subunit vaccines based on the large scale expression of NDV proteins (usually F and/or HN) using baculovirus vectors (Lee *et al.*, 2008); or plants (Berinstein *et al.*, 2005) and the use of DNA vaccines, i.e. plasmid DNA encoding relevant immunogenic NDV proteins (Loke *et al.*, 2005). The establishment of a reverse genetics system for NDV (Peeters *et al.*, 1999) has made it possible to genetically modify the NDV genome and to develop NDV strains with new properties. These include the implementation of serological differentiation (DIVA vaccines (Mebatsion *et al.*, 2002; Peeters *et al.*, 2001) and the incorporation and expression of foreign genes, thereby making NDV itself a vaccine vector for application in poultry (Nakaya *et al.*, 2001) and other species, including primates (Dinapoli *et al.*, 2007).

The desired profile for NDV vaccines include: 1) prevention of transmission; 2) differentiation of infected from vaccinated animals (DIVA); 3) induction of protection with a single dose; 4) maternal antibody override; 5) mass vaccination; 6) cross-protection against variant strains, 7) Increased safety and minimal side effects. Some of the above-mentioned recombinant vaccines reach or surpass the efficiency of conventional vaccines in terms of antibody induction or protection against a virulent challenge strain, and thus they show great promise for future use. Moreover, they offer a number of advantages compared

to conventional NDV live vaccines, such as i) improved safety for vaccinated birds due to the absence of residual virulence, ii) implementation of the DIVA principle, iii) closer immunogenic match with outbreak strains, iv) possibility of an optimally controlled *in ovo* mass vaccination, v) long lasting immunity provided by a single vaccine shot (Palya *et al*., 2014).

Only few of the above-mentioned biotechnological vaccines have been approved in certain countries for application in poultry. A problem for some of the vaccines mentioned here may be that existing immunity against the vector might interfere with generic application of such vaccines in the field. As most vector vaccines are based on viruses that are themselves potential avian pathogens, it is difficult to guarantee complete safety under field circumstances. These problems can be overcome with the apathogenic turkey herpesvirus based recombinant vaccine (Palya *et al.,* 2012). The fact that most of these vaccines are genetically modified organisms (GMO) means that they have to go through a rigorous and tedious testing and registration process. Furthermore, the production of biotechnological vaccines is likely more expensive than that of classical NDV vaccines. As currently used classical vaccines are cheap and adequate, at least for the protection of poultry against clinical signs and death, a real incentive for veterinary pharmaceutical companies to develop new vaccines is lacking. It is likely that poultry farmers would be willing to pay a higher price for a vaccine only if it offers significant advantages over conventional vaccines. It is unlikely that this situation will soon change unless national or international authorities modify the requirements for ND vaccines such as a minimum requirement for the reduction of shedding of challenge virus, interruption of virus transmission or the implementation of the DIVA principle. It must also be considered that the application of new, safe and efficacious vectored vaccines *in ovo* or at 1 day of age, when used alone in countries with a high risk of ND, will not provide the necessary protection. In these epidemiological scenarios early protection must be reinforced by the administration at the hatchery of a live attenuated vaccine followed by a booster vaccination at approximately 2 weeks of age as a protective immunity induced by vector-based vaccines is only achieved some weeks after the administration.

Recurrent outbreaks of ND in the face of vaccination has raised the question whether currently used ND vaccines are still adequate, not only for the protection against clinical disease, but also for the inhibition of virus transmission (Mayers *et al.,* 2017). Some studies have indicated that the extent of homology between vaccine and challenge strain is important in reducing the shedding of virulent virus (Hu *et al*., 2009; Miller *et al*., 2007) but this may not necessarily correlate with disease or transmission. It has been demonstrated that exchanging the F and HN genes of a vaccine strain with the corresponding genes of an outbreak strain resulted in a vaccine that was much better able to reduce virus shedding of the outbreak strain than the unmodified vaccine. However, vaccine failure may be attributable to many factors including importantly, poor vaccination practices. Dortmans *et al.* (2014) demonstrated that susceptibility of vaccinated poultry to NDV infection was not a result of vaccine mismatch, when they challenged field vaccinated chickens with contemporary NDV of different genotypes. Further work is required to fully define and understand factors that will lead to reduced vaccine efficacy in a field environment.

3.2. Special requirements for biotechnological vaccines

Once regulatory approval has been gained, biotechnological vaccines have to fulfil the same or similar requirements as classical vaccines as detailed above (Section C: Requirements for vaccines and diagnostic biologicals).

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NB: There are WOAH Reference Laboratories for Newcastle disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Newcastle disease

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.3.15.

TURKEY RHINOTRACHEITIS (AVIAN METAPNEUMOVIRUS INFECTIONS)

SUMMARY

Avian metapneumovirus (*aMPV*) *principally causes an acute highly contagious upper respiratory tract infection sometimes combined with reproductive disorders, primarily of turkeys, chickens and ducks. The disease produced by aMPV was originally referred to as avian pneumovirus infection and avian rhinotracheitis; it also has been referred to as turkey rhinotracheitis* (*TRT*) *in turkeys and as the triggering pathogen in swollen head syndrome* (*SHS*) *in chickens. aMPV is a single-stranded non-segmented negative-sense RNA virus belonging to the family* Pneumoviridae, *genus* Metapneumovirus*. The disease can cause significant economic losses for the poultry industry, particularly when exacerbated by secondary pathogens. Other avian species known to support the replication of aMPVs, other than turkeys, chickens, Muscovy and Peking ducks, are pheasants, and guinea fowl. The disease has global distribution in poultry-producing regions, with only Oceania and Canada reported to be free of aMPV infection. Four antigenically distinct subgroups, A, B, C and D, of aMPV have been identified by neutralisation with monoclonal antibodies, possible limited cross reactivity in enzyme-linked immunosorbent assay* (*ELISA*), *and sequence analysis of the attachment glyco protein, G. Recent sequence data suggest that additional subgroups may exist in gulls and parakeets.*

Public health significance: *aMPV has not been reported to cause human infections. A human MPV has been identified worldwide as a pathogen causing bronchiolitis in infants, the elderly or the immunocompromised but the two viruses are clearly different.*

Detection of the agent: *Virus isolation in cell cultures, embryonated chicken eggs, and tracheal organ cultures, as well as molecular methods for identification of the nucleic acid, have all been used successfully to detect aMPV. The degree of success depends on the strain of virus, type and timeliness of sample collection, as well as storage and handling of specimens. Electron microscopy, virus neutralisation and molecular techniques can be used to identify the virus. Infectious virus can only usually be isolated for approximately ten days after infection.*

Monoclonal antibodies to the spike glycoprotein, G, have been used in virus neutralisation tests to differentiate subgroups A and B, while neutralisation tests using polyclonal antiserum have shown that subgroups A and B belong to a single serotype. Subgroup C is neutralised poorly by subgroup A or B monospecific antiserum, and not by monoclonal antibodies that differentiate subgroups A and B. These data suggest that subgroup C represents a second serotype of aMPV. Monospecific antiserum and monoclonal antibodies can be used for agent identification by virus neutralisation and immunofluorescence staining of infected cell cultures; however antigenic characteristics need to be considered. The immunodiffusion test has also been used to confirm aMPV isolates.

Molecular procedures based on the nucleoprotein (*N*), *matrix* (*M*), *fusion* (*F*), *small hydrophobic* (*SH*), *G and polymerase L genes of aMPV have been used for the detection and or genomic subgrouping of aMPV. Conventional reverse-transcription polymerase chain reaction* (*RT-PCR*) *procedures can be used for aMPV genomic subgrouping. Different sets of either subgroup-specific or broadly reactive PCR primers have been defined, however, a single set of RT-PCR primers directed to the N gene have been shown to detect subgroups A, B, C and D and could possibly be used as universal primers for the detection of aMPV. A pan-MPV real-time RT-PCR, that detects both aMPV and human MPV* (*see below*) *has also been developed.*

Serological tests: *The most commonly employed method is the ELISA. Other methods that have been used are virus neutralisation* (*VN*), *immunofluorescence and immunodiffusion tests. The VN test*

can be performed in primary tracheal organ cultures, chicken embryo fibroblast (CEF) and chicken embryo liver (CEL); several cell lines such as Vero, MA104 or QT35 have also been used successfully. Numerous commercial ELISA kits, as well as in-house assays, have been developed. For optimal sensitivity, homologous strain of aMPV should be used as antigen because of inter-subgroup variations in antigenicity. In many countries where the disease is endemic, vaccination is also practised, complicating interpretation of the results. Ideally, serum samples from birds in the acute phase of disease and also from convalescent birds should be obtained for testing.

Requirements for vaccines: *Two types of vaccine are commercially available for the control of TRT and SHS: live attenuated vaccines, and inactivated oil-emulsion adjuvanted vaccines.*

A. INTRODUCTION

Avian metapneumovirus (aMPV), previously referred to as avian pneumovirus (APV) and avian rhinotracheitis (ART) virus, causes an acute, highly contagious upper respiratory tract infection of turkeys and chickens and ducks. The onset of clinical signs and spread of infection through a flock can be rapid occurring as quickly as 2–4 hours. In turkeys, the virus causes a disease known as turkey rhinotracheitis (TRT). The aetiological agent is an enveloped virus with an unsegmented single-stranded negative-sense RNA virus of approximately 14 kilo bases contained in a nucleocapsid with a helical symmetry. The virus exhibits some characteristics of a pneumovirus, but differs from mammalian pneumoviruses at the molecular level. aMPV is the type strain of the genus, *Metapneumovirus,* in the family *Pneumoviridae* (Kuhn *et al.,* 2020). Metapneumoviruses have been detected in humans and are associated with respiratory tract infection in children (Van Den Hoogen *et al*., 2001). Avian and human metapneumoviruses have no non-structural NS1 and NS2 proteins and their gene order (3'-N-P-M-F-M2-SH-G-L-5') is different from that of mammalian pneumoviruses (3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5') (Tanaka *et al*., 1995). aMPV has been further classified into four subgroups: A, B, C and D based on reactivity against monoclonal antibodies, cross reactivity in the enzyme-linked immunosorbent assay (ELISA) and neutralisation tests, and nucleotide sequence analysis (Cook *et al*., 1993a). However recent reports from North America suggest the existence of two new isolates that are distinct from A, B, C and D subgroups. Phylogenetic analysis based on L gene sequences show that they are closer to the subgroup C viruses than to A, B and D (Canuti *et al.,* 2019; Retallack *et al.,* 2019). A recent book chapter has been dedicated to these aspects and can be sourced for further reading (Brown & Eterradossi 2019).

Infection with aMPV can occur from a very young age in turkeys and is characterised by snicking, rales, sneezing, nasal discharge, foaming conjunctivitis, swelling of the infraorbital sinuses and submandibular oedema (Pringle, 1998). Secondary adventitious agents can dramatically exacerbate the clinical signs. In an uncomplicated infection, recovery is rapid and the birds appear normal in approximately 14 days. When husbandry is poor or secondary bacterial infection occurs, airsacculitis, pericarditis, pneumonia, and perihepatitis may prolong the disease and there may be an increase in morbidity and mortality (Mekkes & De Wit, 1999). Among secondary agents that have been shown to exacerbate and prolong clinical disease are *Bordetella avium*, *Pasteurella*-like organisms, *Mycoplasma gallisepticum*, *Chlamydophila* and *Ornithobacterium rhinotracheale* (Alkhalaf *et al*., 2002; Jirjis *et al*., 2004; Senne *et al*., 1997; Van Loock *et al*., 2006). In addition, other co-infections with viruses such as infectious laryngotracheitis, infectious bronchitis, paramyxovirus-1 (avian orthoavulavirus-1) or fungi such as *Aspergillus fumigatus* have been reported (Croville *et al.,* 2018). Morbidity can be as high as 100%, with mortality ranging from 0.5% in adult turkeys to 80% in young poults (Van De Zande *et al*., 1999). Clinical signs of infection in chickens include nasal discharge and depression, but they are less characteristic than those in turkeys. Severe respiratory distress may occur in broiler chickens particularly when exacerbated by secondary pathogens such as infectious bronchitis virus, mycoplasmas, and *Escherichia coli* (O'Brien, 1985; Pattison *et al*., 1989). Unlike subgroup A and B, the United States of America (USA) strain – Colorado, or subgroup C – has not been shown to naturally induce disease in chickens, although experimentally infected chickens were shown to be susceptible to a subgroup C turkey isolate of aMPV. Different strains of aMPV have been shown to have a specific tropism for chickens or turkeys (Cook *et al*., 1993b). Other species of birds have been reported to have been infected with aMPV, however clinical signs have rarely been reported. Viruses characterised as subgroup C aMPV and shown to have 75–83% nucleotide identity to the US Colorado subgroup C aMPV have been associated with respiratory signs and decreased egg production in ducks in France (Toquin *et al*., 2006). Retrospective molecular analysis of viruses isolated in the 1980s from turkeys in France indicates the presence of a fourth subgroup of aMPV designated subgroup D.

Most recently a series of experimental studies was published in which the host range of aMPV A, B, C and D was assessed in turkeys, chickens and Muscovy ducks (Brown *et al.,* 2019). Overall these trials showed that aMPV-A, B, Turkey C and D were viruses well adapted to Galliformes, especially turkeys. An aMPV-C duck isolate was well adapted to ducks, however chickens and turkeys seroconverted and were positive by virus isolation. Likewise, the

turkey aMPV-C virus was well adapted to turkeys yet was also isolated from chickens. Other experimental studies suggest that direct contact is necessary for bird-to-bird spread of the disease (Alkhalaf *et al*., 2002). In commercial conditions aerogenous infection following airborne transmission is also likely as the disease is restricted to the respiratory tract. Following experimental infection of 2-week-old turkeys with aMPV alone, the virus was detected in the respiratory tract for only a few days (Bayon-Auboyer *et al*., 1999). However, in birds inoculated with aMPV and *B. avium,* virus was detected for up to 7 days post-inoculation (dpi) (Collins & Gough, 1988; Cook *et al*., 1993b). There is no evidence that aMPV can result in a latent infection and no carrier state is known to exist. Although neonatal turkeys are occasionally infected (Shin *et al*., 2002a), there have been no reports of vertical transmission of aMPV.

In growing turkeys, virus replication is limited to the upper respiratory tract with a short viraemia. Replication of both attenuated and virulent strains of aMPV persists for approximately 10 dpi (Van De Zande *et al*., 1999). Limited replication occurs in the trachea, and lung, but virus has not been shown to replicate in other tissues following natural infection (Cook, 2000). Sequential histopathological and immunocytochemical studies have shown viral replication in the turbinates causing a serous rhinitis with increased glandular activity, epithelial exfoliation, focal loss of cilia, hyperaemia and mild mononuclear infiltration in the submucosa and eosinophilic intracytoplasmic inclusions in the ciliated cells of the turbinates at 2 dpi. A catarrhal rhinitis with mucopurulent exudate, damage to the epithelial layer and a copious mononuclear inflammatory infiltration in the submucosa was seen 3–4 dpi. Transient lesions were seen in the trachea, with little or no lesions present in the conjunctiva and other tissues (Giraud *et al*., 1988; Majo *et al*., 1995). Respiratory infection is less severe in laying turkeys; however, there may be a drop in egg production of up to 70% (Stuart, 1989) and the quality of eggs during the recovery period, up to 3 weeks, may be poor. In experimentally infected laying turkeys, viral replication has been demonstrated in both the respiratory and genital tracts up to 9 dpi.

In chickens, there is strong evidence to suggest aMPV is one of the aetiological agents of swollen head syndrome (SHS). The syndrome is characterised by respiratory disease, apathy, swelling of infraorbital sinuses and unilateral or bilateral periorbital and facial swelling, extending over the head. These signs are frequently followed by cerebral disorientation, torticollis and opisthotonos. Although mortality does not usually exceed 1–2%, morbidity may reach 10%, and egg production is frequently affected (Gough *et al*., 1994; Morley &Thomson, 1984; O'Brien, 1985; Pattison *et al*., 1989; Picault *et al*., 1987; Tanaka *et al*., 1995).

Serological evidence suggests aMPV is widespread throughout the world and of considerable economic importance, particularly in turkeys. Oceania and Canada are the only regions that have not reported aMPV in poultry (Cook, 2000). There is serological and molecular evidence that aMPV occurs in a variety of other avian species, including pheasants, guinea fowl, ostriches, passerines and various waterfowl (Shin *et al*., 2002b), but there is no evidence of disease except in pheasants.

aMPV has not been reported to cause human infections. A human MPV has been identified worldwide as a pathogen causing bronchiolitis in infants, the elderly or the immunocompromised. hMPV is genetically most related to aMPV subgroup C (amino acid identity can be 88% or higher for the most conserved proteins). However hMPV and aMPV-C exhibit striking differences in their SH and G proteins (approximately 30% amino acid identity) demonstrating the two viruses are clearly different.

A more detailed account of the disease and its causal virus may be found in Swayne *et al.* (2020).

B. DIAGNOSTIC TECHNIQUES

Isolation and identification of the agent provide the most certain diagnosis of aMPV, but are not usually attempted for routine diagnostic purposes as the virus may prove difficult to isolate and these tests are labour intensive. Thus indirect tests are more frequent: i) immunological demonstration of specific antibodies to the virus in serum or ii) molecular demonstration of viral RNA in tissues or tissue secretions. Available methods for diagnosis are shown in Table 1 and will have different degrees of usage depending on the objectives.

Table 1. Test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Agent isolation	+[b]	–	–	+[e]	–	–
Virus detection by RT-PCR	+[b]	++[d]	–[b]	+++	–	+[c]
Virus characterisation (nucleotide sequencing)	+	–	–	+++	–	–
Antigen detection in respiratory tissues	+[b]	–	–	+++	–	–
Detection of immune response						
ELISA	+++[b]	+++	+++	+[g]	+++	+++
VN	+[f]	+[f]	–	–	+	++[f]

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction;
ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.
[a]A combination of agent identification methods applied on the same clinical specimen is recommended.
[b]If performed on a large scale and always negative in an area where no live vaccination is performed, or to check for subclinical aMPV.
[c]Could be used post-vaccination to check replication of live vaccine in the respiratory tract.
[d]Could be negative if infection occurred several weeks before testing.
[e]Labour intensive and needs to be complemented with genotyping using full length genome sequences.
[f]Labour intensive however, critical to correlate the presence of detected antibody with protection.
[g]Could be used for case confirmation under certain circumstances, for example if two series of serological samples 3 weeks apart are available.

1. Detection of the agent

To maximise the chances of successfully isolating the virus, a multiple approach to diagnosis is recommended. This is particularly relevant when dealing with different subgroups or genotypes that may require varied *in-vitro* virus isolation methods. This was illustrated in the USA with the failure of the first attempts to isolate subgroup C aMPV. The USA subgroup C has not been associated with ciliostasis, in tracheal organ cultures (Senne *et al*., 1997), and the agent was only cultured following multiple embryo and cell culture passages. This was in contrast to the experience in Europe and elsewhere in which tracheal organ cultures and/or Vero cells were shown to be the most reliable method for the primary isolation of subgroup A, B, C and D of aMPV (Giraud *et al*., 1988).

1.1. Collection and selection of diagnostic specimens

It is very important to take samples for attempted virus isolation in the early stages of infection as the virus may be present only in the sinuses and turbinates for a short period. Ideally, the upper respiratory tract of live birds in the acute phase of the disease should be sampled using sterile swabs (Stuart, 1989). The most successful samples have been nasal exudates, choanal cleft swabs and scrapings of sinus and turbinate tissue. The virus has also been isolated from trachea and lungs, and occasionally viscera of affected turkey poults. Isolation of virus is rarely successful from birds showing severe chronic signs as

the extreme clinical signs are usually due to secondary adventitious agents. This certainly applies to SHS of chickens in which the characteristic signs appear to be due to secondary (e.g. *Escherichia coli*) bacterial infection. Furthermore, for reasons that are unclear, virus isolation from chickens may be more difficult than from turkeys.

It is essential that samples should be sent immediately on ice to the diagnostic laboratory. When delays of more than 3 days are expected, the samples should be frozen prior to dispatch. Swabs for attempted virus isolation should be sent on ice fully immersed in viral transport medium. Swabs for polymerase chain reaction (PCR) analysis can be sent dry, but on ice or frozen.

For virus isolation, a 20% (v/v) suspension of the nasal exudate or homogenised tissue is made in phosphate-buffered saline (PBS) or brain–heart infusion (BHI) broth containing antibiotics, at pH 7.0–7.4. This is then clarified by centrifugation at 1000 ***g*** for 10 minutes and the supernatant is passed through a 450 nm membrane filter.

1.2. Culture and Identification of avian metapneumovirus (aMPV)

The best method for primary virus isolation from infected birds is in tracheal organ cultures or embryonated turkey or chicken eggs with subsequent cultivation in cell cultures; serial passage on Vero cells has also been found to be a sensitive method for the isolation of aMPV (Giraud *et al*., 1988). The original isolation of aMPV in South Africa in the late 1970s and the more recent Colorado aMPV were carried out in embryonated eggs, however subgroup A and B aMPV isolations have routinely been made in tracheal organ cultures. Subgroup C aMPV, do not cause ciliostasis in organ cultures; for this reason: embryonating chicken eggs and subsequent passage on to cell culture are the preferred method for virus isolation (Senne *et al*., 1997). All four aMPV subgroups can be isolated using Vero cells.

Tracheal organ cultures are prepared from turkey embryos or very young turkeys obtained from flocks free of specific antibodies to aMPV. Tracheas from chicken embryo or 1- to 2-day-old chicks may also be used. Transverse sections of trachea are rinsed in PBS (pH 7.2), placed one section per tube in Eagles medium with antibiotics, and held at 37°C. After incubation, the media is removed from the cultures and 0.1 ml of bacteria-free inoculum is added. After incubation for 1 hour at 37°C, growth medium is added and the cultures are incubated at 37°C on a roller apparatus, rotating at 30 revolutions per hour. Cultures are examined daily after agitation on a laboratory mixer to remove debris from the lumen. Ciliostasis may occur within 7 days of inoculation on primary passage, but usually is produced rapidly and consistently only after several blind passages.

For isolation in eggs, 6- to 8-day-old embryonated chicken or turkey eggs from flocks known to be free of aMPV antibodies are inoculated by the yolk-sac route with 0.2–0.3 ml of bacteria-free material from infected birds and incubated at 37°C. If there is no evidence of infection (embryo stunting or mortality) after the first passage, yolk sac material should be processed for a second blind embryo passage. Within 7–10 days, there is usually evidence of stunting of the embryos with few deaths. Serial passage is often required before the agent causes consistent embryo mortality. Isolation in embryonating eggs is a slow, expensive, labour intensive process and requires multiple subsequent cell culture passages for identification.

Various cell cultures have been used for the primary isolation of aMPV, including chicken embryo cells, Vero cells and more recently the QT-35 cells, with varying degrees of success. Primary isolation of the USA subgroup C has been made after multiple (5–6 serial passages) in Vero cell cultures. However, once the virus has been adapted to growth in embryonating eggs or tracheal organ cultures, in which it grows only to low titres, the virus will readily replicate to moderate titres following multiple passages in a variety of primary chicken or turkey embryo cells, Vero cells, and QT-35 cells (Cook, 2000). The primary isolation of all four subgroups of aMPV has proven successful following serial passage on Vero cells. The virus produces a characteristic cytopathic effect (CPE) with syncytial formation within 7 days. Identification of virus-infected cell cultures can be by immunofluorescence staining of infected cells or molecular methods.

Paramyxovirus-like morphology of the virus can be observed by negative-contrast electron microscopy. Pleomorphic fringed particles, roughly spherical and 80–200 nm in diameter are commonly seen. Occasionally much larger filamentous forms are present, which may be up to 1000 nm in length. The surface projections are 13–14 nm in length and the helical nucleocapsid that can sometimes be seen

emerging from disrupted particles, is 14 nm in diameter with an estimated pitch of 7 nm per turn (Collins & Gough, 1988; Giraud *et al*., 1988).

1.3. Molecular identification

Reverse-transcription PCR (RT-PCR) is a significantly more sensitive and rapid method for the detection of aMPV than standard virus isolation methods because of the fastidious nature of aMPV . RT-PCR procedures targeted to the F, M, N and G genes are used for the detection of aMPV; however, because of molecular heterogeneity between aMPV strains, most RT-PCR procedures are subgroup specific or do not detect all subgroups (Bayon-Auboyer *et al*., 1999; Pedersen *et al*., 2000; 2001). Subgroup specific assays are successfully used for the detection and diagnosis of endemic strains (Mase *et al*., 2003; Naylor *et al*., 1997; Pedersen *et al*., 2001). However, limitations of subgroup -specific assays need to be recognised when conducting diagnostic testing for respiratory disease. Primers directed to conserved regions of the N gene have been shown to have broader specificity, detecting representative isolates from A, B, C, and D subgroups (Bayon-Auboyer *et al*., 1999). RT-PCR assays directed to the G gene have also been successfully used for genotype or subgroup identification (Lwamba *et al*., 2005; Mase *et al*., 1996). A variety of RT-PCR techniques have been developed and evaluated and these have been extensively reviewed elsewhere (Njenga *et al*., 2003).

Nasal exudates, choanal cleft swabs, and turbinate specimens collected 2–7 days post-exposure are the preferred specimen (Cook *et al*., 1993b; Pedersen *et al*., 2001; Stuart, 1989). It is imperative to collect specimens when clinical signs are first exhibited as recent studies have shown that the maximum amount of virus is present in the trachea and nasal turbinates at 3 days post-inoculation and viral RNA persists for 9 days in the trachea and up to 14 days in the nasal turbinates (Velayudhan *et al*., 2005). It has been shown that aMPV can be detected from specimens collected 7–10 days post-exposure, however the viral concentration is considerably less thus reducing success of detection (Alkhalaf *et al*., 2002; Pedersen *et al*., 2001). Swabs from a single flock can be pooled in groups of not more than five to allow the processing of samples from a larger number of birds and therefore increasing the potential recovery rate.

Template RNA for RT-PCR can be extracted from homogenised tissue, dry swabs or wet swab pools with silica column or magnetic bead commercial RNA extraction reagents according to the manufacturer's protocol.

Table 2. Example of primers that can be used for the detection of a region of the N gene of subgroups A, B, C and D of aMPV (Bayon-Auboyer et al., 1999; Lemaitre et al., 2018)

Target	Primer ID	Sequence 5' → 3'	Position	Product size	Type of test
N Gene	Nc	5'–TTC-TTT-GAA-TTG-TTT-GAG-AAG-A–3'	632–653	RT primer	End point RT-PCR
	Nx	5'–CAT-GGC-CCA-ACA-TTA-TGT-T–3'	830–812	115	
	Nd	5'–AGC-AGG-ATG-GAG-AGC-CTC-TTT-G–3'	716–737	115	
N Gene	PanMPV /N1fwdA	5'-CTG-TTT-GTG-AAC-ATT-TTY-ATG-CA-3'	718–740 (aMPV A/B/D)		SYBR green real time RT-PCR
	PanMPV /N1AMPVDfwdA	5'-CTG-GTT-GTG-AAC-ATA-TTC-ATG-CA-3'	727–749 (aMPV C)		
	PanMPV /N1RevB	5'-ACA-GAG-ACA-TGG-CCT-AAC-ATD-AT-3'	824–802 (aMPV A/B/D) 833–811 (aMPV C)		

1.3.1. Example protocol (end-point RT-PCR)

i) Synthesis of the cDNA can be carried out in 20 µl volume with the Nc RT primer (or any convenient primer, such as an oligodT or the reverse primer of the primer pair that will be

used in the PCR) and a suitable reverse transcriptase enzyme. Heat 1 µl RT primer (2 pmol), 1 µl dNTP mix (10 mM each), with extracted RNA and sterile distilled water (QS to 20 µl) to 65°C for 5 minutes.

ii) Chill quickly and pulse centrifuge.

iii) Add 4 µl 5× First-Strand buffer, 2 µl 0.1 M DTT, and 1 µl of a suitable RNase.

iv) Heat contents to 42°C for 2 minutes and add 1 µl (200 units) of reverse transcriptase enzyme, mix gently.

v) RT is conducted at 42°C for 50 minutes followed by 70°C for 15 minutes for inactivation of RT enzyme.

vi) PCR amplification can be conducted with a suitable DNA polymerase according to manufacturer's instructions. Amplification conditions are as follows: 94°C for 15 minutes and 30 cycles of 94°C for 20 seconds, 51.0°C for 45 seconds (for the Nd/Nx primer pair, if another pair is used, the annealing temperature should be adapted), 72°C for 45 seconds with a final extension of 72°C for 10 minutes.

Several RT-PCR assays directed to the F, G and M genes have been successfully used for subgroup identification and detection or diagnosis of endemic aMPV (Goyzm *et al*., 2000; Jirjis *et al*., 2004; Majo *et al*., 1995). Nucleotide sequence and phylogenetic analysis of the G gene has been used to genotype subgroup A, B, C and D aMPV and is the recommended procedure for subgroup identification of an unidentified virus. Recommended RT-PCR procedures for sequence analysis of the G gene have been described (Lwamba *et al*., 2005; Toquin *et al*., 2006). A real-time RT-PCR has been demonstrated recently to allow the specific detection, identification and quantification of aMPV subgroups A, B, C and D (Guionie *et al*., 2007) and another protocol was developed for the broad spectrum detection of all MPVs (aMPV and hMPV) (Lemaitre *et al.,* 2018) and could therefore serve for the detection of emerging aMPV of yet-to-be-defined subgroups.

Procedures for the identification of subgroup A and B RNA in diagnostic specimens have also been described (Naylor *et al*., 1997), as have procedures for the detection of subgroup A and C viruses (Pedersen *et al*., 2001). Isolation of aMPV from chickens is difficult and has succeeded only in a limited number of cases; for this reason, molecular tests have been used primarily for the detection of aMPV in chickens (Mase *et al*., 1996). It is important to remember that RT-PCR detects viral RNA, not live virus, so the significance of a positive PCR result in terms of detecting an active infection has to be established.

2. Serological tests

Serology is the most common method of diagnosis of aMPV infections, particularly in unvaccinated flocks, because of difficulties in isolating and identifying aMPV. The most commonly employed method is the ELISA; however, virus neutralisation, microimmunofluorescence and immunodiffusion tests have been used. A number of commercial and in-house ELISA kits are available that are suitable for testing both turkey and chicken serum; however, differences in sensitivity and specificity between commercial kits have been reported (Eterradossi *et al*., 1995; McFarlane-Toms & Jackson, 1998; Mekkes & De Wit, 1999). Competitive or blocking ELISA kits incorporating an aMPV-specific monoclonal antibody have been developed. These kits claim to have a broad spectrum of sensitivity and specificity for all subgroups of aMPV and can be used for testing sera from a variety of avian species. In-house ELISA antigens, as described below, have been prepared in a variety of substrates including various cell cultures and tracheal organ cultures (Chiang *et al*., 2000). Generally, aMPV antibodies are less well detected when a heterologous strain of aMPV is used as antigen, even though the strains appear closely related by virus neutralisation test (Eterradossi *et al*., 1995). The situation is further complicated by discrepancies in the ability of different ELISAs to detect vaccinal antibody when different aMPV strains are used as coating antigens (Eterradossi *et al*., 1995). In-house assays using a homologous antigen have been used extensively for the surveillance of endemic aMPV strains. Ideally, both acute and convalescent serum samples should be obtained for testing. In chickens, the serological response to aMPV infection is weak when compared to the response in turkeys.

2.1. Enzyme-linked immunosorbent assay

The following protocol (Chiang *et al*., 2000), or alternative methods with well documented results (Giraud *et al*., 1987; Grant *et al*., 1987; Gulati *et al*., 2000; 2001; Luo *et al*., 2004), may be used.

Virus is propagated in chicken embryo fibroblast (CEF) or Vero cell cultures until 70–100% of the monolayer is simultaneously infected (3–4 days). The cell culture fluid is decanted and the monolayer washed with PBS (pH 7.2). The monolayer is lysed with 0.5 ml (per 75 cm^2 flask) of a 0.5% non-ionic detergent solution (IGEPAL CA-630 or Nonidet P-40) on a rocking platform for 1 hour at 4°C. Following physical disruption of lysed cells, the whole virus antigen lysate is clarified at 3000 ***g*** for 15 minutes. Uninfected cell cultures are treated in the same manner for a negative control antigen. Serial dilutions of antigen are tested against serial dilutions of anti-species IgG horseradish peroxidase conjugate in a checker-board fashion to determine the optimal antigen/conjugate dilution. A working dilution of the aMPV antigen and normal antigen (100 µl) are coated onto flat-bottom microtitre plates with a carbonate/bicarbonate coating buffer (Chiang *et al*., 2000). Each serum is tested against aMPV and normal antigen for determination of the S/P ratio. Coated plates are incubated at 4°C overnight and washed a total of five times with a Tween 20 wash solution (Chiang *et al*., 2000) prior to use or three times prior to long-term storage at –70°C. Residual wash solution remains on the plate when the plates are frozen. Following storage and equilibration to room temperature, the plates are washed twice and blotted dry prior to use.

2.1.1. Test method

i) Dilute test sera 1/40 in dilution/blocking buffer (Chiang *et al*., 2000).

ii) Apply 50 µl test sera and working dilutions of positive and negative sera to aMPV antigen and normal antigen-coated wells.

iii) Incubate at room temperature for 1 hour.

iv) Wash plates five times with Tween 20 wash solution

v) Apply 50 µl of the working dilution of anti-species IgG horseradish peroxidase conjugate to each well and incubated for 1 hour at room temperature.

vi) Wash plates five times with Tween 20 wash solution

vii) Apply 100 µl of the prepared ortho-phenylenediamine (OPD) chromogen/substrate solution to each well and incubate for 10 minutes in the dark. Combine the following reagents for preparation of OPD in a suitable substrate.

viii) Stop the reaction with 25 µl/well of 2.5 M sulphuric acid.

ix) Read the OD at 490/450 nm.

The results are expressed as the OD difference between the virus antigen-coated and negative control antigen-coated wells. Determine the mean OD_{490} reading for each duplicate set of wells with the positive and negative antigen for each serum. The antigens are usually calibrated so that a sample with an OD_{490} difference between the antigen-coated and negative control antigen-coated wells of more than 0.2 is considered positive (upon development of the method in a laboratory, this threshold may need to be re-evaluated under local conditions, by assessing a panel of negative sera with the newly prepared antigens). Sporadic nonspecific positive reactions are inherent with the ELISA, especially with chicken or duck sera, and immunofluorescence may be used for confirmation testing.

C. REQUIREMENTS FOR VACCINES

1. Background

Two types of vaccine are commercially available for the control of TRT: live attenuated vaccines, or inactivated oil-emulsion adjuvanted vaccines. The possibility exists of developing live recombinant vaccines based on a fowlpox vector expressing the F protein of aMPV (Stuart, 1989), DNA vaccines encoding various aMPV proteins (Tanaka *et al*., 1995) and, more recently, genetically attenuated aMPV produced by reverse genetics.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1.1. Live vaccines: methods of use

Live TRT vaccines are produced from virus strains that have been attenuated by serial passage in embryonating eggs, tracheal organ cultures or cell culture (various cell lines or chicken embryo fibroblasts), or by alternate passages using a combination of these methods. Commercially available live attenuated TRT vaccines have been derived from subgroup A or subgroup B aMPV isolates in Europe, and from a subgroup C aMPV isolate in the USA. The aMPV subgroup to which the vaccine belongs should be mentioned in the vaccine label, as this information is relevant to the development of efficient post-vaccination serological monitoring. Live TRT vaccines are intended for use in young birds to induce an active immune response that will help to prevent the respiratory disease caused by aMPV. Additionally, live TRT vaccines are also used in parent turkeys to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine (see below).

Live TRT vaccines are usually applied several times by coarse spray, in the drinking water, or by oculonasal administration. There is a published report on the use of a single *in-ovo* injection (Shin *et al*., 2002b), but, more often, the first TRT live vaccination is administered to turkeys at day-old or up to 7 days of age. The second TRT live vaccine is either applied around 6 weeks of age (when only two vaccinations are performed), or around 3 weeks of age (when there is a third application) or after 6 weeks of age. The rationale for these repeated vaccinations is linked first to the difficulties of inducing a prolonged antibody response lasting for the whole life of the meat turkeys, and second to the need to avoid TRT vaccination in young turkeys when they have recently been vaccinated against haemorrhagic enteritis (vaccines against haemorrhagic enteritis virus [HEV], are usually administered at around 28 days of age to avoid interference with maternally derived antibodies [MDA] to HEV). Although it has been published that MDA to TRTV do not prevent infection of day-old turkeys by virulent aMPV strains (Toquin *et al*., 2003), it has been observed that some interference between MDA and some live TRT vaccines may occur and result in lower vaccine take in young turkeys with higher MDA levels. Clinical cross-protection between live vaccine and challenge virus belonging to subgroups A or B (and *vice versa*) has been reported (Cook *et al*., 1993b; Velayudhan *et al*., 2005). Protective immunity was also observed when birds immunised against aMPV subgroups A or B were subsequently challenged with a subgroup C virulent virus, but not in the converse experiment .

Avian metapneumoviruses are very easily neutralised in the environment by physical and chemical agents and thus ensuring good live vaccination against these viruses may be demanding. If the vaccine is given in the drinking water, clean water with a neutral pH must be used and it must be free from smell or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per litre. Care must be taken to ensure that all birds receive their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the medicated water is made available and care must be taken that no residual water remains in the water adduction pipes or in the drinkers. If the vaccine is given by spray, high quality water with a neutral pH and with no disinfectant residues should be used. A specific nebuliser should be used that will be used for no other purpose but vaccination. This apparatus should ideally allow for constant pressure throughout the vaccination process (and thus for a constant size of the vaccine droplets). The turkeys to be vaccinated should be grouped together prior to vaccination and several passes with the nebuliser should be performed to ensure that all birds are indeed exposed to the spray. The ventilation and heating of the poultry house should be turned as low as practical, so that the nebulised vaccine is neither eliminated by ventilation, nor inactivated by overheating (heating moreover favours evaporation, which decreases the size of the nebulised vaccine droplets and cause an increase proportion of the vaccine to reach the lower respiratory tract, a phenomenon that has been suspected to contribute to adverse reactions to live vaccination). It is important that the birds are allowed to calm down immediately after spraying as a non-negligible amount of the vaccine may be absorbed when the birds preen their feathers after being exposed to the vaccine spray.

aMPV vaccines have been reported not to interfere with Newcastle disease vaccines in chickens (Van De Zande *et al*., 1999; Yu *et al*., 1992); however the compatibility of TRT vaccines is not documented in turkeys. As with other vaccines, only healthy birds should be vaccinated. Vials of lyophilised vaccine should be kept at temperatures between 2°C and 8°C up to the time of use.

1.2. Inactivated vaccines: method of use

Inactivated aMPV vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeder turkeys that have previously been primed by live vaccine or by natural exposure to

field virus during rearing. As the rationale to use inactivated vaccines in breeders is to improve their protection not only against the respiratory signs of TRT, but also against the reproductive signs (egg-drops) associated with aMPV infection, it is not uncommon that the inactivated aMPV vaccines also associate this virus with several other viruses also involved in respiratory and/or reproductive disorders. The usual programme is to administer the inactivated vaccine at least 4–6 weeks after the last live vaccination, up to 28 weeks of age in turkeys, avoiding the 4 last weeks before lay. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular in the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous route. A multidose syringe may be used, subject to the apparatus being in full working order and in accordance with manufacturers' instructions and recommended hygiene practices. All equipment should be cleaned and sterilised between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. The vaccine should not be frozen; it should be stored at between 4°C and 8°C instead (but should be allowed to reach room temperature before injection). Inactivated vaccines should not be exposed to bright light or high temperatures.

Only healthy birds, known to be sensitised by previous exposure to aMPV, should be vaccinated. Used in this way the inactivated vaccine should produce a good antibody response that will protect the breeders against respiratory and reproductive signs during the period of lay (Van De Zande *et al*., 2000). The precise level and duration of immunity conferred by inactivated vaccines will depend mainly on the concentration of antigen present per dose. The manufacturing objective should be to obtain a high antigen concentration and hence a highly potent vaccine.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

See also Chapter 1.1.8 Principles of veterinary vaccine production and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

2.1.1. Biological characteristics of the master seed

i) Live vaccines

The identity of live aMPV vaccines of subgroups A, B or C that are kept as master seeds for vaccine production should ideally be confirmed by deep sequencing so that any subsequent contamination by adventitious aMPV strains can be detected in the purity checks.

ii) Inactivated vaccines

For inactivated vaccines, the most important characteristics are high yield and good antigenicity.

2.1.2. Quality criteria

i) Purity

The seed live vaccine virus or inactivated vaccine must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly from avian pathogens. This includes freedom from contamination with other strains of aMPV.

Seed virus must be shown to be stable, with no tendency to revert to virulence. This can be confirmed by carrying out at least five consecutive turkey-to-turkey passages at 2- to 6-day intervals. Use turkeys not older than 3 weeks and free of MDAs against aMPV. Passage may be achieved by natural spreading or by inoculating a suspension prepared from the mucosa of the turbinates and upper trachea of the previously inoculated birds, or from tracheal swabs. Care must be taken to avoid contamination by viruses from previous passages. It must be shown that the virus was transmitted. The stability should be evaluated by demonstrating that there is no indication of an increased severity in the clinical signs when comparing the maximally passaged virus with the unpassaged vaccine. A scoring system may be used to quantify the severity of the signs.

2.1.3. Validation as a vaccine strain

Data on efficacy should be obtained before bulk manufacture of vaccine begins. The vaccine should be administered to birds in the way in which it will be used in the field. Live vaccine can be given to young birds and the response measured serologically and by resistance to experimental challenge. In the case of killed vaccines, a test must be carried out in older birds that go on to lay, using the recommended vaccination schedule, so that the prolonged seroconversion can be demonstrated. A scoring system may be used to quantify the severity of the signs

i) Live vaccine

Safety: Ten field doses of the vaccine candidate are administered by the oculonasal route to each of 10 turkeys of the minimum age recommended for vaccination and free from antibodies to aMPV. Observe the turkeys at least daily for 21 days. The vaccine fails the test if any turkey dies or shows signs of disease attributable to the vaccine. If more than two turkeys show abnormal clinical signs or die due to causes not related to the vaccine, the test must be repeated. This test is performed on each batch of final vaccine.

Efficacy test: efficacy should be tested for each of the recommended routes of vaccination. Use turkeys that are not older than the minimum age recommended for vaccination and are free of antibodies against aMPV. Administer one field dose of vaccine of the minimum recommended titre by one of the recommended routes to each of 20 turkeys, keeping 10 turkeys as non-vaccinated controls. After 21 days, challenge all turkeys by oculonasal administration of a suitable dose of a virulent strain of aMPV (suitable challenge viruses can be provided by the WOAH Reference Laboratory for TRT[1]). Observe the turkeys daily for 10 days and register their clinical signs individually. The vaccine fails the test unless at least 90% of the vaccinated turkeys survive without showing either clinical signs or lesions evocative of aMPV infection. A scoring system may be used to quantify the severity of the signs. If less than 80% of the non-vaccinated turkeys exhibit clinical signs following challenge, or more than 10% of the control or inoculated birds die from causes not attributable to the test, the test is invalid. Providing results are satisfactory, this test needs to be carried out on only one batch of all those batches prepared from the same seed lot.

ii) Inactivated vaccine

Safety of the inactivated vaccine should be tested for all recommended administration routes and with a batch of vaccine whose activity is at least the maximal activity of future commercial batches. One dose, or a double dose to ensure maximal activity, of vaccine is administered to specific antibody negative (SAN) or specific pathogen free (SPF) turkeys. Clinical signs in vaccinated turkeys are checked daily and for 14 days. The vaccine passes the test if no signs are observed and no death can be attributed to the vaccine. The test is invalid if nonspecific death occurs.

Efficacy test: as drops in egg production are not easily reproduced experimentally, vaccine-induced protection against egg drop following virulent aMPV challenge may be difficult to document and thus protocols aimed at demonstrating the reduction in excretion levels are also acceptable. Alternatively, the induction of a long-lasting immune response following injection of the inactivated vaccine may also be used. For the latter experiment, at least 20 unprimed turkeys are given one dose of vaccine at the recommended age (near to point-of-lay) by one of the recommended routes, and the antibody response is measured between 4 and 6 weeks after vaccination by ELISA or serum neutralisation. If a primary vaccination with a live vaccine is recommended, an additional group of turkeys is given only the primary vaccination so that the actual effect of the inactivated vaccine can be indeed assessed individually.

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

2.2. Methods of manufacture

2.2.1. Procedure

Seed virus may be propagated in various cell culture systems. The bulk is distributed in aliquots and freeze-dried in sealed containers.

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry.

Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. Specific pathogen free eggs must be used for all materials employed in propagation and testing of the vaccine. Live vaccines can be produced in eggs or cell cultures. Inactivated vaccines may be made using virulent virus grown in cell culture or embryonating eggs. A high virus concentration is required. These vaccines are made as water-in-oil emulsions. A typical formulation is to use 80% mineral oil to 20% virus suspension, with suitable emulsifying and preservative agents.

2.2.2. Requirements for ingredients

i) Ingredients of animal origin

All ingredients of animal origin, including serum and cells, must be checked for the presence of viable bacteria, viruses, fungi or mycoplasma. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

SPF eggs must be used for all materials employed in propagation and testing of the vaccine.

ii) Preservatives

A preservative may be required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its efficacy until the end of the shelf life should be checked. A suitable preservative already established for such purposes should be used.

2.2.3. In-process control

i) Antigen content

Having grown the virus to a high concentration, its titre should be assayed by use of tracheal organ culture or cell cultures, as appropriate, to the strain of virus being used. The antigen content or infectious titre required to produce satisfactory batches of vaccine should be based on determinations made on test vaccine that has been shown to be effective in laboratory and field trials.

ii) Inactivation of inactivated vaccines

Inactivation is often done with either ß-propiolactone or formalin. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine virus and any potential contaminants, e.g. bacteria that may arise from the starting materials.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation of the vaccine should be carried out on each batch of both the bulk harvest after inactivation and the final product. The test selected should be appropriate to the vaccine virus being used and should consist of at least two passages in susceptible cell cultures, embryos or turkeys, with ten replicates per passage. No evidence of the presence of any live virus or microorganism should be observed.

iii) Sterility of inactivated vaccines

Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are

carried out on each batch of final vaccine as described, for example, in the European Pharmacopoeia.

2.2.4. Final product batch test

i) Identity

The identity of a live aMPV vaccine can be confirmed at the batch level by incubating an appropriate dilution of the vaccine with a monospecific anti-aMPV antiserum, then inoculating the mix to susceptible SAN or SPF eggs, or susceptible tracheal organ or cell cultures. The neutralised vaccine should not exhibit any infectivity.

The identity of inactivated aMPV vaccine can be confirmed at the batch level by administrating the vaccine to SAN or SPF chickens, and demonstrating that the vaccine does induce aMPV-specific antibodies. In some instances, this test can be combined with the potency test in order to reduce the number of animals used in the experiments.

ii) Sterility and absence of extraneous agents

Tests for sterility and freedom from contamination of biological materials by bacteria, fungi, mycoplasma and extraneous agents are described in Chapter 2.3.4 *Minimum requirements for the production and quality control of vaccines*.

iii) Safety

a) Live vaccine safety test

As described under Section C.2.1.3.i *Live vaccine* (safety), ten field doses of vaccine are administered by the oculonasal route to each of 10 turkeys of the minimum age recommended for vaccination and free from antibodies to aMPV. Observe the turkeys at least daily for 21 days. The vaccine fails the test if any turkey dies or shows signs of disease attributable to the vaccine. If more than two turkeys show abnormal clinical signs or die due to causes not related to the vaccine, the test must be repeated. This test is performed on each batch of final vaccine.

b) Extraneous agents in inactivated vaccines

Ten SPF turkeys, free of maternal antibodies to aMPV and 14–28 days of age, are inoculated by the recommended routes with twice the field dose. The birds are observed for 3 weeks. No abnormal local or systemic reaction should develop. The test is performed on each batch of final vaccine, unless controls at earlier production stages complemented by implementation of good manufacturing practices advocate for the safety of the overall process.

iv) Residual live vaccine in inactivated vaccines

The process described in Section C.2.2.3.ii *In process controls* may be performed on each batch of final product.

v) Potency

a) Live vaccine potency test

A potency test (virus titration) in embryonating eggs, tracheal organ cultures or suitable cell cultures, as appropriate to the vaccine virus, must be carried out on each serial (batch) of vaccine produced. The vaccine titre at the time of issue must be high enough to guarantee that the minimum virus titre per dose will be maintained at least until the expiry date. In addition, the method described in Section C.2.1.3.i *Live vaccine* (efficacy test) must be used and yield satisfactory results on one batch representative of all the batches prepared from the same seed lot.

b) Inactivated vaccine potency test

The potency test for inactivated vaccines is developed from the results of the efficacy test on a representative batch of vaccine the master seed virus, by measuring antibody production.

As explained in Section C.2.1.3.ii *Live vaccine*, the following protocol may be followed: at least 20 unprimed turkeys are given one dose of vaccine at the recommended age (near to point-of-lay) by one of the recommended routes, and the antibody response is measured between 4 and 6 weeks after vaccination by ELISA or serum neutralisation. If a primary vaccination with a live vaccine is recommended, an additional group of turkeys is given only the primary vaccination so that the actual effect of the inactivated vaccine can be indeed assessed individually.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning the manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and C.2.2 *Methods of manufacture*) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Live attenuated aMPV vaccines of subgroup A, B and C (galliforms origin) will infect both turkeys and chickens, but ducks are not susceptible to these viruses (see Section A. *Introduction* and Brown *et al.,* 2019).

No interaction of live aMPV vaccines with non-target avian species has been documented so far. Any information regarding a negative effect in a non-target animal species should be provided in the vaccine instructions for use.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

It is critical that the potential of live attenuated aMPV vaccines to revert to virulence is assessed prior to regulatory approval (See Section C.2.1.2.i *Quality criteria* [purity] above).

Environmental considerations to be taken into account in the regulatory approval process include the knowledge of the aMPV strains that circulate in the area where the licensed vaccine will be used, as this knowledge may help i) in selecting the vaccines suitable for controlling these strains and ii) in deciding whether it is justified or not to introduce a live attenuated aMPV vaccine strain possibly significantly different from the local aMPV strains.

iii) Precautions (hazards)

aMPV is not recognised as a zoonotic agent, however precaution should be implemented in the manufacturing steps or during vaccination to minimise the exposure of staff to vaccine aerosols. Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident the person should go at once to a hospital, taking the vaccine package and manufacturer's datasheet with them. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injury.

2.3.3. Efficacy requirements

The tests, challenge models and criteria used to assess the efficacy of aMPV vaccines are described in Sections C.2.1.3.i *Live vaccine* and C.2.1.3.ii *Inactivated vaccine*. When assessing efficacy in an aMPV challenge model, it is advisable that the selected challenge virus be representative of contemporary aMPV strains that circulate in the area where the licensed vaccine will be used.

2.3.4. Vaccines permitting a DIVA strategy

No DIVA (detection of infection in vaccinated animals) vaccines are commercially available for aMPV.

2.3.5. Duration of immunity

The cell-mediated response to aMPV infection has been reported to be the main line of defence and protection has been reported to last as long as 22 weeks under experimental conditions following vaccination (Bao *et al.*, 2020; Williams *et al.,* 1991). However, in the field repeat vaccinations (2 – 3 times) are practiced in order to stimulate the cell mediated response in the respiratory tract (Rautenschlein, 2020)

2.3.6. Stability

Evidence should be provided on at least one representative batch of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.

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NB: There is a WOAH Reference Laboratory for turkey rhinotracheitis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for turkey rhinotracheitis

NB: FIRST ADOPTED IN 2008. MOST RECENT UPDATES ADOPTED IN 2022.

SECTION 3.4.

BOVINAE

CHAPTER 3.4.1.

BOVINE ANAPLASMOSIS

SUMMARY

Definition of the disease: *Bovine anaplasmosis results from infection with* Anaplasma marginale*. A second species,* A. centrale*, has long been recognised and usually causes benign infections.* Anaplasma marginale *is responsible for almost all outbreaks of clinical disease.* Anaplasma phagocytophilum *and* A. bovis*, which infect cattle, have been recently included within the genus but they are not reported to cause clinical disease. The organism is classified in the genus* Anaplasma *belonging to the family* Anaplasmataceae *of the order* Rickettsiales*.*

Description of the disease: *Anaemia, jaundice and sudden death are characteristic signs of anaplasmosis. Other signs include rapid loss of milk production and weight, but the clinical disease can only be confirmed by identifying the organism. Once infected, cattle may remain carriers for life, and identification of these animals depends on the detection of specific antibodies using serological tests, or of rickettsial DNA using molecular amplification techniques. The disease is typically transmitted by tick vectors, but mechanical transmission by biting insects or by needle can occur.*

Detection of the agent: *Microscopic examination of blood or organ smears stained with Giemsa stain is the most common method of identifying* Anaplasma *in clinically affected animals. In these smears,* A. marginale *organisms appear as dense, rounded, intraerythrocytic bodies approximately 0.3–1.0 μm in diameter situated on or near the margin of the erythrocyte.* Anaplasma centrale *is similar in appearance, but most of the organisms are situated toward the centre of the erythrocyte. It can be difficult to differentiate* A. marginale *from* A. centrale *in a stained smear, particularly with low levels of rickettsaemia. Commercial stains that give very rapid staining of* Anaplasma *are available in some countries.* Anaplasma phagocytophilum *and* A. bovis *can only be observed infecting granulocytes, mainly neutrophils.*

It is important that smears be well prepared and free from foreign matter. Smears from live cattle should preferably be prepared from blood drawn from the jugular vein or another large vessel. For post-mortem diagnosis, smears should be prepared from internal organs (including liver, kidney, heart and lungs) and from blood retained in peripheral vessels. The latter are particularly desirable if post-mortem decomposition is advanced.

Serological tests: *A competitive enzyme-linked immunosorbent assay (C-ELISA) has been demonstrated to have good sensitivity in detecting carrier animals. Card agglutination is the next most frequently used assay. The complement fixation test (CFT) is no longer considered a reliable test for disease certification of individual animals due to variable sensitivity. Cross reactivity between* Anaplasma *spp. can complicate interpretation of serological tests. In general, the C-ELISA has the best specificity, with cross-reactivity described between* A. marginale, A. centrale, A. phagocytophilum *and* Ehrlichia *spp. Alternatively, an indirect ELISA using the CFT with modifications is a reliable test used in many laboratories and can be prepared in-house for routine diagnosis of anaplasmosis.*

Nucleic-acid-based tests *have been used experimentally, and are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. A nested reaction is necessary to identify low-level carriers using conventional polymerase chain reaction (PCR) and nonspecific*

amplification can occur. Recently, real-time PCR assays with analytical sensitivity equivalent to nested conventional PCR have been described.

Requirements for vaccines: *Live vaccines are used in several countries to protect cattle against* A. marginale *infection. A vaccine consisting of live* A. centrale *is most widely used and gives partial protection against challenge with virulent* A. marginale*.*

Anaplasma centrale *vaccine is provided in chilled or frozen forms. Quality control is very important as other blood-borne agents that may be present in donor cattle can contaminate vaccines and be disseminated broadly. For this reason, frozen vaccine is recommended as it allows thorough post-production quality control, which limits the risk of contamination with other pathogens.*

Anaplasma centrale *vaccine is not entirely safe. A practical recommendation is to restrict its use, as far as possible, to calves, as nonspecific immunity will minimise the risk of some vaccine reactions that may require treatment with tetracycline or imidocarb. Partial immunity develops in 6–8 weeks and lasts for several years after a single vaccination. In countries where* A. centrale *is exotic, it cannot be used as vaccine against* A. marginale*.*

A. INTRODUCTION

Outbreaks of bovine anaplasmosis are due to infection with *Anaplasma marginale. Anaplasma centrale* is capable of producing a moderate degree of anaemia, but clinical outbreaks in the field are extremely rare. New species of *Anaplasma*, *A. phagocytophilum* and *A. bovis* (Dumler *et al*., 2001), with a primary reservoir in rodents, have been reported to infect cattle, but do not cause clinical disease (Dreher *et al*., 2005; Hofmann-Lehmann *et al.*, 2004).

The most marked clinical signs of anaplasmosis are anaemia and jaundice, the latter occurring late in the disease. Haemoglobinaemia and haemoglobinuria are not present, and this may assist in the differential diagnosis of anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can only be confirmed, however, by identification of the organism.

Anaplasma marginale occurs in most tropical and subtropical countries, and in some more temperate regions. *Anaplasma centrale* was first described from South Africa. The organism has since been imported by other countries – including Australia and some countries in South America, South-East Asia and the Middle East – for use as a vaccine against *A. marginale.*

Anaplasma species were originally regarded as protozoan parasites, but further research showed they had no significant attributes to justify this description. Since the last major accepted revision of the taxonomy in 2001 (Dumler *et al*., 2001), the Family *Anaplasmataceae* (Order *Rickettsiales*) is now composed of four genera, *Anaplasma*, *Ehrlichia, Neorickettsia,* and *Wolbachia*. The genus *Aegyptianella* is retained within the Family *Anaplasmataceae* as genus *incertae sedis*. The revised genus *Anaplasma* now contains *Anaplasma marginale* as the type species, *A. phagocytophilum* the agent of human granulocytic ehrlichiosis (formerly *Ehrlichia phagocytophila* and *E. equi*) *A. platys,* and *A. bovis. Haemobartonella* and *Eperythrozoon* are now considered most closely related to the mycoplasmas.

Anaplasma species are transmitted either mechanically or biologically by arthropod vectors. Reviews based on careful study of reported transmission experiments list up to 19 different ticks as capable of transmitting *A. marginale* (Kocan *et al*., 2004). These are: *Argas persicus, Ornithodoros lahorensis, Dermacentor albipictus, D. andersoni, D. hunteri, D. occidentalis, D. variabilis, Hyalomma excavatum, H. rufipes, Ixodes ricinus, I. scapularis, Rhipicephalus annulatus* (formerly *Boophilus annulatus), R. bursa, R. calcaratus, R. decoloratus, R. evertsi, R. microplus, R. sanguineus* and *R. simus.* However, the classification of several ticks in these reports has been questioned. Intrastadial or transstadial transmission is the usual mode, even in the one-host *Rhipicephalus* species. Male ticks may be particularly important as vectors; they can become persistently infected and serve as a reservoir for infection. Experimental demonstration of vector competence does not necessarily imply a role in transmission in the field. However, *Rhipicephalus* species are clearly important vectors of anaplasmosis in countries such as Australia and countries in Africa, and Latin America, and some species of *Dermacentor* are efficient vectors in the United States of America (USA).

Various other biting arthropods have been implicated as mechanical vectors, particularly in the USA. Experimental transmission has been demonstrated with a number of species of *Tabanus* (horseflies), and with mosquitoes of the genus *Psorophora* (Kocan *et al*., 2004). The importance of biting insects in the natural

transmission of anaplasmosis appears to vary greatly from region to region. *Anaplasma marginale* also can be readily transmitted during vaccination against other diseases unless a fresh or sterilised needle is used for injecting each animal. Similar transmission by means of unsterilised surgical instruments has been described (Reinbold *et al.*, 2010a).

The main biological vectors of *A. centrale* appear to be multihost ticks endemic in Africa, including *R. simus.* The common cattle tick (*R. microplus*) has not been shown to be a vector. This is of relevance where *A. centrale* is used as a vaccine in *R. microplus*-infested regions.

Anaplasma marginale infection has not been reported in humans. Thus, there is no risk of field or laboratory transmission to workers and laboratories working with *A. marginale* may operate at the lowest biosafety level, equivalent to BSL1.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of bovine anaplasmosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Microscopic examination	–	+	–	+++	–	–
Detection of the agent[(a)]						
PCR	–	+++	–	+++	–	–
Detection of immune response						
CAT	–	–	–	–	+-	+
ELISA	+++	+	+++	–	+++	+++
IFAT	+	–	–	–	++	++
CFT	–	–	–	–	+	-

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
Agent id. = agent identification; CAT = card agglutination test; CFT = complement fixation test; ELISA = enzyme-linked immunosorbent assay; IFAT = indirect fluorescent antibody test; PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

1.1. Microscopic examination

Samples from live cattle should include thin blood smears and blood collected into an anticoagulant. Air-dried thin blood smears can be kept satisfactorily at room temperature for at least 1 week. The blood sample in anticoagulant should be held and transferred at 4°C, unless it can reach the laboratory within a few hours. This sample is useful for preparing fresh smears if those submitted are not satisfactory. In addition, a low packed cell volume and/or erythrocyte count can help to substantiate the involvement of *A. marginale* when only small numbers of the parasites are detected in smears, for example during the recovery stage of the disease.

In contrast to *Babesia bovis, A. marginale* does not accumulate in capillaries, so blood drawn from the jugular or other large vessel is satisfactory. Because of the rather indistinctive morphology of

Anaplasma, it is essential that smears be well prepared and free from foreign matter, as specks of debris can confuse diagnosis. Thick blood films as are used sometimes for the diagnosis of babesiosis are not appropriate for the diagnosis of anaplasmosis, as *Anaplasma* are difficult to identify once they become dissociated from erythrocytes.

Samples from dead animals should include air-dried thin smears from the liver, kidney, heart and lungs and from a peripheral blood vessel. The latter is particularly recommended should there be a significant delay before post-mortem examination because, under these circumstances, bacterial contamination of organ smears often makes identification of *Anaplasma* equivocal. Brain smears, which are useful for the diagnosis of some forms of babesiosis, are of no direct value for diagnosing anaplasmosis, but should be included for differential diagnosis where appropriate.

Blood from organs, rather than organ tissues *per se*, is required for smear preparation, as the aim is to be able to examine microscopically intact erythrocytes for the presence of *Anaplasma.* Organ-derived blood smears can be stored satisfactorily at room temperature for several days.

Both blood and organ smears can be stained in 10% Giemsa stain for approximately 30 minutes after fixation in absolute methanol for 1 minute. After staining, the smears are rinsed three or four times with tap water to remove excess stain, and are then air-dried. Conditions for Giemsa staining vary from laboratory to laboratory, but distilled water is not recommended for dilution of Giemsa stock. Water should be pH 7.2–7.4 to attain best resolution with Giemsa stain. Commercial stains that give very rapid staining of *Anaplasma* are available in some countries. Smears are examined under oil immersion at a magnification of ×700–1000.

Anaplasma marginale appear as dense, rounded and deeply stained intraerythrocytic bodies, approximately 0.3–1.0 μm in diameter. Most of these bodies are located on or near the margin of the erythrocyte. This feature distinguishes *A. marginale* from *A. centrale,* as in the latter most of the organisms have a more central location in the erythrocyte. However, particularly at low levels of rickettsaemia, differentiation of these two species in smears can be difficult. Appendages associated with the *Anaplasma* body have been described in some isolates of *A. marginale* (Kreier & Ristic, 1963; Stich *et al*., 2004).

The percentage of infected erythrocytes varies with the stage and severity of the disease. Maximum rickettsaemias in excess of 50% may occur with *A. marginale.* Multiple infections of individual erythrocytes are common during periods of high rickettsaemias.

The infection becomes visible microscopically 2–6 weeks following transmission. During the course of clinical disease, the rickettsaemia approximately doubles each day for up to about 10 days, and then decreases at a similar rate. Severe anaemia may persist for some weeks after the parasites have become virtually undetectable in blood smears. Following recovery from initial infection, cattle remain latently infected for life.

1.2. Polymerase chain reaction

Nucleic-acid-based tests to detect *A. marginale* infection in carrier cattle have been developed although not yet fully validated. The analytical sensitivity of polymerase chain reaction (PCR)-based methods has been estimated at 0.0001% infected erythrocytes, but at this level only a proportion of carrier cattle would be detected. A nested PCR has been used to identify *A. marginale* carrier cattle with a capability of identifying as few as 30 infected erythrocytes per ml of blood, well below the lowest levels in carriers. However, nested PCR poses significant quality control and specificity problems for routine use (Torioni De Echaide *et al*., 1998). Real-time PCR has also been described for identification of *A. marginale* (Carelli *et al*., 2007; Decaro *et al*., 2008; Reinbold *et al*., 2010b), and should be considered instead of the nested PCR. Two advantages of this technique, which uses a single closed tube for amplification and analysis, are reduced opportunity for amplicon contamination and a semi-quantitative assay result. Equipment needed for real-time PCR is expensive, requires preventive maintenance, and may be beyond the capabilities of some laboratories. Real-time PCR assays may target one of several genes (Carelli *et al*., 2007; Decaro *et al*., 2008), or 16S rRNA (Reinbold *et al*., 2010b), and are reported to achieve a level of analytical sensitivity equivalent to nested conventional PCR (Carelli *et al*., 2007; Decaro *et al*., 2008; Reinbold *et al*., 2010b).

2. Serological tests

In general, unless animals have been treated or are at a very early stage of infection (<14 days), serology using the competitive enzyme-linked immunosorbent assay (C-ELISA), indirect ELISA (I-ELISA) or card agglutination test (CAT) (see below) may be the preferred methods of identifying infected animals in most laboratories. *Anaplasma* infections usually persist for the life of the animal. However, except for occasional small recrudescences, *Anaplasma* cannot readily be detected in blood smears after acute rickettsaemia and, even end-point PCR may not detect the presence of *Anaplasma* in blood samples from asymptomatic carriers. Thus, a number of serological tests have been developed with the aim of detecting persistently infected animals.

A feature of the serological diagnosis of anaplasmosis is the highly variable results with regard to both sensitivity and specificity reported for many of the tests from different laboratories. This is due at least in part to inadequate evaluation of the tests using significant numbers of known positive and negative animals. Importantly, the capacity of several assays to detect known infections of long-standing duration has been inadequately addressed. An exception is C-ELISA (see below), which has been validated using true positive and negative animals defined by nested PCR (Torioni De Echaide *et al.*, 1998), and the card agglutination assay, for which relative sensitivity and specificity in comparison with the C-ELISA has been evaluated (Molloy *et al.*, 1999). Therefore, while most of the tests described in this section are useful for obtaining broad-based epidemiological data, caution is advised on their use for disease certification. The C-ELISA, I-ELISA and CAT are described in detail below.

It should be noted that there is a high degree of cross-reactivity between *A. marginale* and *A. centrale*, as well as cross-reactivity with both *A. phagocytophilum* and *Ehrlichia* spp. in serological tests (Al-Adhami *et al.*, 2011; Dreher *et al.*, 2005). While the infecting species can sometimes be identified using antigens from homologous and heterologous species, equivocal results are obtained on many occasions.

2.1. Competitive enzyme-linked immunosorbent assay

A C-ELISA using a recombinant antigen termed rMSP5 and MSP5-specific monoclonal antibody (MAb) has proven very sensitive and specific for detection of *Anaplasma*-infected animals (Hofmann-Lehmann *et al.*, 2004; Reinbold *et al.*, 2010b; Strik *et al.*, 2007). All *A. marginale* strains tested, along with *A. ovis* and *A. centrale*, express the MSP5 antigen and induce antibodies against the immunodominant epitope recognised by the MSP5-specific MAb. A recent report suggests that antibodies from cattle experimentally infected with *A. phagocytophilum* will test positive in the C-ELISA (Dreher *et al.*, 2005). However, in another study no cross-reactivity could be demonstrated, and the MAb used in the assay did not react with *A. phagocytophilum* MSP5 in direct binding assays (Strik *et al.*, 2007). Cross reactivity has been demonstrated between *A. marginale* and *Ehrlichia* spp, in naturally and experimentally infected cattle (Al-Adhami *et al*, 2011). Earlier studies had shown that the C-ELISA was 100% specific using 261 known negative sera from a non-endemic region, detecting acutely infected cattle as early as 16 days after experimental tick or blood inoculation, and was demonstrated to detect cattle that have been experimentally infected as long as 6 years previously (Knowles *et al.*, 1996). In detecting persistently infected cattle from an anaplasmosis-endemic region that were defined as true positive or negative using a nested PCR procedure, the rMSP5 C-ELISA had a sensitivity of 96% and a specificity of 95% (Torioni De Echaide *et al.*, 1998).

Test results using the rMSP5 C-ELISA are available in less than 2.5 hours. A test kit available commercially contains specific instructions. In general, however, it is conducted as follows.

2.1.1. Kit reagents

A 96-well microtitre plate coated with rMSP5 antigen,

A 96-well coated adsorption/transfer plate for serum adsorption to reduce background binding,

100×MAb/peroxidase conjugate,

10× wash solution and ready-to-use conjugate-diluting buffer,

Ready-to-use substrate and stop solutions,

Positive and negative controls

2.1.2. Test procedure

i) Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and incubate at room temperature for 30 minutes.

ii) Transfer 50 µl per well of the adsorbed serum to the rMSP5-coated plate and incubate at room temperature for 60 minutes.

iii) Discard the serum and wash the plate twice using diluted wash solution.

iv) Add 50 µl per well of the 1× diluted MAb/peroxidase conjugate to the rMSP5-coated plate, and incubate at room temperature for 20 minutes.

v) Discard the 1×diluted MAb/peroxidase conjugate and wash the plate four times using diluted wash solution.

vi) Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate for 20 minutes at room temperature.

vii) Add 50 µl per well of stop solution to the substrate solution already in the wells and gently tap the sides of the plate to mix the wells.

viii) Immediately read the plate in the plate reader at 620 nm.

2.1.3. Test validation

The mean optical density (OD) of the negative control must range from 0.40 to 2.10. The per cent inhibition of the positive control must be ≥30%.

2.1.4. Interpretation of the results

The % inhibition is calculated as follows:

$$100 - \frac{\text{Sample OD} \times 100}{\text{Mean negative control OD}} = \text{Per cent inhibition}$$

Samples with <30% inhibition are negative. Samples with ≥30% inhibition are positive.

Specificity of the MSP5 C-ELISA may be increased by using a higher percentage inhibition cut-off value (Bradway *et al*., 2001); however the effect of this change on sensitivity has not been thoroughly evaluated.

Recently, an improved MSP5 C-ELISA was developed by replacing rMBP-MSP5 with rGST-MSP5 in addition to an improvement in the antigen-coating method by using a specific catcher system. The new rMSP5-GST C-ELISA was faster, simpler, had a higher specificity and an improved resolution compared with the rMSP5-MBP C-ELISA with MBP adsorption (Chung *et al*., 2014).

2.2. Indirect enzyme-linked immunosorbent assay

An I-ELISA was first developed using the CAT antigen (see below) and it can be implemented where the commercial C-ELISA is not available. Unlike the C-ELISA, most reagents, such as buffers and ready-to dissolve substrates, are available commercially in many countries. Any laboratory can prepare the antigen using local strains of *A. marginale* I-ELISA uses small amounts of serum and antigen, and the sensitivity and specificity of the test standardised with true positive and negative sera is as good as for the C-ELISA. As it can be prepared in each laboratory, only the general procedure is described here (Barry *et al.*, 1986). For commercial kits, the manufacturer's instructions should be followed. In the case of in-house I-ELISA, refer to Barry *et al.* 1986. Initial bodies and membranes are obtained as for the complement fixation test (Rogers *et al.,* 1964). This antigen is treated with 0.1% sodium dodecyl sulphate for 30 minutes prior to fixing the antigen to the microtitre plate. For each laboratory, the specific amount of antigen has to be adjusted to obtain the best reading and the least expenditure.

Test results using the I-ELISA are available in about 4 to 5 hours. It is conducted as follows:

2.2.1. Test reagents

A 96-well microtitre plate coated with crude *A. marginale* antigen,

PBS/Tween buffer, (PBS 0.1 M, pH 7.2, Tween 20 0.05%),

Blocking reagent (e.g. commercial dried skim milk)

Tris buffer 0.1M, $MgCl_2$, 0.1M, NaCl .005 M, pH 9.8

Substrate *p*-Nitrophenyl phosphate disodium hexahydrate

Positive and negative controls.

2.2.2. Test procedure (this test is run in triplicates)

i) Plates can be prepared ahead of time and kept under airtight conditions at –20°C

ii) Carefully remove the plastic packaging before using plates, being careful not to touch the bottom of them as this can distort the optical density reading.

iii) Remove the lid and deposit 200 µl PBST20 solution in each well and incubate for 5 minutes at room temperature (RT).

iv) For one plate, dissolve 1.1 g of skim milk (blocking agent) in 22 ml of PBST20.

v) Remove the plate contents and deposit in each well, 200 µl of blocking solution put the lid and incubate for 60 minutes at 37°C.

vi) Wash the plate three times for 5 minutes with PBST20.

vii) Dilute all serum samples including controls 1/100 in PBST20 solution;

viii) Remove the contents of the plate and deposit 200 µl of diluted serum in each of the three wells for each dilution, starting with the positive and negative and blank controls.

ix) Incubate plate at 37°C covered for 60 minutes.

x) Wash three times as described in subsection vi.

xi) Dilute 1/1000 anti-IgG bovine alkaline phosphatase conjugate in PBST20 solution; Add 200 µl of the diluted conjugate per well; incubate the covered plate at 37°C for 60 minutes.

xii) Remove the lid and make three washes with PBST20.

xiii) Remove the contents of the plate and deposit 195 µl of 0.075% *p*-Nitrophenyl phosphate disodium hexahydrate in Tris buffer and incubate for 60 minutes at 37°C

xiv) The reaction is quantified by a microplate reader spectrophotometer, adjusted to 405 nm wavelength. The data are expressed in optical density (OD).

2.2.3. Data analysis

Analysis of results should take into account the following parameters.

i) The mean value of the blank wells.

ii) The mean value of the positive wells with their respective standard deviations.

iii) The mean value of negative wells with their respective standard deviations.

iv) The mean value of the blank wells is subtracted from the mean of all the other samples if not automatically subtracted by the ELISA reader.

v) Control sera are titrated to give optical density values ranging from 0.90 to 1.50 for the positive and, 0.15 to 0.30 for the negative control

Positive values are those above the cut-off calculated value which is the sum of the average of the negative and two times the standard deviation.

For purposes of assessing the consistency of the test operator, the error "E" must also be estimated; this is calculated by determining the percentage represented by the standard deviation of any against their mean serum.

2.3. Card agglutination test

The advantages of the CAT are that it is sensitive, may be undertaken either in the laboratory or in the field, and gives a result within a few minutes. Nonspecific reactions may be a problem, and subjectivity in interpreting assay reactions can result in variability in test interpretation. In addition, the CAT antigen, which is a suspension of *A. marginale* particles, can be difficult to prepare and can vary from batch to batch and laboratory to laboratory. Splenectomised calves are infected by intravenous inoculation with blood containing *Anaplasma*-infected erythrocytes. When the rickettsaemia exceeds 50%, the animal is exsanguinated, the infected erythrocytes are washed, lysed, and the erythrocyte ghosts and *Anaplasma* particles are pelleted. The pellets are sonicated, washed, and then resuspended in a stain solution to produce the antigen suspension.

A test procedure that has been slightly modified from that originally described (Amerault & Roby, 1968; Amerault *et al.*, 1972) is as follows, and is based on controlled conditions in a laboratory setting:

2.3.1. Test procedure

i) Ensure all test components are at a temperature of 25–26°C before use (this constant temperature is critical for the test).

ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with circles that are 18 mm in diameter), place next to each other, but not touching, 10 µl of bovine serum factor (BSF), 10 µl of test serum, and 5 µl of CAT antigen[1]. Negative and low positive control sera must be tested on each card.

BSF is serum from a selected animal with high known conglutinin level. If the conglutinin level is unknown, fresh serum from a healthy animal known to be free from *Anaplasma* can be used. The BSF must be stored at –70°C in small aliquots, a fresh aliquot being used each time the tests are performed. The inclusion of BSF improves the sensitivity of the test.

iii) Mix well with a glass stirrer. After mixing each test, wipe the stirrer with clean tissue to prevent cross-contamination.

iv) Place the test card in a humid chamber and rock at 100–110 rpm for 7 minutes.

v) Read immediately against a backlight. Characteristic clumping of the antigen (graded from +1 to +3) is considered to be a positive result. The test is considered to give a negative result when there is no characteristic clumping.

2.4. Complement fixation test

The complement fixation (CF) test has been used extensively for many years; however, it shows variable sensitivity (ranging from 20 to 60%), possibly reflecting differences in techniques for antigen production, and poor reproducibility. In addition, it has been demonstrated that the CF assay fails to detect a significant proportion of carrier cattle (Bradway *et al.*, 2001). It is also uncertain as to whether or not the CF test can identify antibodies in acutely infected animals prior to other assays (Coetzee *et al.*, 2007; Molloy *et al.*, 1999). Therefore, the CF test is no longer recommended as a reliable assay for detecting infected animals.

2.5. Indirect fluorescent antibody test

Because of the limitations on the number of indirect fluorescent antibody (IFA) tests that can be performed daily by one operator, other serological tests are generally preferred to the IFA test. The IFA test is performed as described for bovine babesiosis in chapter 3.4.2, except that *A. marginale* infected blood is used for the preparation of antigen smears. A serious problem encountered with the test is nonspecific fluorescence. Antigen made from blood collected as soon as adequate rickettsaemia (5–

1 The test as conducted in the USA and Mexico uses larger volumes of reagents: antigen (15 µl), serum (30 µl), and bovine serum factor (30 µl), and a 4-minute reaction time (see step iv).

10%) occurs is most likely to be suitable. Nonspecific fluorescence due to antibodies adhering to infected erythrocytes can be reduced by washing the erythrocytes in an acidic glycine buffer before antigen smears are prepared. Infected erythrocytes are washed twice in 0.1 M glycine buffer (pH 3.0, centrifuged at 1000 ***g*** for 15 minutes at 4°C) and then once in PBS, pH 7.4. Recently published data show that the IFA, like the C-ELISA, can cross react with other members of the *Anaplasmataceae* family (Al-Adhami *et al.,* 2011).

C. REQUIREMENTS FOR VACCINES

1. Background

Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is endemic, but none is ideal to date (McHardy, 1984). A review of *A. marginale* vaccines and antigens has been published (Kocan *et al.*, 2003) Use of the less pathogenic *A. centrale,* which gives partial cross-protection against *A. marginale,* is the most widely accepted method, although not used in many countries where the disease is exotic, including north America.

In this section, the production of live *A. centrale* vaccine is described. It involves infection of a susceptible, splenectomised calf and the use of its blood as a vaccine. Detailed accounts of the production procedure are available and reference should be made to these publications for details of the procedures outlined here (Bock *et al.*, 2004; de Vos & Jorgensen, 1992; Pipano, 1995).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Anaplasma centrale vaccine can be provided in either frozen or chilled form depending on demand, transport networks, and the availability of liquid nitrogen or dry ice supplies. Frozen vaccine is recommended in most instances, as it allows for thorough post-production quality control of each batch. It is, however, more costly to produce and more difficult to transport than chilled vaccine. The risk of contamination makes post-production control essential, but may be prohibitively expensive.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Anaplasma centrale was isolated in 1911 in South Africa, and has been used as a vaccine in South America, Australia, Africa, the Middle East, and South-East Asia. It affords only partial, but adequate, protection in regions where the challenging strains are of moderate virulence (e.g. Australia) (Bock & de Vos, 2001). In the humid tropics where *A. marginale* appears to be a very virulent rickettsia, the protection afforded by *A. centrale* may be inadequate to prevent disease in some animals.

Anaplasma centrale usually causes benign infections, especially if used in calves under 9 months of age. Severe reactions following vaccination have been reported when adult cattle are inoculated. The suitability of an isolate of *A. centrale* as a vaccine can be determined by inoculating susceptible cattle, monitoring the subsequent reactions, and then challenging the animals and susceptible controls with a virulent local strain of *A. marginale.* Both safety and efficacy can be judged by monitoring rickettsaemias in stained blood films and the depression of packed cell volumes of inoculated cattle during the vaccination and challenge reaction periods.

Infective material for preparing the vaccine is readily stored as frozen stabilates of infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone M.W. 40,000 (Bock *et al.*, 2004) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the stabilate. A detailed account of the freezing technique using DMSO is reported elsewhere (Mellors *et al.,* 1982), but briefly involves the

following: infected blood is collected, chilled to 4°C, and cold cryoprotectant (4 M DMSO in PBS) is added slowly with stirring to a final blood:protectant ratio of 1:1, to give a final concentration of 2 M DMSO. The entire dilution procedure is carried out in an ice bath and the diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as possible, in the vapour phase of a liquid nitrogen container.

2.1.2. Quality criteria

Evidence of purity of the *A. centrale* isolate can be determined by serological testing of paired sera from the cattle used in the safety test for possible contaminants that may be present (Bock *et al.*, 2004; Pipano, 1997). Donor calves used to expand the seed for vaccine production should be examined for all blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*, *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected. The absence of other infective agents should also be confirmed. These may include the agents of enzootic bovine leukosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, foot and mouth disease, and rinderpest. The testing procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera at the very least and, in some cases, virus isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano, 1981; 1997).

2.2. Method of manufacture

2.2.1. Procedure

i) Production of frozen vaccine

Quantities of the frozen stabilate (5–10 ml) are thawed by immersing the vials in water preheated to 40°C. The thawed material is kept on ice and used as soon as possible (within 30 minutes) to infect a susceptible, splenectomised calf by intravenous inoculation.

The rickettsaemia of the donor calf is monitored daily by examining stained films of jugular blood, and the blood is collected for vaccine production when suitable rickettsaemias are reached. A rickettsaemia of 1×10^8/ml (approximately 2% rickettsaemia in jugular blood) is the minimum required for production of vaccine as this is the dose to vaccinate a bovine. If a suitable rickettsaemia is not obtained, passage of the strain by subinoculation of 100–200 ml of blood to a second splenectomised calf may be necessary.

Blood from the donor is collected by aseptic jugular or carotid cannulation using heparin as an anticoagulant (5 International Units [IU] heparin/ml blood). The use of blood collection units for human use are also suitable and guaranty sterility and obviate the need to prepare glass flask that make the procedure more cumbersome.

In the laboratory, the infective blood is mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose at 37°C (final concentration of glycerol 1.5 M). The mixture is then equilibrated at 37°C for 30 minutes and dispensed into suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (Bock *et al.,* 2004).

DMSO can be used as a cryoprotectant in the place of glycerol. This is done in the same way as outlined for the preparation of seed stabilate (Mellors *et al.,* 1982; Pipano, 1981).

If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and 5 mM glucose (Jorgensen *et al.,* 1989). Vaccine cryopreserved with DMSO should be diluted with diluent containing the same concentration of DMSO as in the original cryopreserved blood (Pipano *et al.,* 1986).

ii) Production of chilled vaccine

Infective material for chilled vaccine is prepared in the same way as for frozen vaccine, but it must be issued and used as soon as possible after collection. The infective blood can be

diluted to provide 1×10^7 parasites per dose of vaccine. A suitable diluent is 10% sterile bovine serum in a glucose/balanced salt solution containing the following quantities per litre: NaCl (7.00 g), $MgCl_2.6H_2O$ (0.34 g), glucose (1.00 g), Na_2HPO_4(2.52 g), KH_2PO_4(0.90 g), and $NaHCO_3$(0.52 g).

If diluent is not available, acid citrate dextrose (20% [v/v]) or citrate phosphate dextrose (20% [v/v]) should be used as anticoagulant to provide the glucose necessary for survival of the organisms.

iii) Use of vaccine

In the case of frozen vaccine, vials should be thawed by immersion in water, preheated to 37°C to 40°C, and the contents mixed with suitable diluent to the required dilution. If glycerolised vaccine is prepared, it should be kept cool and used within 8 hours (Bock *et al.,* 2004). If DMSO is used as a cryoprotectant, the prepared vaccine should be kept on ice and used within 15–30 minutes (Pipano, 1981). The vaccine is most commonly administered subcutaneously.

iv) Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation.

The strain of *A. centrale* used in vaccine is of reduced virulence, but is not entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant close attention, and should be observed daily for 3 weeks post-vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the manufacturers. Protective immunity develops in 6–8 weeks and usually lasts for several years.

Anaplasmosis and babesiosis vaccines are often used concurrently, but it is not advisable to use any other vaccines at the same time (Bock *et al.,* 2004).

2.2.2. Requirements for substrates and media

Anaplasma centrale cannot be cultured in vitro. No substrates or media other than buffers and diluents are used in vaccine production. DMSO or glycerol should be purchased from reputable companies.

2.2.3. In-process controls

i) Source and maintenance of vaccine donors

A source of calves free from natural infections of *Anaplasma* and other tick-borne diseases should be identified. If a suitable source is not available, it may be necessary to breed the calves under tick-free conditions specifically for the purpose of vaccine production.

The calves should be maintained under conditions that will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production of vaccine weighed against the possible adverse consequences of spreading disease (Bock *et al.*, 2004).

ii) Surgery

Donor calves should be splenectomised to allow maximum yield of organisms for production of vaccine. This is best carried out in young calves and under general anesthesia.

iii) Screening of vaccine donors before inoculation

As for preparation of seed stabilate, donor calves for vaccine production should be examined for all blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*, *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be done by

routine examination of stained blood films after splenectomy, and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected. The absence of other infective agents should also be confirmed. These may include the agents of enzootic bovine leukosis, bovine viral diarrhoea, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, and foot and mouth disease. The testing procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera at the very least and, in some cases, virus isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano, 1981; 1997).

iv) Monitoring of rickettsaemias following inoculation

It is necessary to determine the concentration of rickettsia in blood being collected for vaccine. The rickettsial concentration can be estimated from the erythrocyte count and the rickettsaemia (percentage of infected erythrocytes).

v) Collection of blood for vaccine

All equipment should be sterilised before use (e.g. by autoclaving). Once the required rickettsaemia is reached, the blood is collected in heparin using strict aseptic techniques. This is best done if the calf is sedated and with the use of a closed-circuit collection system.

Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live, the transfusion of a similar amount of blood from a suitable donor is indicated. Alternatively, the calf should be killed immediately after collection of the blood.

vi) Dispensing of vaccine

All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of blood and diluent throughout the dispensing process. Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of dispensing.

2.2.4. Final product batch tests

The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, and specifications for frozen vaccine depend on the country involved. The following are the specifications for frozen vaccine produced in Australia.

i) Sterility and purity

Standard tests for sterility are employed for each batch of vaccine and diluent (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

The absence of contaminants is determined by doing appropriate serological testing of donor cattle, by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral infection, and by inoculating cattle and monitoring them serologically for infectious agents that could potentially contaminate the vaccine. Cattle inoculated during the test for potency (see Section C.2.2.4.iii) are suitable for the purpose. Depending on the country of origin of the vaccine, these agents include the causative organisms of enzootic bovine leukosis, infectious bovine rhinotracheitis, bovine viral diarrhoea, ephemeral fever, Akabane disease, Aino virus, bluetongue, parainfluenza, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, contagious bovine pleuropneumonia, Jembrana disease, heartwater, pathogenic *Theileria* and *Trypanosoma spp*., *Brucella abortus*, *Coxiella*, and *Leptospira* (Bock *et al.*, 2004; Pipano, 1981; 1997).Other pathogens to consider include the causal agents of bovine tuberculosis and brucellosis as they may spread through contaminated blood used for vaccine production. Most of these agents can be tested by means of specific PCR and there are many publications describing primers, and assay conditions for any particular disease.

ii) Safety

Vaccine reactions of the cattle inoculated in the test for potency (see Chapter 1.1.8 *Principles of veterinary vaccine production*) are monitored by measuring rickettsaemia and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use.

iii) Potency

Vaccine is thawed and diluted 1/5 with a suitable diluent (Bock *et al.*, 2004). The diluted vaccine is then incubated for 8 hours at 4°C, and five cattle are inoculated subcutaneously with 2 ml doses. The inoculated cattle are monitored for the presence of infections by examination of stained blood smears. All should become infected for a batch to be accepted. A batch proving to be infective is recommended for use at a dilution of 1/5 with isotonic diluent.

2.3. Requirements for authorisation

2.3.1. Safety

The strain of *A. centrale* used in vaccine is of reduced virulence, but is not entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant close attention, and should be observed daily for 3 weeks post-vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the manufacturers.

Anaplasma centrale is not infective to other species, and the vaccine is not considered to have other adverse environmental effects. The vaccine is not infective for humans. When the product is stored in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of deep-frozen material applies.

2.3.2. Efficacy requirements

Partial but long-lasting immunity results from one inoculation. There is no evidence that repeated vaccination will have a boosting effect. The vaccine is used for control of clinical anaplasmosis in endemic areas. It will not provide sterile immunity, and should not be used for eradication of *A. marginale*.

2.3.3. Stability

The vaccine can be kept for 5 years when stored in liquid nitrogen. Once thawed, it rapidly loses its potency. Thawed vaccine cannot be refrozen.

3. Vaccines based on biotechnology

There are no vaccines based on biotechnology available for anaplasmosis.

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NB: There is a WOAH Reference Laboratory for anaplasmosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratory for any further information on diagnostic tests, reagents and vaccines for bovine anaplasmosis

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2015.

CHAPTER 3.4.2.

BOVINE BABESIOSIS

SUMMARY

Bovine babesiosis is a tick-borne disease of cattle caused by protozoan parasites including Babesia bovis, B. bigemina and B. divergens. Rhipicephalus (Boophilus) microplus*, the principal vector of* B. bovis *and* B. bigemina*, is widespread in tropical and subtropical countries. The major vector of* B. divergens *is* Ixodes ricinus*. There are other important vectors that can transmit these pathogens, including* Haemaphysalis *and other* Rhipicephalus *spp.*

Detection of the agent: *In the case of live animals, thick and thin films of blood should be taken from a peripheral blood vessel or small capillary bed, for example, the tip of the tail. Demonstration of parasites in dead animals is possible by microscopic examination of smears of peripheral blood, brain, kidney, heart muscle, spleen and liver, provided decomposition is not advanced. The smears are fixed with methanol, stained with 10% Giemsa for 15–30 minutes, and examined at ×800–1000 magnification under oil immersion. Sensitive polymerase chain reaction assays are available that can detect and differentiate* Babesia *species in cattle.*

Serological tests: *Enzyme-linked immunosorbent assays (ELISAs) and competitive ELISA (C-ELISA) using recombinant* B. bovis *and* B. bigemina *merozoite proteins have largely replaced the indirect fluorescent antibody test (IFAT) for the detection of antibodies to* Babesia spp.*, because of processing efficiency and objectivity in interpretation of results. The IFA test has been widely used in the past, but serological cross-reactions make species diagnosis difficult in* B. bigemina*. Immunochromatographic tests (ICT) using recombinant* B. bovis *and* B. bigemina *merozoite proteins have also been developed and used for epidemiological surveys of those infections.*

Requirements for vaccines: *Vaccines consisting of live, attenuated strains of* B. bovis, B. bigemina *or* B. divergens *are produced in several countries either from the blood of infected donor animals or by* in-vitro *culture. The vaccines are provided in frozen or chilled forms. Frozen vaccine has the advantage of allowing post-production control of each batch, but has a much reduced post-thaw shelf life compared with chilled vaccine. The risk of contamination of the blood-derived vaccines makes thorough quality control essential, but this may be prohibitively expensive.*

Whilst in-vitro *production methods offer obvious advantages in terms of animal welfare, vaccine can also be successfully produced using* in-vivo *production systems under strict animal welfare guidelines. With either* in-vivo *or* in-vitro *systems, strict adherence to production protocols is essential to ensure consistency of vaccine and to avoid potential changes in virulence, immunogenicity and consequent protectiveness associated with continued passage of* Babesia *spp. organisms in either culture or splenectomised calves.*

With live Babesia *vaccines, it is prudent, for safety reasons, to limit use to calves less than one year of age, as these animals likely have nonspecific immunity. Older vaccinated animals should be held under surveillance and treated with a babesiacide if adverse events occur.*

Protective immunity develops in 3–4 weeks. A single vaccination usually provides lifelong immunity.

A. INTRODUCTION

Bovine babesiosis is caused by protozoan parasites of the genus *Babesia*, order Piroplasmida, phylum Apicomplexa. Of the species affecting cattle, two – *Babesia bovis* and *B. bigemina* – are widely distributed and of major importance in Africa, Asia, Australia, and the American continent, from the south of the United States to the north of Argentina. *Babesia divergens* is economically important in some parts of Europe. A recent study reported

severe clinical babesiosis caused by *Babesia* sp. Mymensingh in a cow (Sivakumar *et al.,* 2018). However, further studies are essential to confirm experimentally the virulence of this novel *Babesia* species and to determine its geographical distribution.

Tick species are the vectors of *Babesia* (Bock *et al.,* 2008). *Rhipicephalus* (*Boophilus*) *microplus* is the principal vector of *B. bigemina* and *B. bovis* and is widespread in the tropics and subtropics. The vector of *B. divergens* is *Ixodes ricinus*. Other important vectors include *Haemaphysalis* and other species of *Rhipicephalus*.

Babesia bigemina has the widest distribution but *B. bovis* is generally more pathogenic than *B. bigemina* or *B. divergens*. *Babesia bovis* infections are characterised by high fever, ataxia, anorexia, general circulatory shock, and sometimes also nervous signs as a result of sequestration of infected erythrocytes in cerebral capillaries. Anaemia and haemoglobinuria may appear later in the course of the disease. In acute cases, the maximum parasitaemia (percentage of infected erythrocytes) in circulating blood is less than 1%. This is in contrast to *B. bigemina* infections where the parasitaemia often exceeds 10% and may be as high as 30%. In *B. bigemina* infections, the major signs include fever, haemoglobinuria and anaemia. Intravascular sequestration of infected erythrocytes does not occur with *B. bigemina* infections. The parasitaemia and clinical appearance of *B. divergens* infections are somewhat similar to *B. bigemina* infections (Zintl *et al.,* 2003).

Infected animals develop a life-long immunity against reinfection with the same species. There is also evidence of a degree of cross-protection in *B. bigemina*-immune animals against subsequent *B. bovis* infections. Calves rarely show clinical signs of disease after infection regardless of the *Babesia* spp. involved or the immune status of the dams (Bock *et al.,* 2008; Zintl *et al.,* 2003).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of babesiosis in cattle and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Microscopic examination	–	–	–	+++	+	–
PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
ELISA	+++	+++	+++	–	+++	+++
IFAT	++	++	++	–	++	+++
ICT	–	–	–	–	++	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
IFAT = indirect fluorescent antibody test, ICT = immunochromatographic test.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

1.1. Direct microscopic examination

The traditional method of identifying the agent in infected animals is by microscopic examination of thick and thin blood films stained with Giemsa, a Romanowsky type stain (10% Giemsa in phosphate buffered saline (PBS) or Sörenson's buffer at pH 7.4). The sensitivity of thick films is such that it can detect

parasitaemias as low as 1 parasite in 10^6 red blood cells (RBCs). Species differentiation is good in thin films but poor in the more sensitive thick films. This technique is usually adequate for detection of acute infections, but not for detection of carriers where the parasitaemias are mostly very low. Parasite identification and differentiation can be improved by using a fluorescent dye, such as acridine orange, instead of Giemsa.

Samples from live animals should preferably be films made from fresh blood taken from capillaries, such as those in the tip of the ear or tip of the tail, as *B. bovis* is more common in capillary blood. *Babesia bigemina* and *B. divergens* parasites are uniformly distributed through the vasculature. If it is not possible to make fresh films from capillary blood, sterile jugular blood should be collected into an anticoagulant such as lithium heparin or ethylene diamine tetra-acetic acid (EDTA). The sample should be kept cool, preferably at 5°C, until delivery to the laboratory. Thin blood films are air-dried, fixed in absolute methanol for 10–60 seconds and then stained with 10% Giemsa for 15–30 minutes. It is preferable to stain blood films as soon as possible after preparation to ensure proper stain definition. Thick films are made by placing a small droplet of blood (approximately 50 µl) on to a clean glass slide and spreading this over a small area using a circular motion with the corner of another slide. This droplet is not fixed in methanol, but simply air-dried, heat-fixed at 80°C for 5 minutes, and stained in 10% Giemsa. This is a more sensitive technique for the detection of *Babesia* spp., as RBCs are lysed and parasites concentrated, but species differentiation is more difficult. Unstained blood films should not be stored with formalin solutions as formalin fumes affect staining quality. Moisture also affects staining quality.

Samples from dead animals should consist of thin blood films, as well as smears from cerebral cortex, kidney (freshly dead), spleen (when decomposition is evident), heart muscle, lung, and liver (Bock *et al.,* 2006). Organ smears are made by pressing a clean slide on to a freshly cut surface of the organ or by crushing a small sample of the tissue (particularly cerebral cortex) between two clean microscope slides drawn lengthwise to leave a film of tissue on each slide. The smear is then air-dried (assisted by gentle warming in humid climates), fixed for 10–60 seconds in absolute methanol, and stained for 15–30 minutes in 10% Giemsa. This method is especially suitable for the diagnosis of *B. bovis* infections using smears of cerebral cortex, but is unreliable if samples are taken 24 hours or longer after death has occurred, especially in warmer weather. Parasites can sometimes be detected in capillary blood taken from the lower limb region one or more days after death.

All stained films are examined under oil immersion using (as a minimum) a ×10 eyepiece and a ×100 objective lens. *Babesia bovis* is a small parasite, usually centrally located in the erythrocyte. It measures approximately 1–1.5 µm long and 0.5–1.0 µm wide, and is often found as pairs that are at an obtuse angle to each other. *Babesia divergens* is also a small parasite and is very similar morphologically to *B. bovis*. However, obtuse-angled pairs are often located at the rim of the erythrocyte. *Babesia bigemina* is typically pear-shaped, but many diverse single forms are found. It is usually a much bigger parasite (3–3.5 µm long and 1–1.5 µm wide), and is often found as pairs at an acute angle to each other or almost parallel. In acute cases, the parasitaemia of *B. bovis* seldom reaches 1% (measured in general circulation, rather than capillary blood), but with *B. bigemina* and *B. divergens* much higher parasitaemias are usual.

1.2. Nucleic acid-based diagnostic assays

Nucleic acid-based diagnostic assays are very sensitive particularly in detecting *B. bovis* and *B. bigemina* in carrier cattle (Buling *et al.,* 2007; Criado-Fornelio, 2007). Polymerase chain reaction (PCR-based techniques are reported to be as much as 1000 times more sensitive than microscopy for detection of *Babesia* spp., with detection at parasitaemia levels ranging from 0.001% to 0.0000001% (1 parasite in 10^9 RBCs) (Criado-Fornelio, 2007). A number of PCR techniques have been described that can detect and differentiate species of *Babesia* in carrier infections (Buling *et al.,* 2007; Criado-Fornelio, 2007). PCR assays to differentiate isolates of *B. bovis* have also been described. The application of the reverse line blot procedure, in which PCR products are hybridised to membrane-bound, species-specific oligonucleotide probes, to *Babesia* and, more recently, two quantitative PCR methods (Criado-Fornelio *et al.,* 2009) have enabled the simultaneous detection of multiple species, even in carrier state infections. However, current PCR assays generally do not lend themselves well to large-scale testing and at this time are unlikely to supplant serological tests as the method of choice for epidemiological studies. PCR assays are useful as confirmatory tests and in some cases for regulatory testing. Recently, loop-mediated isothermal amplification (LAMP) and multiplex LAMP methods have been developed (Iseki *et al*., 2007; Liu *et al.,* 2012) with higher sensitivity than that of PCR, although optimisation for detection of strains from different parts of the world needs to be addressed. This latter method requires expensive or

sophisticated equipment, while the LAMP method only requires an ordinary water bath and the results can be read with the naked eye. The LAMP method is, therefore, cost-effective, simple, and a rapid DNA amplification method that is suitable for pen-side diagnostics. However, care should be taken to avoid cross-contamination as sensitivity of the LAMP is usually very high.

1.2.1. Nested polymerase chain reaction for the simultaneous detection of *B. bovis* and *B. bigemina*

As the sensitivity of nested PCR is higher than that of PCR, nested PCR is a suitable assay for international trade, especially for *B. bovis,* which usually shows low parasitaemia or carrier status. Although there are several genes used for nested PCR, RAP-1 and AMA-1 have been widely used for *B. bovis* and *B. bigemina*, respectively.

i) DNA extraction

a) Whole blood sample is collected into a vacuum blood collection tube containing EDTA.

b) 200 µl bovine blood is placed in a 1.5 ml microfuge tube

c) 1 ml cold PBS is added and the mixture is centrifuged at 1000 ***g*** for 5 minutes at 4°C. This step is repeated three times.

d) The supernatant is discarded and the pellet is resuspended in 200 µl PBS.

e) After measuring DNA concentrations using a spectrophotometer, the samples can be stored at –20°C.

Note that the DNA samples can also be prepared from infected blood samples using commercial DNA extraction kits.

ii) Multiplex nested PCR

The following test procedure is adapted from Figueroa *et al.* (1993) with modifications for the multiplex nested PCR detection of *B. bovis* and *B. bigemina* based on *RAP-1* and *Spe*I-*Ava*I, respectively.

a) For the first round of PCR, 9 µl reaction mixture that includes 1 µl of 10 × reaction buffer, 200 µM of each dNTPs, 0.5 µM of outer forward (BoF and BilA) and reverse (BoR and BilB) primers, 0.5 units of Taq DNA polymerase (Applied Biosystems), and double distilled water is prepared.

b) 1 µl of the extracted DNA sample is added to the reaction mixture and then subjected to the following PCR cycling conditions. The initial enzyme activation at 95°C for 5 minutes is followed by 35 cycles, each of them consisting of a denaturing step at 95°C for 30 seconds, an annealing step at 55°C for 1 minute, and an extension step at 72°C for 1 minute. The final elongation step is at 72°C for 10 minutes.

c) For the nested round of PCR, 1 µl of the first PCR product is transferred to a new PCR tube that contains a reaction mixture with the same composition as that of the first PCR except for the outer primers, which were replaced with the inner forward (BoFN and BilAN) and reverse (BoRN and BilBN) primers.

iii) List of PCR primers

Parasite	PCR	Primer	Sequence (5'–3')
B. bovis	Primary	BoF	CAC-GAG-GAA-GGA-ACT-ACC-GAT-GTT-GA
		BoR	CCA-AGG-AGC-TTC-AAC-GTA-CGA-GGT-CA
	Nested	BoFN	TCA-ACA-AGG-TAC-TCT-ATA-TGG-CTA-CC
		BoRN	CTA-CCG-AGC-AGA-ACC-TTC-TTC-ACC-AT

Parasite	PCR	Primer	Sequence (5'–3')
B. bigemina	Primary	BilA	CAT-CTA-ATT-TCT-CTC-CAT-ACC-CCT-CC
		BilB	CCT-CGG-CTT-CAA-CTC-TGA-TGC-CAA-AG
	Nested	BilAN	CGC-AAG-CCC-AGC-ACG-CCC-CGG-TGC
		BilBN	CCG-ACC-TGG-ATA-GGC-TGT-GTG-ATG

a) PCR products are separated by electrophoresis (100 V) in 1.5 % agarose gels and 0.5 × Tris/borate/EDTA (TBE) buffer. Gels are then stained with a fluorescent DNA stain, visualised under ultraviolet light, and photographed.

iv) Interpretation of the results

a) Positive samples should have PCR products of the expected size (170 bp for *B. bigemina* and 298 bp *B. bovis*) similar to those of positive controls.

b) The assay must be repeated if the positive control remained negative, or if the negative controls are positive.

Note: Although the above described procedure is for the multiplex PCR assay, simplex nested PCR can also be performed to detect either *B. bovis* or *B. bigemina* using only the respective PCR primers.

Sivakumar *et al.* (2012) noted that the primers that target the *B. bigemina Spe*I-*Ava*I restriction fragment can also amplify *B. ovata* DNA. Therefore, care should be taken when testing DNA samples that were sourced from *B. ovata*-endemic regions.

Recently, a new, improved method for diagnostics based on apocytochrome b genes (CYTb) of *B. bovis* and *B. bigemina* was developed (Romero-Salas *et al.,* 2016), allowing the detection of as little as 0.1 fg DNA of each *Babesia* pathogen, and showing 100 and 1000 times higher sensitivity for *B. bovis* and *B. bigemina*, respectively, as compared with those described by Figueroa *et al.* (1993).

1.3. *In-vitro* culture

In-vitro culture methods have been used to demonstrate the presence of carrier infections of *Babesia* spp. (Holman *et al.,* 1993), and *B. bovis* has also been cloned in culture. The minimum parasitaemia detectable by this method will depend, to a large extent, on the facilities available and the skills of the operator, but could be as low as 10^{-10} (Friedhoff & Bose, 1994), making it a very sensitive method, with 100% specificity, for the demonstration of infection.

2. Serological tests

The indirect fluorescent antibody test (IFAT) was widely used in the past to detect antibodies to *Babesia* spp., but the *B. bigemina* test has poor specificity. Cross-reactions with antibodies to *B. bovis* in the *B. bigemina* IFAT were a particular problem in areas where the two parasites coexist. The IFA test also has the disadvantages of low sample throughput and subjectivity. The complement fixation test is no longer used for diagnosis of these infections.

Enzyme-linked immunosorbent assays (ELISA) have largely replaced the IFAT as the diagnostic test of choice for *Babesia* spp. because of the objectivity in interpretation of results and capacity to process high numbers of samples daily. An ELISA for the diagnosis of *B. bovis* infection that uses a whole merozoite antigen has undergone extensive evaluation (Molloy *et al.,* 1998). High sensitivity and specificity of this test was demonstrated in both Australia and Zimbabwe, although threshold values varied between laboratories (Molloy *et al.,* 1998). Indirect (Bono *et al.,* 2008; Boonchit *et al*., 2006) and competitive ELISAs (Goff *et al.,* 2003) using recombinant merozoite surface and rhoptry-associated antigens of *B. bovis* have also been developed. The competitive ELISA has been more widely validated in different laboratories, with the antigen recognised by antibody from diverse regions around the world (Goff *et al.,* 2006). Reduction in specificity of the indirect *B. bovis* ELISA using recombinant antigens has been noted in some situations (Bono *et al.,* 2008).

There is still no well validated ELISA available for *B. bigemina* despite the efforts of several investigators in different laboratories. ELISAs for antibodies to *B. bigemina* crude antigen typically have poor specificity. Competitive ELISAs developed and validated in Australia and USA (Goff *et al.,* 2008) are apparently the only ELISAs in routine use. Unlike *B. bovis* where animals are thought to remain carriers for life after infection, *B. bigemina* may clear infection and antibody levels may decline below the negative threshold within months after infection (Goff *et al.,* 2008). Inconclusive results may occur around the negative threshold values; and this phenomenon can provide a diagnostic challenge in animals where titres are declining if the animal clears infection.

ELISAs have also been developed for *B. divergens* using antigen derived from culture, *Meriones* or cattle, but there does not appear to be one that has been validated internationally (Zintl *et al.,* 2003).

The immunochromatographic test (ICT) is a rapid diagnostic method that detects antibodies against a specific antigen by means of specific antibodies and antigens impregnated on a nitrocellulose membrane-based test strip. ICT is a rapid, easy-to-read assay, very much needed under field situations, particularly in developing countries where equipment and electricity are limited. An ICT for simultaneous rapid serodiagnosis of bovine babesiosis caused by *B. bovis* and *B. bigemina* has been developed (Kim *et al.,* 2008).

2.1. *Babesia bovis* indirect enzyme-linked immunosorbent assay

Antigen preparation is based on a technique described by Waltisbuhl *et al.* (1987). Infected blood (usually 5–10% parasitaemia) is collected from a splenectomised calf into EDTA. The blood is first centrifuged and the plasma removed and stored for later use. Infected RBCs can also be obtained from *in-vitro* cultures. The RBCs are then washed three times in five volumes of phosphate-buffered saline (PBS), and infected cells are concentrated by differential lysis of uninfected cells in hypotonic saline solution. Infected cells are more resistant to lysis in hypotonic saline solutions than are uninfected cells.

To find the best concentration for particular infected blood, a series of hypotonic saline solutions are prepared, ranging from 0.35% to 0.50% NaCl in 0.025% increments. Five volumes of each saline solution are then added to one volume of packed RBCs, gently mixed and allowed to stand for 5 minutes. The mixtures are then centrifuged and the supernatants aspirated. An equal volume of plasma (retained from the original blood) is added to each tube containing packed RBCs, and the contents of the tubes are mixed. Thin blood films are prepared from each of these resuspended blood cell mixtures, fixed in methanol, and stained with Giemsa. These films are examined under a microscope to determine which saline solution lyses most uninfected RBCs but leaves infected RBCs intact. It should be possible to achieve >95% infection in the remaining intact RBCs.

The bulk of the packed RBCs are then differentially lysed with the optimal saline solution, centrifuged and the supernatant removed. The sediment (>95% infected RBCs) is lysed in distilled water at 4°C, and parasites are pelleted at 12,000 ***g*** for 30 minutes. The pellet is washed at least three times in PBS by resuspension and centrifugation at 4°C until minimal haemoglobin is in the supernatant. It is then resuspended in one to two volumes of PBS at 4°C, and sonicated in appropriate volumes using medium power for 60–90 seconds. The sonicated material is ultra-centrifuged (105,000 ***g*** for 60 minutes at 4°C) and the supernatant containing the solubilised merozoite antigen is retained. The supernatant is mixed with an equal volume of glycerol and stored in 2–5 ml aliquots at –70°C. Short-term storage at –20°C is acceptable for the working aliquot.

2.1.1. Test procedure

The following test procedure is based on that described by Molloy *et al.* (1998) with some modification.

i) 100 µl of antigen solution (with the antigen typically diluted in the range from 1/400 to 1/1600 in 0.1 M carbonate buffer (pH 9.6) is added to each well of a polystyrene 96-well microtitre plate. The plate is covered and incubated overnight at 4°C.

ii) The solution containing any unbound antigen is removed and the wells are then blocked for 1 hour at 20–25°C by the addition of 200 µl of a 2% solution of sodium caseinate in carbonate buffer (pH 9.6).

iii) After blocking, the wells are rinsed three times with PBS containing 0.1% Tween 20 (PBST); then 100 µl of diluted test and control bovine serum (diluted 1/100 in PBST containing 2%

skim milk powder) is added into each well, and the plates are incubated for 30 minutes at 20–25°C with shaking.

iv) The washing step consists of five rinses with PBST. During the last rinse, the plate is shaken for 5 minutes.

v) Next, 100 µl of peroxidase-labelled anti-bovine IgG diluted in PBST containing 2% skim milk powder is added and the plates are shaken for a further 30 minutes at 20–25°C. (NB: some batches of skim milk powder may contain immunoglobulins that can interfere with anti-bovine IgG conjugates and must be tested for suitability prior to use).

vi) Wells are washed as described in step iv above, and to each well is added 100 µl of peroxidase substrate/chromagen (ABTS [2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)]) 0.3 g/litre in a glycerine/citric acid buffer; with H_2O_2 concentration of 0.01%). The substrate reaction is allowed to continue until the absorbance of a strong positive control serum included on each plate approaches 1.

vii) At this point, the reaction is stopped using an equal volume (100 µl) of ABTS Peroxidase Stop Solution (working concentration of 1% sodium dodecyl sulphate). Absorbance at 414 nm is read on a microtitre plate reader within 30 minutes. 3,3',5,5'-Tetramethyl benzidine (TMB) is also a suitable chromagen, but is stopped with equal volumes of 1 M phosphoric acid (H_3PO_4) and is read at wavelength 450 nm.

To control for inter-plate variation, known positive and negative sera are included in each plate. Test sera are then ranked relative to the positive control. ELISA absorbance results are expressed as a percentage of this positive control (per cent positivity). Positive and negative threshold values should be determined in each laboratory by testing as many known positive and negative sera as possible.

Each batch of antigen and conjugate should be titrated using a checkerboard layout. With this test, it is possible to detect antibodies at least 4 years after a single infection. There should be 95–100% positive reactions with *B. bovis*-immune animals, 1–2% false-positive reactions with negative sera, and <2% false-positive reactions with *B. bigemina*-immune animals.

2.2. *Babesia bovis* and *Babesia bigemina* competitive enzyme-linked immunosorbent assays

The format of these tests is based on that described by Goff *et al.* (2003). The tests are described together because of the similarity of the processes. They have also been developed with the intention of validation as international standard tests and include purified recombinant antigen dried onto microtitre wells for ease of standardisation, handling and distribution, and inter-laboratory use and comparison under varying conditions (Goff *et al.,* 2006; 2008). The assays are based on a species-specific, broadly conserved, and tandemly repeated B-cell epitope within the C terminus of the rhoptry-associated protein 1 (RAP 1), expressed as a histidine-tagged thioredoxin fusion peptide. The expressed purified antigen is coated and then dried onto microtitre wells; optimal concentration of antigen and monoclonal antibody (MAb) is determined by block titration. In the case of *B. bovis*, positive sera inhibit the binding of the epitope-specific MAb BABB'75A4; in the case of *B. bigemina*, positive sera inhibit the binding of MAb 64/04.10.3.

The specificity, sensitivity and predictive values of these competitive ELISAs have been calculated, and the test reliability compared across laboratories. For *B. bovis* (Goff *et al.,* 2006), based on random operator receiver (ROC) analysis, 21% inhibition was chosen as the threshold value to define positive and negative samples. Using this value, specificity was 100%, sensitivity 91.1%, and positive predictive value 100%; negative predictive value varied with prevalence, ranging from 99% at 10% prevalence to 55.6% at a prevalence of 90%. A competitive ELISA using recombinant merozoite surface antigen 2c (rMSA-2c) was evaluated in *Babesia bovis* in Argentina and showed 98% specificity and 96.2% sensitivity (Dominguez *et al.,* 2012). For *B. bigemina* (Goff *et al.,* 2008), using a hypothetical prevalence rate of 25% and threshold inhibition for a negative value at 16%, the assay had a specificity of 98.3% and sensitivity of 94.7%. When threshold inhibition was increased to 21%, specificity was 100% but sensitivity reduced to 87.2%; negative predictive value at 25% prevalence reduced from 98.2% to 95.9%; and positive predictive value increased to 100% from 94.9%. At 21% inhibition, negative predictive value varied from 97.0% at 10% prevalence to 48.2% with a prevalence of 95%; positive predictive values were 90.7% (10% prevalence),

95.7% (15% prevalence) and 100% at all higher prevalence rates. The attributes of both tests appear to meet standards required for international application.

2.3. Indirect fluorescent antibody test

2.3.1. Antigen preparation

Antigen slides are made from jugular blood or *in-vitro* culture, ideally when the parasitaemia is between 2% and 5%.

Blood is collected into a suitable anticoagulant (sodium citrate or EDTA), and is then washed at least three times in five to ten volumes of PBS to remove contaminating plasma proteins and, in particular, host immunoglobulins. After washing, the infected RBCs are resuspended in two volumes of PBS to which 1% bovine serum albumin (BSA) has been added. The BSA is used to enable RBCs to adhere to the glass slide. By preference, single-layered blood films are made by placing a drop of blood on to a clean glass slide, which is then spun in a cytocentrifuge. This produces very uniform smears. Alternatively, thin blood films may be made by the conventional manual technique (dragging with the end of another slide). Commercially available microscope slides containing wells can also be conveniently used. The films are air-dried and fixed for 5 minutes in an oven at 80°C or cold dry acetone for 1 minute at –20°C. Fixed blood films are then covered (e.g. with aluminium foil or brown paper sticking tape) so as to be airtight, and stored at –70°C until required (maximum 5 years).

2.3.2. Test procedure

Test sera are diluted 1/80 in PBS. Sera may be used with or without heat inactivation at 56°C for 30 minutes. The slides are marked into 8–10 divisions with an oil pen to produce hydrophobic divisions. In each test square, 5–10 µl of each serum dilution is added to a filter paper disc using a fine pipette. The preparations are then incubated at 37°C for 30 minutes, in a humid chamber. For controls, negative and weak positive sera (at the same /80 dilution) are used on each test slide.

After incubation, the slides are rinsed with a gentle stream of PBS to remove the filter paper discs. The slides are soaked for 10 minutes in racks in PBS followed by 10 minutes in water. The PBS and water are circulated using a magnetic stirrer. Diluted anti-bovine IgG antibody labelled with fluorescein isothiocyanate (FITC) is then added to each test square. The appropriate dilution is based on titration of every new batch of conjugate, with the working range usually being between 1/400 and 1/1200. Conjugated rabbit, donkey and chicken antibodies are usually more suitable for this purpose than goat antibodies. Currently, newer fluorochromes offer better options due to their improved photostability and brightness, and a wider pH range (for more information contact the WOAH Reference Laboratories). The slides with the conjugate are incubated again at 20–25°C for 30 minutes, and washed as above. The wet slides are mounted with cover-slips in a solution containing 1 part glycerol and 1 part PBS, and examined by standard fluorescence microscopy. A competent operator can examine approximately 150 samples per day.

2.4. Other tests

Other serological tests have been described in recent years, and include a dot ELISA, a slide ELISA, latex and card agglutination tests and an immunochromatographic test (Kim *et al.,* 2008). These tests show acceptable levels of sensitivity and specificity for *B. bovis* and, in the case of the dot ELISA and ICT, also for *B. bigemina*. However, none of these tests appears to have been adopted for routine diagnostic use in laboratories other than those in which the original development and validation took place. Adaptability of these tests to routine diagnostic laboratories should be therefore evaluated in the future.

C. REQUIREMENTS FOR VACCINES

1. Background

Cattle develop a durable, long-lasting immunity after a single infection with *B. bovis, B. divergens* or *B. bigemina*. This feature has been exploited in some countries to immunise cattle against babesiosis (Bock *et al.,* 2008; Mangold

et al., 1996; Pipano, 1997). Most of these live vaccines contain specially selected strains of *Babesia*, mainly *B. bovis* and *B. bigemina*, and are produced in government-supported production facilities as a service to the livestock industries, in particular in Australia, Argentina, South Africa and Israel. Some other countries possess the ability to produce vaccine on a smaller scale. An experimental *B. divergens* vaccine prepared from the blood of infected *Meriones unguiculatus* (mongolian gerbil) has also been used successfully in Ireland (Zintl *et al.,* 2003).

A killed *B. divergens* vaccine has also been prepared from the blood of infected calves (Zintl *et al.,* 2003), but little information is available on the level and duration of the conferred immunity. Other experimental vaccines containing *Babesia* spp. antigens produced *in vitro* have also been developed (Montenegro-James *et al.,* 1992), but the level and duration of protection against heterologous challenge are unclear. Despite the characterisation of various parasite proteins and the *B. bovis* genome (Brayton *et al.,* 2007) and considerable effort worldwide directed towards identification of candidate vaccine antigens, the prospects for recombinant vaccines against *Babesia* spp. remain challenging (Brown *et al.,* 2006). To date no effective subunit vaccine is available commercially.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and those in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

This section will deal with the production of live babesiosis vaccines, mainly those against *B. bovis* and *B. bigemina* infections in cattle. Production involves infection of calves with selected strains, and use of the infected RBCs as vaccine (Bock *et al.,* 2008); or *in-vitro* culture methods to produce parasites for vaccine (Mangold *et al.,* 1996). Calves used for infection with these strains or, in the case of *in vitro* methods, as a source of serum and RBCs for culture, must be free of infectious agents that can be transmitted by products derived from their blood. In the case of *B. divergens*, blood of infected gerbils (*Meriones unguiculatus*) can be used instead of bovine blood. Evidence that changes in immunogenicity occur with repeat passages in calves, and that possible antigenic drift occurs during long-term maintenance of *B. bovis* in culture, must be managed by limiting the number of repeat passages or subcultures made before returning to the vaccine working seed stabilate. Whilst *in-vitro* production methods offer obvious advantages in terms of animal welfare, vaccine can also be successfully produced using *in-vivo* production systems under strict animal welfare guidelines. Close to 400,000 doses of vaccine per year have been successfully produced in Argentina under authority of SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria, National Service for Agrifood Health and Quality) using *in-vitro* culture, and up to 850,000 doses per year have been successfully produced in Australia under authority of APVMA (Australian Pesticides and Veterinary Medicines Authority) using *in-vivo* techniques.

Babesia bovis and *B. bigemina* vaccines can be prepared in either frozen or chilled form depending on demand, transport networks and the availability of liquid nitrogen or dry ice supplies. Preparation of frozen vaccine (Bock *et al.,* 2008; Mangold *et al.,* 1996; Pipano, 1997) allows for thorough post-production testing of each batch. However, it has a much reduced shelf-life once thawed, is more costly to produce and more difficult to transport than chilled vaccine. The potential risk of contamination of this blood-derived vaccine makes pre- and post-production quality control essential, but may put production beyond the financial means of some countries in endemic regions.

2. Outline of vaccine production

2.1. Characteristics of the seed

2.1.1. Internationally available strains

Attenuated Australian strains of *B. bovis* and *B. bigemina* have been used effectively to immunise cattle in Africa, South America and South-East Asia (Bock *et al.,* 2008). Tick-transmissible and non-transmissible strains are available. A strain of *B. divergens* with reduced virulence for *Meriones* has also been developed (Zintl *et al.,* 2003).

2.1.2. Isolation and purification of local strains

Strains of *B. bovis*, *B. divergens* and *B. bigemina* that are free of extraneous agents, such as *Anaplasma*, *Eperythrozoon, Theileria, Trypanosoma* and various viral and bacterial agents are most readily isolated by feeding infected ticks on susceptible splenectomised cattle. The vectors and modes of transmission of the species differ, and these features can be used to separate the species (Friedhoff & Bose, 1994).

Babesia spp. can also be isolated from infected cattle by subinoculation of blood into susceptible splenectomised calves. A major disadvantage of this method is the difficulty of separating the *Babesia* spp. from contaminants such as *Anaplasma* and *Eperythrozoon*. Isolation of *B. divergens* is a relatively simple process because of the susceptibility of *Meriones* (Zintl *et al.,* 2003). Maintenance of isolated strains *in vitro* (Jorgensen & Waldron, 1994) can be used to eliminate most contaminants, but not to separate *Babesia* spp. Selective chemotherapy (for example, 1% trypan blue to eliminate *B. bigemina*) can be used to obtain pure *B. bovis* from a mixed *Babesia* infection, while rapid passage in susceptible calves will allow isolation of *B. bigemina*.

2.1.3. Attenuation of strains

Various ways of attenuating *Babesia* spp. have been reported. The most reliable method of reducing the virulence of *B. bovis* involves rapid passage of the strain through susceptible splenectomised calves. Attenuation is not guaranteed, but usually follows after 8 to 20 calf passages (Bock *et al.,* 2008). The virulence of *B. bigemina* decreases during prolonged residence of the parasite in latently infected animals. This feature has been used to obtain avirulent strains by infecting calves, splenectomising them 6–12 weeks after inoculation and then using the ensuing relapse parasites to repeat the procedure (Bock *et al.,* 2008). Attenuation of *B. divergens* in *M. unguiculatus* followed long-term maintenance *in vitro* has been achieved (Zintl *et al.,* 2003).

Attenuation of *Babesia* spp. with irradiation has been attempted, but the results were variable. Similarly, maintenance in vitro in modified media has been used experimentally.

Avirulent strains should be stored as stabilate for safety testing and for future use as master seed in the production of vaccine.

2.1.4. Preparation and storage of master seed

Avirulent strains are readily stored as frozen infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone (PVP) MW 40,000 (Bock *et al.,* 2008) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the master seed.

For the DMSO procedure, infected blood is collected and chilled to 4°C. Cold cryoprotectant (4 M DMSO in PBS) is then added, while stirring slowly, to a final blood: cryoprotectant ratio of 1:1 (final concentration of DMSO is 2 M). This dilution procedure is carried out in an ice bath, and the diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as possible, in the vapour phase of a liquid nitrogen container. The vials are stored in the liquid phase in a designated tank to prevent loss of viability and contamination. Stored in this way, master seed lots of *Babesia* spp. have been known to remain viable for 20 years.

Unlike DMSO, it has not been found necessary to work with stabilates containing PVP in an ice-bath (Standfast & Jorgensen, 1997). Pre- and post-thaw storage at 20–25°C have not affected infectivity. PVP is a complex polymer that does not permeate intact cell membranes. It has low toxicity for vertebrates and parasites and stabilate-containing PVP is infective when inoculated intravenously. PVP with a molecular weight of 40,000 is made up to a 20% solution with PBS and autoclaved to sterilise. Blood from an infected calf is slowly mixed with an equal volume of the 20% PVP in PBS solution to produce a final concentration of 10% PVP. The mixture is then dispensed into 5 ml cryovials, frozen in the vapour phase of liquid nitrogen by cooling at a rate of about 10°C per minute for 15 minutes and then stored in liquid nitrogen (Standfast & Jorgensen, 1997).

In-vitro cultures are based on the microaerophilous stationary phase method (Levy & Ristic, 1980). Blood infected with avirulent strains of *B. bovis* or *B. bigemina* is harvested from splenectomised calves and washed with VYM phosphate-buffered saline solution (Vega *et al.,* 1985) to remove the plasma and buffy coat. The VYM solution is composed of $CaCl_2.2H_2O$ (16.0 mg), KCl (400.0 mg), KH_2PO_4 (1415.4 mg), $MgSO_4.7H_2O$ (154.0 mg), $Na_2HPO_4.7H_2O$ (1450 mg), NaCl (7077.0 mg) and dextrose (20.5 g) in 1 litre of double-distilled deionised water containing 0.25 mM adenine and 0.50 mM guanosine. Individual 5% suspensions of infected and uninfected RBCs are then prepared in basic culture medium consisting of commercial M199 medium and normal bovine serum (60/40). The basic medium is supplemented with 18 mM HEPES (4-[2-Hydroxyethyl] piperazine-1-ethanesolfonic acid), 10 mM $NaHCO_2$, 100 µg/ml streptomycin sulphate and

100 U/ml penicillin G. The parasitised and normal RBC suspensions are mixed (1/1), dispensed into culture flasks and incubated under an atmosphere of 90% N_2, 5% O_2 and 5% CO_2 at 37°C. After 8 to 10 subcultures in different size culture flasks, the final complete cultures are spun at 1200 ***g*** for 10 minutes at 4°C and the supernatant removed. Packed parasitised RBCs are gently mixed with an equal volume (1/1) of 20% PVP in VYM solution, and dispensed in 2 ml cryovials. The parasitised RBCs are frozen in the vapour phase of liquid nitrogen by cooling at a rate of about 10°C per minute for 15 minutes and then stored in liquid nitrogen (Standfast & Jorgensen, 1997). Normal blood from donor cattle, which is used as a source of serum and uninfected RBCs for culture medium, is defibrinated with glass balls. The RBCs are washed and stored for up to 3 weeks in VYM solution at 4°C and normal serum is stored frozen at –20°C until use.

2.1.5. Preparation and storage of working seed

Working seed is prepared in the same way as master seed (Section C.2.1) using master seed as starting material.

2.1.6. Validation of safety and efficacy of working seed

The suitability of a working seed is determined by repeatability of infectivity in splenectomised calves, or in initiating cultures, and its safety and efficacy in non-splenectomised cattle. Repeatability is determined by inoculating several susceptible splenectomised calves and monitoring parasite progression by stained blood smears. The prepatent period and parasite progression should be relatively consistent between calves to allow inoculations to be scheduled with a degree of certainty.

In-vitro prepared working seed vials are thawed by immersing in water preheated to 40°C and directly dispensed in culture media. The *in-vitro* multiplication process starts with a 5% RBC suspension, which is progressively increased up to 10%. Working seed is considered acceptable when continuous cultures derived from it achieve 8–12% of RBCs parasitised by morphologically normal merozoites/trophozoites after the third subculture and growth in an atmosphere of 5% CO_2 in air at 37°C.

The safety and efficacy of the vaccine strain is determined by inoculating suitable numbers of susceptible adult cattle with vaccine prepared from RBCs from a calf that has been inoculated with the strain, or from an *in-vitro* culture process. Safety can be judged by monitoring body temperature, parasitaemia in stained blood films, and PCV depression following vaccination. Efficacy is judged by monitoring the same parameters following the inoculation of the vaccinated cattle with a heterologous strain. The purity of the working seed is tested by monitoring the cattle used in the safety test for evidence of possible extraneous agents or by thorough testing of the calf from which the stabilate was produced (see Section C.2.2.3). Bovine donors of uninfected blood used for *in-vitro* cultures are maintained in isolated pens and their health status monitored.

2.2. Method of manufacture

2.2.1. Production of frozen vaccine concentrate

First, 5–10 ml quantities of working seed are rapidly thawed by immersing the vials in water preheated to 37°C. The thawed material is used as soon as possible to infect a susceptible, splenectomised calf (free of potential vaccine extraneous agents) by intravenous inoculation. If DMSO is used as the cryopreservative, the thawed working seed must be kept on ice and inoculated within 30 minutes of thawing.

Blood suitable for vaccine is obtained by monitoring films of jugular blood and collecting the required volume of blood when a suitable parasitaemia is reached. A parasitaemia of 3.5×10^8/ml for *B. bovis* in jugular blood, or 3×10^7/ml for *B. bigemina*, is usually adequate for production of frozen vaccine. If a suitable *B. bovis* parasitaemia is not obtained, passage of the strain by subinoculation of 100–800 ml of blood into a second splenectomised calf may be necessary. Passage of *B. bigemina* through splenectomised calves is not recommended because of the potential for the attenuated strain to increase in virulence.

Blood from the infected donor calf is collected by jugular cannulation using preservative-free heparin as anticoagulant (5 IU heparin/ml blood).

In the laboratory, the parasitised blood is held at 20–25°C and mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose (final concentration of glycerol in blood mixture is 1.5 M) held at 37°C. The mixture is then equilibrated at 37°C for 30 minutes, and dispensed into suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (Bock *et al.,* 2008).

DMSO can be used as cryoprotectant in the place of glycerol. This is carried out in the same way as outlined for the preparation of master seed (Pipano, 1997).

When glycerolised frozen vaccine is diluted for use as vaccine, the diluent should be iso-osmotic and consist of PBS containing 1.5 M glycerol and 5 mM glucose. Similarly, the diluent used in vaccine cryopreserved with DMSO should be iso-osmotic, and should contain the same concentration of DMSO in PBS as the concentration of DMSO in the vaccine concentrate.

Frozen vaccine containing both *B. bovis* and *B. bigemina* can be prepared by mixing equal volumes of blood containing each of the parasites obtained from different donors (Mangold *et al.,* 1996). A trivalent vaccine containing RBCs infected with *B. bovis*, *B. bigemina* and *Anaplasma centrale* is also made in Australia. RBCs from three donors (one for each parasite) are concentrated by centrifugation and mixed with glycerol solution to produce the trivalent concentrate, which is thawed and mixed with a diluent before use (Bock *et al.,* 2008).

The recommended dose volume of vaccine after reconstitution and dilution ranges from 1 to 2 ml depending on local practices and requirements, but aims to deliver a minimum infective dose of parasites, based on the parasitaemia prior to freezing.

2.2.2. Production of chilled vaccine

Infective material used in the production of chilled vaccine is obtained in the same way as for frozen vaccine, but should be issued and used as soon as possible after collection. If it is necessary to obtain the maximum number of doses per calf, the infective material can be diluted to provide the required number of parasites per dose (usually from 2.5 to 10 $\times 10^6$). A suitable diluent is 10% sterile bovine serum in a balanced salt solution containing the following ingredients per litre: NaCl (7.00 g), $MgCl_2.6H_2O$ (0.34 g), glucose (1.00 g), Na_2HPO_4 (2.52 g), KH_2PO_4 (0.90 g), and $NaHCO_3$ (0.52 g).

In-vitro multiplication is carried out in 225 cm^2 tissue culture flasks, where 115 ml of complete culture medium is dispensed to achieve a depth of 5.0–5.2 mm. Ninety ml of supernatant is replaced daily by fresh medium and 50–75% of parasitised RBCs are replaced every 48 hours by uninfected RBCs (subculture). Parasitised RBCs containing *Babesia* spp. are harvested when the parasites show typical morphology and achieve the maximum parasitaemia still inside the RBCs. Ninety per cent of the basic medium from each flask is removed without disturbing the settled RBCs. The *Babesia*-parasitised RBCs, still suspended in the remaining medium, are then mixed 1/1 with balanced salt solution, dispensed into one bottle and refrigerated to 5°C until use. The suspensions of each *Babesia* species, both with a high concentration of parasites, are finally diluted with the same balanced salt solution enriched with 10% bovine serum to achieve a concentration of 10^7 *B. bovis and* 10^7 *B. bigemina* parasitised RBCs per 2 ml dose.

Where anaplasmosis is of concern, *Anaplasma centrale* may also be incorporated into the vaccine to make a trivalent vaccine effective against *B. bovis*, *B. bigemina* and *Anaplasma marginale*.

2.2.3. In-process control

i) Sources and maintenance of vaccine donors

A source of donors free of natural infections with *Babesia*, other tick-borne diseases, and other infectious agents transmissible with blood, should be identified. If a suitable source is not available, it may be necessary to breed donor calves under tick-free conditions specifically for the purpose.

Donor calves should be maintained under conditions that will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production of vaccine (as opposed to importation of a suitable product) should be weighed against the possible adverse consequences of spreading disease (Bock *et al.,* 2008).

ii) Surgery

Calves to be used as vaccine donors should be splenectomised to allow maximum yield of parasites for production of vaccine. This is easier in calves less than 3 months of age and must be performed under general anaesthesia.

iii) Screening of vaccine donors before inoculation

Donor calves should be examined for agents of all blood-borne infections prevalent in the country, including *Babesia, Anaplasma, Theileria*, *Eperythrozoon* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serological testing pre- and post-quarantine. Calves showing evidence of natural infections with any of these agents should be rejected or have infections chemically sterilised. The absence of other infective agents endemic in the country should also be confirmed; this may include the agents of enzootic bovine leucosis, bovine immunodeficiency virus, bovine pestivirus, bovine syncitial virus, infectious bovine rhinotracheitis, Akabane disease, Aino virus, ephemeral fever, bluetongue, foot and mouth disease, bluetongue, *Brucella abortus*, *Leptospira* spp., heartwater, Jembrana disease, Rift Valley fever, rabies, lumpy skin disease, contagious bovine pleuropneumonia and rinderpest. The test procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera and, in some cases, virus isolation or antigen or DNA detection (Bock *et al.,* 2008; Pipano, 1997).

iv) Monitoring of parasitaemias following inoculation

It is necessary to determine the concentration of parasites in blood collected for vaccine or in RBCs harvested from culture. There are accurate techniques for determining the parasite count, but the parasite concentration can be adequately estimated from the RBC count and the parasitaemia (% infected RBCs).

v) Collection of blood for vaccine

All equipment should be sterilised before use (e.g. by autoclaving). The blood is collected in heparin using strict aseptic techniques in a closed-circuit collection system, when the required parasitaemia is reached. The calf should be sedated (for example, with xylazine).

Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live, the transfusion of a similar amount of blood from a suitable donor (or blood previously collected from the donor itself) is indicated. Alternatively, the calf should be killed immediately after collection of the blood.

Using *in-vitro* culture, a 225 cm^2 flask can routinely provide 1800 doses. Starting from one 225 cm^2 flask containing 11 ml of 8–10% parasitised RBCs, it is possible to harvest about 45,000 doses after 6 days of continuous growth.

vi) Dispensing of vaccine

All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of infected RBCs and diluent throughout the dispensing process.

2.2.4. Batch control

The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, except by thorough testing of vaccine donors and adherence to the principles of good

manufacturing practice. Specifications of frozen vaccine depend on the code of practice of the country involved. The following are the specifications for frozen vaccine produced in Australia.

i) Sterility and freedom from contaminants

Standard tests for sterility are employed for each batch of vaccine and diluent. The absence of contaminants is determined by doing appropriate serological and molecular diagnostic testing of donor cattle for evidence of viral and bacterial infection. Potential contaminants include those agents listed in Section C.2.2.3.

ii) Safety

Adverse reactions of the cattle inoculated in the test for potency may be monitored by measuring parasitaemia, temperature and packed cell volume; or based on regular observation of the health and demeanour of vaccinated animals. Detailed monitoring is more usually associated with the development and testing of parasite strains as potential candidates for vaccine production. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use. Vaccine is preferably used in calves less than 1 year of age.

Non-target species are of no concern. Some attenuated vaccine strains of *B. bovis* are tick transmissible and evidence suggests that they may return to virulence after transmission by ticks. This is of little consequence in endemic situations.

No withholding periods for milk or meat are necessary following use of the vaccine, unless required by local legislation.

iii) Potency

Frozen, glycerolised vaccine concentrate is thawed and diluted 1/10 with isotonic diluent (Bock *et al.,* 2008; Pipano, 1997).The prepared vaccine is then stored for 8 hours at 4°C, and 5 to 25 susceptible cattle (held in cattle tick-free areas) are each inoculated subcutaneously with a 2 ml dose of that vaccine batch. The inoculated cattle are then monitored for the presence of viable *Babesia* spp. infections by examination of stained blood smears, by PCR techniques or by evidence of seroconversion. Only batches with acceptable infectivity are released for use at a working dilution of 1/10. Greater than 95% of vaccinated cattle would be expected to develop immunity to *Babesia* spp. after a single inoculation with an adequate dose (1×10^7 parasites) in a chilled or frozen vaccine prepared, stored and transported according to appropriate protocols.

iv) Duration of immunity

Long-lasting immunity usually results from one inoculation. Protective immunity develops in 3–4 weeks, and lasts at least 4 years in most cases (Bock & de Vos, 2001). Evidence of *B. bovis* vaccine failures have been reported and are related to the choice of vaccine strain, the presence of heterologous field strains and host factors (Bock *et al.,* 2008). There is little evidence of time-related waning of immunity (Bock & de Vos, 2001).

v) Stability

When stored in liquid nitrogen, the frozen vaccine can be kept for 5 years. Sterile diluent can be kept for 2 years in a refrigerator. Thawed vaccine rapidly loses potency and cannot be refrozen.

vi) Preservatives

Benzylpenicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine concentrate prior to dispensing into cryotubes. No preservative is used.

vii) Use of vaccine

In the case of frozen vaccine, vials should be thawed by immersion in water preheated to 37°C. Glycerolised vaccine should be kept cool and used within 8 hours (Bock *et al.,* 2008),

while vaccine with DMSO as cryoprotectant should be kept on ice and used within 15–30 minutes of thawing (Pipano, 1997).

Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation, depending on the viability of the parasites and the recommendation of the vaccine production facility.

The strains of *B. bovis, B. divergens* and *B. bigemina* used in the vaccine may be of reduced virulence, but may not be entirely safe. A practical recommendation is therefore to limit the use of vaccine to calves under the age of 1 year, when nonspecific immunity will minimise the risk of adverse reactions. If older animals are to be vaccinated, there is a greater risk of vaccine reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals warrant due attention and should be observed daily for 3 weeks after vaccination. Ideally, rectal temperatures of vaccinated cattle should be taken and the animals should be treated if significant fever develops. Reactions to *B. bigemina* and *B. divergens* are usually seen by day 6–8 and those to *B. bovis* by day14–18 (Bock *et al.,* 2008).

Babesiosis and anaplasmosis vaccines are often used concurrently, but it is preferable not to use any other vaccines at the same time (Bock *et al.,* 2008).

viii) Precautions

Babesia bovis and *B. bigemina* vaccines are not infective for humans. However, cases of *B. divergens* have been reported in splenectomised individuals. When the vaccine is stored in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of liquid nitrogen and deep-frozen material applies.

2.3. Requirements for regulatory approval

Issues of safety, potency, stability of vaccine strains, non-target species and reversion to virulence are dealt with in preceding sections. The vaccine is only used to control babesiosis. Eradication of babesiosis is only undertaken through eradication of the tick vector and/or intensive chemotherapeutic regimes.

3. Vaccines based on biotechnology

No biotechnology-based vaccines are currently available.

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NB: There are WOAH Reference Laboratories for babesiosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for babesiosis

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.4.3.

BOVINE CYSTICERCOSIS

See Chapter 3.10.3. Cysticercosis (*including infection with* Taenia solium).

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CHAPTER 3.4.4.

BOVINE GENITAL CAMPYLOBACTERIOSIS

SUMMARY

Description of the disease: *Bovine genital campylobacteriosis (BGC) is a venereal disease also known as bovine venereal campylobacteriosis. The causal agent of this sexually transmissible disease is* Campylobacter fetus *subsp.* venerealis, *one of three subspecies of* C. fetus. *Bovine infections with* C. fetus *subsp.* venerealis *are associated with infertility, early embryonic death, and abortion with considerable economic losses. Bovine infections with* C. fetus *subsp.* fetus *are associated with abortion and have a more sporadic occurrence.*

Campylobacter fetus *subsp.* venerealis *has a pronounced tropism for the male and female genital systems of cattle. Bacterial transmission takes place mainly during natural mating. The disease may also spread through artificial insemination with semen from infected bulls.*

Detection of the agent: *Samples taken from bulls, cows or aborted fetuses can be analysed for the presence of the causal organism. The organism is a thin Gram-negative curved rod that may form S-shapes, flying seagull silhouette shapes and spirals, and can be cultured on selective media at 37°C after at least 2 days in a microaerobic atmosphere. Confirmation of organism and discrimination between the* C. fetus *subspecies can be achieved using biochemical or molecular methods. Immunofluorescence may also be used to identify the organism, but it will not differentiate between different subspecies.*

A monoclonal antibody-based capture enzyme-linked immunosorbent assay (ELISA) may also be used to detect C. fetus *from Clarke's transport enrichment medium. Like immunofluorescence, the ELISA cannot differentiate adequately between the two* C. fetus *subspecies and is limited to a screening method, with traditional cultural methods to confirm identification of any positive ELISA result. However, compared with immunofluorescence, it has the advantage of higher sensitivity and specificity for* C. fetus *identification and much higher throughput for both negative and positive results where larger volume testing is conducted.*

Serological tests: *ELISA can be used for evaluating herd immunity, but is not suitable for diagnosis of the infection in individual animals. This test cannot differentiate between infections caused by the two subspecies* C. fetus *subsp.* venerealis *and* C. fetus *subsp.* fetus.

Requirements for vaccines: *While there are commercial vaccines available, and autologous vaccines based on isolated strains from farms can be used, there is a lack of scientific evidence overall of the effectiveness of vaccination.*

A. INTRODUCTION

1. Description and impact of the disease

Bovine genital campylobacteriosis (BGC, also known as bovine venereal campylobacteriosis) is a venereal disease characterised by infertility, early embryonic death, and abortion in cattle. The causal agent of this sexually transmissible disease is *Campylobacter fetus* subsp. *venerealis* (Cfv). It can be isolated from the genital tract of cattle (e.g. preputial smegma, vaginal mucus) or internal organs of aborted fetuses and causes fertility problems with considerable economic losses. Bulls are important reservoirs and should therefore be targeted for diagnostic testing and disease control. *Campylobacter fetus* subsp. *fetus* (Cff) can be recovered from the intestinal tract of cattle and other animal species (Garcia *et al*., 1983); it can be isolated from aborted bovine fetuses indicating that it

has clinical relevance in cattle. Cff is associated with sporadic cases of bovine abortion whereas Cfv is associated with endemic abortion and fertility problems in certain areas.

Although *C. fetus* is primarily recognised as a pathogen in animals, Cff is occasionally diagnosed as an opportunistic pathogen in humans. Infections usually occur in pregnant or immuno-compromised individuals and are often systemic with a variety of complications depending on the site of infection (Wagenaar *et al*., 2014). Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

2. Taxonomy

Campylobacter fetus is one of the 33 currently recognised species within the genus *Campylobacter* (http://www.bacterio.net/campylobacter.html). Three subspecies of *C. fetus* have been recognised: *C. fetus* subspecies *testudinum* (Cft), *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, of which Cft is reptile-associated and both Cff and Cfv are mammal-associated. Cfv includes a variant designated Cfv biovar *intermedius* (Florent *et al*., 1959; Veron & Chatelain, 1973). Although the clinical signs related to infections by Cfv and Cff overlap, they were originally defined by the differences in clinical presentation (Florent *et al.,* 1959; Veron & Chatelain, 1973).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of bovine genital campylobacteriosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Culture (including subspecies identification)	+++	+++	+++	+++	+++	–
IFAT	++	++	++	++	++	–
MAb-based capture ELISA	++	++	++	++	++	–
Detection of immune response						
Antibody ELISA	–	–	–	–	–	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
IFAT = immunofluorescent antibody test; MAb = monoclonal antibody; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

Culturing and identification of the organism is a suitable test for certifying individual animal prior to movement. The validated sensitive and specific monoclonal antibody (MAb)-based capture enzyme-linked immunosorbent assay (ELISA) screening procedure (Brooks *et al.,* 2004; Devenish *et al.,* 2005) can accurately detect *C. fetus* subsp. from incubated Clarke's transport enrichment medium (TEM). Only positive ELISA results are cultured for confirmation of the specific subspecies using standard isolation and identification methods. This procedure has the advantage of more rapid turnaround times for reporting, and ease, compared with traditional cultural methods.

Cfv and Cff can be differentiated by the 1% glycine tolerance test and H_2S production in cysteine-rich medium; Cfv is not able to grow in the presence of 1% glycine and not able to produce H_2S from cysteine-rich medium, whereas

Cff is 1% glycine tolerant and H_2S production positive (Florent *et al.*, 1959; Veron & Chatelain, 1973). Neither DNA–DNA hybridisation (Harvey & Greenwood, 1983) nor protein-banding patterns using polyacrylamide gel electrophoresis (PAGE) of whole cell proteins (Vandamme *et al*., 1990) revealed any major difference between Cfv and Cff. MALDI-TOF (matrix assisted laser desorption ionisation–time of flight) mass spectrometry can be used to identify *C. fetus* at species level (Bessede *et al*., 2011), but this assay is not able to differentiate between Cff and Cfv. Of all described molecular tests, only polymerase chain reaction (PCR) assays targeting the *nahE* gene, both conventional and real-time (Abril *et al*., 2007; van der Graaf-van Bloois *et al*., 2013), multilocus sequence typing (MLST) and amplified fragment length polymorphism (AFLP) (van Bergen *et al.*, 2005a; Wagenaar *et al*., 2001) are able to identify *C. fetus* reliably at the species level. Several molecular methods have been described claiming to differentiate between Cff and Cfv, including PCR (Abril *et al.,* 2007; Hum *et al*., 1997; Tu *et al*., 2005; van Bergen *et al*., 2005c; Wang *et al*., 2002), pulsed-field gel electrophoresis (PFGE) (On & Harrington, 2001), MLST (van Bergen *et al*., 2005a) and AFLP (Wagenaar *et al*., 2001) (see also Section B.1.9), but none of these molecular tests is able to identify the *C. fetus* isolates reliably to subspecies level (Iraola *et al*., 2015; van der Graaf-van Bloois *et al*., 2013). Whole genome sequencing can be used to differentiate the mammal-associated *C. fetus* strains based on their core genomes (van der Graaf-van Bloois *et al.*, 2014), but this method is not in full concordance with the phenotypic subspecies identification of *C. fetus* strains.

1. Isolation and detection of the agent

1.1. Collection of specimens

1.1.1. Male: preputial smegma and semen

In bulls, smegma may be obtained by different methods (da Silva Silveira *et al*., 2018): scraping (Tedesco *et al*., 1977), aspiration (Campero *et al*., 2003), and washing (Clarke & Dufty, 1978). Note that the same sample, collected using the same sampling method, can be used for the detection of *Trichomonas foetus* (Waldner *et al.,* 2017*)* (see Chapter 3.4.16 *Trichomonosis*).

Smegma can be used for isolation of the organism or is rinsed into a tube with approximately 5 ml phosphate buffered saline (PBS) with 1% formalin for diagnosis by immunofluorescence. Smegma can also be collected from the artificial vagina after semen collection, by washing the artificial vagina with 20–30 ml of PBS.

For preputial washing, 20–30 ml of PBS is introduced into the preputial sac. After vigorous massage for 15–20 seconds, the infused liquid is collected.

Semen is collected under conditions that are as aseptic as possible. Semen samples must be diluted with PBS and are inoculated directly on to culture medium or into transport and enrichment medium.

1.1.2. Female: cervico vaginal mucus

Samples may be obtained by aspiration or washing the vaginal cavity. Hollow plastic scraping tools can also be used. The samples can be used for *Trichomonas foetus* detection (see chapter 3.4.16)

For aspiration, the vulva region is cleaned, and an artificial insemination (AI) pipette or Cassou pipette (blue sheath type) is inserted into the vaginal cavity so that the anterior reaches the cervix (Campero *et al*., 2003). Gentle suction is applied while moving the pipette gently backwards and forwards. The pipette is removed, and the collected mucus is inoculated directly on to culture medium or into transport and enrichment medium.

Cervico-vaginal mucus (CVM) may also be collected by washing the vaginal cavity: 20–30 ml of PBS is infused into the cavity through a syringe attached to an AI pipette. The fluid is sucked out and re-infused four to five times before being collected and spread directly on to culture medium or added to transport and enrichment medium. Washing fluid in the vaginal cavity may also be collected by a tampon or gauze held inside the vagina for 5–10 minutes after PBS infusion. Samples of CVM obtained by suction may be diluted with PBS, or sown directly on to culture medium or transport and enrichment medium.

CVM is transferred into approximately 5 ml of PBS with 1% of formalin for immunofluorescence testing.

1.1.3. Aborted fetuses, placentas

The placenta as well as the liver, lungs and stomach contents of the fetus provide the best samples for isolation of the causative bacteria. Samples are collected aseptically and inoculated into transport and enrichment medium, or into PBS with 1% formalin for IFA testing.

1.2. Transport of specimens

The use of a transport medium is essential if the specimens are not processed in the laboratory within the same day after collection. For dispatch to the laboratory, specimens must be placed in an insulated container (within the temperature range 4–8°C) protected from light and shipped to reach the laboratory as soon as possible, preferably on the same day as sampling.

Various transport and enrichment media are available, such as Clark's, Lander's, SBL, Foley's and Clark's, Weybridge's, Cary-Blair's (Garcia *et al*., 1984; Hum *et al*., 1994; Monke *et al*., 2002), Thomann's transport and enrichment (TTE) medium (Harwood *et al.,* 2009).

Some of the transport and enrichment media mentioned above contain cycloheximide. Because of its potential toxicity, amphotericin B can be used as an alternative.

1.3. Treatment of specimens

On arrival at the laboratory, specimens should be inoculated directly on to culture medium, or, if required immediately, processed further.

1.3.1. Genital tract samples

The total volume of the preputia washings may be centrifuged (3500 ***g***) to concentrate the sample. The final sample (reduced to 500 µl) may be inoculated on to the culture medium (directly on Skirrow agar plate, using the filter method, and to TTE medium (Harwood *et al.,* 2009).

If the CVM is not very viscous it can be inoculated directly or diluted with an equal volume of PBS. When the CVM is very viscous, it may be necessary to liquefy it by adding an equal volume of L-cysteine solution (100 mM *N*-Acetyl-L-cysteine, CAS number 616-91-1[1], dissolved in PBS and sterilised by membrane filtration). After 15–20 minutes, the diluted and liquefied mucus can then be inoculated on to isolation medium.

For the filter method; sterile cellulose acetate membranes of 0.65 µm pore size are placed on the surface of non-selective agar supplemented with 5% blood. A suspension of the sample is made in in PBS or saline, and 10–15 drops of this suspension are placed on top of the membrane and allowed to filter passively at 37°C (microaerobic conditions are not required). After 30–40 minutes, the filter membranes are removed and the culture plates incubated for 1–2 days at 37°C under microaerobic conditions (Steele & McDermott, 1984).

1.3.2. Aborted fetuses, placentas

Fetal stomach contents are inoculated directly on to culture medium. Internal organs or pieces of organs are flamed to disinfect the surface, and are subsequently homogenised. The homogenate is inoculated on to culture medium.

After washing placental membranes with PBS to eliminate the majority of the surface contamination, the chorionic villi are scraped and the scrapings are transferred to culture medium.

1 This is the unique number for this chemical assigned by Chemical Abstracts Service (CAS).

1.4. Isolation of *Campylobacter fetus*

1.4.1. Culture media for isolation

Many media are currently in use for the bacteriological diagnosis of BGC. It should be noted that several media used for the isolation of *Campylobacter* spp. are not suitable for the isolation of *C. fetus* because of antimicrobials (e.g. cephalosporins) that may inhibit C. *fetus* growth (van Bergen *et al*., 2005b). Most culture media contain cycloheximide. Because of its potential toxicity, this antifungal agent can be replaced by amphotericin B. The recommended selective medium for isolation of *C. fetus* is TTE (Harwood *et al.*, 2009). This is based on Skirrow's medium, but with the selective supplements reduced to 75% of the original recommendations, i.e.: polymyxin B suphate (1.9 IU/ml), trimethoprim (3.8 μg/ml), vancomycin (7.5 μg/ml) and 5 mg/litre amphotericin B. After 48 hours of enrichment, the enrichment broth can be plated on either Skirrow's selective plates or non-selective plates using the filtration method. Skirrow's selective plates are a blood-based agar containing 5–7% (lysed) defibrinated blood and Skirrow selective supplement.

Alternatively, a non-selective blood-based (5–7% blood) medium in combination with filtration (0.65 μm) can be used; however, it may be less sensitive when compared with a selective medium.

Quality control of each batch of media should be performed using control strains.

1.4.2. Incubation conditions

Plates or tubes with TTE medium are incubated at 37°C and under microaerobic atmosphere of 5–10% oxygen, 5–10% carbon dioxide and preferably 5–9% hydrogen for optimal growth (Vandamme, 2000). Appropriate microaerobic conditions may be produced by a variety of methods. In some laboratories the suitable atmosphere is created by a gas replacement in a jar. Gas generator kits are also available from commercial sources. Variable atmosphere incubators can also be used.

Conditions of culture and incubation are systematically verified by using control strains of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Such controls should be set up for each isolation attempt.

1.5. Identification of *Campylobacter* species

1.5.1. Colony morphology

Colonies of *C. fetus* usually appear on culture media after 2–5 days. To prevent overgrowth of specific colonies by contaminants, it is recommended that the media be evaluated daily and suspicious colonies be subcultured. After 3–5 days of incubation, colonies measure 1–3 mm in diameter. They are slightly grey-pink, round, convex, smooth and shiny, with a regular edge.

1.5.2. Macroscopic morphology

Campylobacter is motile, a property that may disappear during sub-culturing. *Campylobacter* often takes the form of a thin, curved bacillus, 0.3–0.4 μm wide and 0.5–8.0 μm long. Short forms (comma-shaped), medium forms (S-shaped/flying seagull-shaped), and long forms (helical with several spirals) may be observed simultaneously in the living state. Old cultures may contain coccoid bacteria.

1.5.3. Biochemical tests

See Table 2.

1.5.4. Atmosphere

Campylobacter does not grow under aerobic conditions.

1.6. Detection of *Campylobacter fetus* by the immunofluorescence method

The immunofluorescence antibody test (IFAT) can be applied to detect the organism directly from samples or to confirm the identification of a strain after isolation. This test was able to detect *C. fetus* in contaminated field samples. The detection limit of this test is 10^4 and 10^2 colony-forming units/ml in non-centrifuged and centrifuged preputial washings, respectively (da Silva Silveira *et al*., 2018). IFAT cannot differentiate between different subspecies.

1.6.1. Preparation of immune sera

Campylobacter strains, preferably standard strains from recognised culture collections (*C. fetus* subsp. *venerealis* or *C. fetus* subsp. *fetus*), are grown on blood-based medium at 37°C under microaerobic conditions for 3 days. The organisms are harvested into PBS, and washed twice by centrifugation. Rabbits aged 3 months are inoculated intramuscularly with 2 ml of 10^{11} organisms/ml of a *C. fetus* subspecies resuspended in PBS and Freund's incomplete adjuvant. Inocula are administered at four sites, 0.5 ml at each site. The animals are bled before inoculation and at weekly intervals thereafter. When the serum titres reach high levels, as estimated by the immunofluorescence test or agglutination test, 0.1–1.0 ml of 10^{10} viable organisms/ml are injected intravenously. The rabbits are bled for serum 7 days later. The rabbit sera are pooled. A conjugate prepared from chicken IgY was described as an alternative to rabbit antibodies. MAbs that can be used for immunodiagnostic detection of *C. fetus* have been described (Brooks *et al*., 2002).

1.6.2. Preparation of conjugates

Conjugates are prepared as described by Harlow & Lane (1988). The working dilution of the conjugate is determined by checkerboard titration against smears of a *C. fetus* culture using positive and negative control dilutions, and selecting twice the lowest concentration that produces brilliant fluorescence with *C. fetus* bacteria.

1.6.3. Sample preparation

The genital fluid (fetal abomasal content, preputial smegma or CVM) samples are rinsed into approximately 5 ml PBS + 1% formalin. Two centrifugation steps are carried out. First, samples are centrifuged at 600 ***g*** for 10 minutes at 4°C to remove debris. Subsequently, the supernatant is centrifuged at 8000 ***g*** for 30 minutes at 4°C. The pellet is dissolved in ~100 µl remaining supernatant.

1.6.4. Immunofluorescence test (Mellick *et al.*, 1965)

The sample (20 µl) is applied in duplicate to microscope slides. The material is air-dried and fixed in acetone at –20°C for 30 minutes or ethanol at 18–25°C for 30 minutes. Glass slides will be air-dried and the fluorescein isothiocyanate isomer (FITC)-conjugated antiserum is added at the appropriate dilution. Staining is carried out in a humid chamber at 37°C for 30 minutes in dark condition. The slides are then washed three times for 10 minutes in PBS. The slides are mounted in buffered glycerol (90% glycerol: 10% PBS). The cover-slips are sealed to prevent drying, and the slides are examined under ultraviolet light in a fluorescent microscope. Positive and negative control slides will be used each time the test is done. *Campylobacter fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* reference strains are used as positive controls, and another *Campylobacter* species is used as negative control. Samples that show fluorescent bacteria presenting the typical morphology of *C. fetus* are considered positive.

1.7. Biochemical identification of *Campylobacter fetus* subspecies

Tests described in Table 2 must be done on pure cultures.

Table 2. Differential characteristics of Campylobacter *species potentially isolated from the bovine genital tract and aborted fetuses (according to Bergey's Manual 2nd edition, 2005)*

	25°C	42°C	Oxidase	Catalase	NaCl 3.5%	Glycine 1%	H_2S cysteine
C. fetus subsp. *venerealis*	V	V[(a)]	+	V	–	–	–
C. fetus subsp. *fetus*	V	V[(a)]	+	+	–	+	+
C. jejuni	–	V[(b)]	+	V[(c)]	–	V	+
C. hyointestinalis	–	+	+	+	–	V	n.d.
C. sputorum	–	+	+	V	+	+	n.d.

[(a)]Although *C. fetus* does not belong to the thermophilic *Campylobacter*s, a considerable number of strains of this species grows at 42°C;
[(b)]*C. jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is negative;
[(c)]*C. jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is variable;
+ = positive reaction or growth and – = negative reaction or absence of growth of the strain on an appropriate medium under specified conditions (see Section B.1.4); V = variable results; n.d. = not determined.

1.7.1. Growth at 25°C and 42°C

A cell-suspension (~McFarland No. 1) is inoculated on to two blood-based medium-plates. Each plate is incubated under the specified atmospheric conditions (see Section B.1.4.2) at 25°C and 42°C. Control strains are tested in parallel.

1.7.2. Oxidase and catalase

Tests are performed according to a standard bacteriological protocol. Control strains are tested in parallel.

1.7.3. Growth in the presence of sodium chloride

A cell-suspension is inoculated on to blood medium containing 3.5% NaCl (15 ml of blood medium + 2.04 ml of 5 M sodium chloride solution), and on to plain blood medium. Incubation is performed under the specified atmospheric conditions (see Section B.1.4.2). Control strains are tested in parallel.

1.7.4. Growth in the presence of 1% glycine

A cell-suspension (~McFarland No. 1) is inoculated on to a glycine medium (15 ml of blood-based medium + 1.65 ml of 10% aqueous solution of filter sterilised glycine), and on to the same medium without glycine. Incubation is performed under the specified atmospheric conditions (see Section B.1.4.2). Two control strains (Cff and Cfv) are tested in parallel. As all strains are fastidious, small changes in media can be important, and lack of growth in the presence of glycine should be considered to be a presumptive test for *C. fetus* subsp. *venerealis*. The reproducibility of the assay is poor and intermediate strains have been described (Salama *et al*., 1992 and Van Bergen *et al.,* 2005a). Furthermore, the reliability of the 1% glycine tolerance test can be influenced by the fact that glycine tolerance can be transduced by phages (Chang & Ogg, 1971).

1.7.5. Hydrogen sulphide production in cysteine medium

The H_2S test is done in a *Brucella* broth medium containing 0.02% cysteine. A cell suspension (~McFarland No. 1) is inoculated into the medium. H_2S production is detected by a lead acetate strip that is attached inside the top of the tube during 72 hours. Blackening of the lead acetate strip is considered as a positive reaction. Control strains are tested in parallel.

1.8. Monoclonal antibody-based capture ELISA

The MAb-based capture ELISA procedure can be used to detect the presence of *C. fetus* species cultured in Clarke's TEM. The validated procedure and reagents are described by Brooks *et al.* (2004) and Devenish *et al.* (2005). Briefly, the samples (preputial washes, vaginal mucus, fetal fluids, placental

tissues, liver tissue) are placed in Clarke's TEM and incubated for 4–5 days. Approximately 1.5 ml of the TEM fluids is withdrawn, heated and tested by ELISA. A rabbit polyclonal antiserum (to six different *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* serotype A and B strains) is used to capture antigen from the TEM fluid. The detection of any captured antigen is accomplished with further testing using four mouse MAbs specific to lipopolysaccharide (LPS) epitopes; *C. fetus* subsp. core LPS (1 MAb: M1825), serotype A-specific side chain LPS (2 MAbs: M1177 and M1194) and serotype B-specific side chain LPS (1 MAb: M1183). The test has been shown to be 100% sensitive and 99.5% specific for the detection of *C. fetus* species from the TEM fluids, and large numbers of samples can be tested at the same time.

1.8.1. Test procedure

i) *Solid phase:* ELISA plate wells are coated with an optimal dilution of a rabbit polyclonal anti-*C. fetus* subsp. serum (in 0.06 M carbonate buffer, pH 9.6) and incubated for 18 hours at room temperature (RT). Coated ELISA plates can be sealed and stored at –20°C for 1 month. Prior to testing, ELISA plates, at RT, are washed four times using 0.01 M PBS containing 0.15 M NaCl and 0.05% Tween 20 (PBST).

ii) *Controls:* There are three control antigens: 1) *C. fetus* subsp. *fetus* (ATCC 27374), a serotype B strong positive control that binds with the LPS core MAb (M1825) and the single serotype B specific MAb (M1183); 2) *C. fetus* subsp. *venerealis* (ATCC 19438), a weak serotype A positive control that binds to the LPS core MAb (M1825) and the two serotype A-specific MAbs (M1177 and M1194); and 3) *C. sputorum* biovar *sputorum,* a negative control that does not bind with any of the four MAbs. Lots of the three control antigens are grown, washed, concentrated and stored at –20°C.

iii) *Testing samples:* After 4–5 days' incubation at 37°C of inoculated TEM samples, approximately 1.5 ml of fluid is withdrawn from the TEM vials. Optimally diluted control antigens and undiluted TEM fluid samples are heated at 100°C for 15 minutes and cooled to RT. Each heated test fluid sample is added in duplicate and each heated control antigen is inoculated in quadruple to appropriate wells in the solid-phase ELISA plate and incubated for 1 hour at RT.

iv) *Detector:* To economise on reagents, screening of fluids is done initially with the core MAb M1825. Diluted MAb M1825 (optimally in PBST) is added to each well of the ELISA plates and the plates are incubated for 1 hour at RT. Fluids that are positive using MAb M1825 are further tested using all four MAbs.

v) *Conjugate:* Horseradish-peroxidase goat anti-mouse immunoglobulin G (heavy and light chains) conjugate, optimally diluted in PBST, is added to all wells and plates are incubated for 1 hour at RT.

vi) *Substrate:* 3,3',5,5'-tetramethylbenzidine-hydrogen peroxide substrate is added to wells and the plates are placed on an orbital shaker for 10 minutes at RT. Optical densities (OD) are immediately measured at 620 nm (OD_{620}) using an ELISA reader.

All reagent lots, including capture antiserum, control antigens, mouse MAbs and conjugate are tested beforehand by checkerboard titration to determine optimal dilutions used in routine testing of sample fluids. Throughout the test procedure, 100 µl volumes are used and plates are washed four times between stages using PBST.

vii) *Interpretation of the results:* To be ELISA positive, the fluid must be positive against not only the core MAb but also to at least one of the serotype-specific MAbs. In initial screening using MAb M1825, the mean OD_{620} of the each test sample is divided by the mean OD_{620} obtained from strong positive *C. fetus* subsp. *fetus* serotype B control antigen and multiplied by 100% to obtain a per cent positivity (%P). Any test fluid with a %P greater than or equal to 14% is considered positive and tested further with all four MAbs in the procedure. A repeat positive with M1825 and an OD_{620} value greater than or equal to 0.2 with at least one of the other three serotype-specific MAb is a positive ELISA result for the detection of *C. fetus* ssp. in the original test fluid sample. All TEM vials corresponding to ELISA positive fluid samples are cultured as described in Section B.1.4 *Isolation of* Campylobacter fetus for confirmation and to determine the subspecies of the cultured isolate.

1.9. Molecular identification of *Campylobacter fetus* subspecies

The use of clinical samples for direct PCR has not been successfully demonstrated and can result in nonspecific amplification. The method is therefore not recommended for primary diagnosis. It is however possible to perform PCR from TTE medium after enrichment.

Several molecular methods for the identification of *C. fetus* subspecies have been described, including 16S sequencing (Gorkiewicz *et al*., 2003; On & Harrington, 2001), PFGE (On & Harrington, 2001), AFLP (Wagenaar *et al*., 2001), and MLST (van Bergen *et al*., 2005a). MLST was recommended for the differentiation of Cff and Cfv strains; a recent study however described a Cff strain that was isolated with the Cfv-associated MLST ST-4 genotype (Iraola *et al*., 2015), showing that MLST is also not fully reliable for *C. fetus* subspecies differentiation. Whole genome sequencing can be used for a very reliable characterisation of *C. fetus* strains (van der Graaf-van Bloois *et al*., 2014), but this method is expensive and currently not widely used in diagnostic laboratories.

Routine diagnostic laboratories would be served best by a PCR assay. Several PCRs have been claimed to be subspecies specific including those developed by Hum *et al*. (1997), Wang *et al*. (2002), Tu *et al*. (2005), van Bergen *et al*. (2005c) and Abril *et al.* (2007). However, a PCR assay that identifies *C. fetus* isolates reliably to subspecies level is not available (van der Graaf-van Bloois *et al*., 2013) (Table 3).

Table 3. Sensitivity and specificity of C. fetus (sub)species identification of PCR assays

PCR assay	Identification	Target	Sensitivity[a]	Specificity[a]
Abril *et al*., 2007	*C. fetus* Cfv	*nahE* ISC*fe1*	100% (143/143) 97% (58/60)	100% (12/12) 100% (95/95)
Van Bergen *et al*., 2005c	Cfv	unknown	45% (27/60)	100% (95/95)
Hum *et al*., 1997	*C. fetus* Cfv	*cstA* *parA*	100% (143/143) 58% (46/70)	100% (12/12) 83% (79/95)
McMillen *et al*., 2006	Cfv	*parA*	53% (32/60)	100% (95/95)
Wang *et al*., 2002	Cff	*sapB2*	76% (63/83)	72% (52/72)
Van der Graaf-van Bloois *et al*., 2013	*C. fetus*	*nahE*	100% (143/143)	100% (12/12)

[a]Represented as percentage (number of correct identified strains/total number of strains). Study included; 143 *C. fetus* strains, 60 Cfv strains, 83 Cff strains, 12 non-fetus *Campylobacter* strains, 95 non-Cfv strains and 72 non-Cff strains (van der Graaf-van Bloois *et al*., 2013).

The multiplex PCR assay described by Abril *et al.* (2007) proved to be reliable for the correct identification of *C. fetus* species, with 100% sensitivity and 100% specificity, though the Cfv-specific target (IS-element) described for this PCR is 97% sensitive and cannot be used for a reliable differentiation of Cff and Cfv isolates (van der Graaf-van Bloois *et al*., 2013). The *C. fetus* target of this PCR, gene *nahE*, can be used to identify *C. fetus* species and has also been developed as a real-time assay (McGoldrick *et al.,* 2013; van der Graaf-van Bloois *et al*., 2013).

The PCR described by Van Bergen *et al*. (2005c) is able to detect Cfv strains as defined by AFLP, but the assay does not identify the *C. fetus* subsp. *venerealis* biovar intermedius. Therefore, this PCR is not suitable for diagnostic purposes (van der Graaf-van Bloois *et al*., 2013).

The multiplex PCR described by Hum *et al*. (1997) enables the amplification of a *C. fetus*-specific DNA fragment (approximately 200 bp smaller than the 960 bp described in the original publication), as well as a *C. fetus* subsp. *venerealis*-specific fragment. Comparison of this PCR against AFLP and MLST (van Bergen *et al*., 2005a) and against the glycine test (Willoughby *et al*., 2005) confirms that PCR can give false positive and negative reactions (van der Graaf-van Bloois *et al*., 2013). In a recent study, this PCR showed a positive result with a *C. hyointestinalis* strain isolated from a bull (Spence *et al*., 2011). This observation renders the Cfv-specific target gene *parA,* and consequently all other PCR assays using this target, unsuitable for diagnostic purposes.

The PCR described by Wang *et al*. (2002) reveals only a *C. fetus* subsp. *fetus*-specific product. These results were obtained only for a very limited number of strains. Evaluations of its value for subspecies differentiation using larger sets of strains yielded both false positive and negative reactions (Van Bergen *et al*., 2005c; Van der Graaf-van Bloois *et al*., 2013).

The random amplification of polymorphic DNA (RAPD)-PCRs were described by Tu *et al*. (2005) but apparently have been evaluated with a very limited number of *C. fetus* subsp. *venerealis* strains. In a recent study, the sensitivity of this assay was shown to be very low (Van der Graaf-van Bloois *et al*., 2013).

2. Serological tests – antibody detection

An ELISA is available for the detection of antigen-specific secretory IgA antibodies in the vaginal mucus following abortion due to *C. fetus* subsp. *venerealis*. These antibodies are long lasting, and their concentration remains constant in the vaginal mucus for several months (Hum *et al*., 1991). This assay has been used to screen for BGC in cattle herds with infertility and abortions; the specificity was found to be 98.5%. The sensitivity could not be estimated (da Silva Silveira *et al*., 2018) so it is not yet considered as validated. The ELISA does not differentiate an antibody response to the different subspecies.

Initial sampling can be done after the early involution period (usually 1 week after abortion) when mucus becomes clear.

2.1. Antigen preparation and coating

Cultures are transferred to PBS with 0.5% formalin for 1 hour, centrifuged at 17,000 ***g***, washed twice with PBS, and then resuspended in 0.05 M carbonate buffer, pH 9.6. The final absorbance is adjusted to $OD_{610\,nm}$ = 0.21. Flat-bottomed polystyrene microtitre plates coated with 10 µl of antigen are left overnight at 4°C, and then stored at –20°C. Before use, the plates are rinsed twice with distilled water and then tapped gently to remove moisture.

2.1.1. Test procedure

i) Diluted vaginal mucus (100 µl) is added to each well, and the plate is incubated at 37°C for 2 hours. The plates are then washed as before, and 100 µl of rabbit anti-bovine IgA is added. After 2 hours incubation at 37°C, the plates are washed and 100 µl of goat anti-rabbit IgG conjugated to horseradish peroxidase is added to each well. After a further 2 hours incubation at 37°C, the plates are washed, and 100 µl of substrate is added (0.8 mg/µl 5 amino-salicylic acid; pH 6.0), immediately activated by the addition of 2% 1 M hydrogen peroxide). The plates are left at room temperature for 30 minutes and the reaction is stopped by the addition of 50 µl of 3 M sodium hydroxide. The absorbance is measured on an ELISA reader at 450 nm. Each sample is tested in duplicate, and positive and negative controls are included in each plate. The absorbance measurements yielded by the test sample are corrected for the absorbance measurement of positive and negative controls according to the formula:

$$\text{Result} = \frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{negative control}}}{\text{Absorbance}_{\text{positive control}} - \text{Absorbance}_{\text{negative control}}} \times 100$$

ii) The test is considered to be positive if the result is above 40. Vaccinated animals will not react to IgA ELISA as their vaginal mucus contains only IgG isotype antibodies.

An ELISA for the detection of the serum humoral IgG response after vaccination has also been described (Cobo *et al*., 2004).

C. REQUIREMENTS FOR VACCINES

There are several commercial vaccines available around the world, however vaccine efficacies have either not been reported for many decades or have not been reported favourably (Cobo *et al.,* 2003). Certain jurisdictions advocate

the use of these vaccines, however WOAH is not currently in a position to recommend an international standard for such products.

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NB: There is a WOAH Reference Laboratory for campylobacteriosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine genital campylobacteriosis

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.4.5.

BOVINE SPONGIFORM ENCEPHALOPATHY

SUMMARY

Description of the disease: *Bovine spongiform encephalopathy (BSE) is a fatal neurological disease of adult cattle* (Bos taurus *and* B. indicus) *that was first recognised in Great Britain (GB) and that has been identified in classical (C-type BSE) and atypical forms (H- and L-type). Both C-type BSE and atypical forms have been detected in cattle indigenous to most European countries, the Americas, and Asia and the Pacific. BSE is a transmissible spongiform encephalopathy (TSE), or prion disease. The archetype for this group of diseases is scrapie of sheep and goats (see Chapter 3.8.11* Scrapie).

The C-type BSE epizootic resulted from oral exposure to prions in the ruminant-derived protein of meat-and-bone meal and milk replacers included in animal feedstuffs. As a result of control measures, C-type BSE epizootics are in decline. Atypical forms of BSE are believed to occur spontaneously in all cattle populations at a very low rate, and have only been identified in older cattle. They have been detected in many countries, but only as an incidental finding when conducting intensive surveillance for C-type BSE.

Experimental transmissibility of all forms of BSE to cattle has been demonstrated. The C-type BSE agent is also believed to be the common source, via dietary routes, of TSEs in other ruminant species and felidae. There is evidence of a causal link between the C-type BSE agent and variant Creutzfeldt-Jakob disease (vCJD) in humans. Recommendations for handling BSE-infected material assume that BSE is a zoonosis and manipulations with potentially contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis.

Detection of the agent: *Clinical C-type BSE had a peak incidence in cattle aged between 4 and 5 years during the height of the epizootic. The clinical course is variable but can extend to several months. Overt clinical signs are distinctive, if differential diagnoses can be eliminated. Early clinical signs may be subtle and mostly behavioural, and may lead to disposal of affected animals before suspicion of BSE is triggered. In countries with a statutory policy toward the disease, clinically suspect cases must be killed, the brain examined and the carcass destroyed. Now, in most countries involved, active surveillance at abattoirs and the screening of fallen stock identifies preclinical cases and cases in which there may have been unrecognised clinical signs. No diagnostic test is currently available for live animals.*

A disease-specific partially protease-resistant, misfolded isoform of a membrane protein PrP^{c}, originally designated PrP^{Sc}, has a critical importance in disease pathogenesis. According to the prion hypothesis, PrP^{Sc} is the principal or sole component of the infectious agent. Confirmation of the diagnosis is reached by immunohistochemical (IHC) and/or immunochemical detection of PrP^{Sc} in brain tissue. The discrimination of atypical phenotypes from the classical BSE type is based on Western immunoblot banding pattern and bioassay characterisation.

Commercial diagnostic kits for BSE are available and are used in many countries; similarly, a number of anti-PrP antibodies form the basis of many diagnostic methods. Some are available commercially, or from WOAH Reference Laboratories or other laboratories with active TSE surveillance programmes.

Serological tests: *Specific immune responses have not been detected in TSEs.*

Requirements for vaccines: *There are no vaccines available.*

A. INTRODUCTION

For up-to-date information on the distribution of the disease, please consult the WOAH World Animal Health Information Database[1].

1. Description of the disease

Bovine spongiform encephalopathy (BSE) is a fatal prion disease of cattle (*Bos taurus* and *B. indicus*) and may present clinically with signs of central nervous disease, such as apprehension, hyper-reactivity and ataxia. Confirmatory diagnosis relies on post mortem IHC and immunochemical detection of misfolded prion proteins in the brain.

Transmission of classical BSE (C-type BSE) occurs through the feeding of BSE prion contaminated meat and bone meal and animal feedstuffs containing meat and bone meal (Wells & Wilesmith, 1995). There is no evidence of horizontal transmission and little data to support vertical transmission (Prince *et al.,* 2003). Epidemiological data and experimental transmission studies indicate that the incubation period is at least 2 years and may extend beyond a decade. The course of disease is usually subacute to chronic, and affected animals display progressive neurological signs. There is no effective treatment and affected animals will inevitably die if the disease is left to run its course.

Clinical C-type BSE occurs in adult cattle (ranging between 20 months and 22 years in the UK). During the main epizootic most cases were observed in dairy cattle aged 4–6 years. Subsequently the impact of effective controls has been reflected in an increasing age at onset of clinical disease. BSE has an insidious onset and usually a slowly progressive course (Konold *et al.,* 2004; Wilesmith *et al.*, 1992). Occasionally, a case will present with acute signs and then deteriorate rapidly, although frequency of observation is a significant factor in determining early clinical signs. Presenting signs, though variable, usually include changes in behaviour and temperament, hyper-reactivity and incoordination. Affected cows may be reluctant to enter the milking parlour or may kick vigorously during milking, which is often the first observed sign. In dry cows especially, hind-limb incoordination and weakness can be the first clinical features to be noticed. The most commonly reported nervous signs have been apprehension, pelvic limb ataxia, and hyperaesthesia to touch and sound. Startle responses to external stimuli, which are repeatable, are frequent and thus used to support the clinical diagnosis in suspect BSE cases (Konold *et al.*, 2004). Affected cows will sometimes stand with low head carriage and the neck extended, an arched back or wide-based hind limbs. A tremor of the head may also be visible. Abnormalities of gait, such as incoordination and hypermetria, are usually confined to the hind limbs and are most readily appreciated when cattle are observed at pasture. With advancing severity of locomotor signs, generalised weakness, resulting in falling and recumbency, can dominate the clinical picture. General clinical features of loss of bodily condition and reduction in milk yield often accompany nervous signs as the disease progresses. There has been no change in the clinical picture of C-type BSE over the course of the epizootic in the UK (Konold *et al.,* 2004; Wilesmith *et al.,* 1992). Clinical signs are essentially similar in other countries where C-type BSE has occurred. The protracted clinical course, extending usually over a period of weeks or months, would eventually require culling on welfare considerations. However, a statutory policy to determine the BSE status of a country requires compulsory notification and diagnostic investigation of clinically suspect cases, their culling and post-mortem examination of the brain. Early in the disease course, the signs may be subtle, variable and nonspecific, and thus may prevent clinical diagnosis on an initial examination. Continued observation of such equivocal cases, together with appropriate clinical pathology procedures to eliminate differential diagnoses, especially metabolic disorders, will establish the essential progression of signs. Subtle signs may sometimes be exacerbated following stress, such as that caused by transport.

Video clips of cattle affected by BSE may be downloaded from the web site of the WOAH Reference Laboratory for BSE in the UK[2], which also provides electronic footage upon request.

Little is known about the clinical presentation of naturally occurring atypical BSE as most of these cases were detected by active surveillance of fallen stock or apparently healthy cattle, and correlation of laboratory diagnostic data with clinical histories is lacking. An interesting common feature is that cases of atypical BSE were almost exclusively detected in cattle older than 8 years. Data from experimental intracerebral inoculation of cattle suggest

1 http://www.woah.org/en/animal-health-in-the-world/the-world-animal-health-information-system/the-world-animal-health-information-system/

2 https://science.vla.gov.uk/tseglobalnet/

that the clinical signs in H- and L-type BSE may include those observed in C-type BSE, but dullness and difficulty in rising are also prominent features of the clinical presentation in these animals (Konold *et al.,* 2012).

2. Causal pathogen

Without an isolatable causal agent, cases can only be conclusively confirmed post-mortem by the accumulation in the central nervous system (CNS) of abnormal prion protein (PrP^{Sc}, PrP^{d} or PrP^{res}), a partially protease-resistant isoform of a host-encoded protein (PrP^{C}). The prion hypothesis proposes that the agent is composed entirely of PrP^{Sc}, which is capable of inducing conversion of PrP^{C}. Pathological and bioassay characterisation showed that the BSE epizootic was sustained by a single strain, and consistently distinctive neuropathology and PrP^{Sc} molecular profiles in clinically affected animals were the basis for the case definition of C-type BSE. Since 2003, reports of variant pathology or molecular characteristics in aged cattle from many countries have indicated agent strain variation (Biacabe *et al.,* 2004; Casalone *et al.,* 2004).

So far, more than 100 atypical BSE cases have been recognised that differ in their molecular profiles by Western immunoblotting from C-type BSE. Bioassay data support the hypothesis that these strains are biologically distinct from C-type BSE (Beringue *et al.,* 2006; Lombardi *et al.,* 2008; Okada *et al.,* 2011). The two atypical forms are operationally defined as BASE (bovine amyloidotic spongiform encephalopathy) or L-type, and H-type BSE based on the lower or higher mass respectively of the unglycosylated PrP^{Sc} fragment in Western immunoblots compared to C-type BSE (Casalone *et al.,* 2004; Jacob *et al.,* 2007).

3. Zoonotic risk and biosafety requirements

The emergence of variant Creutzfeldt-Jakob disease (vCJD) in humans has been causally linked to ingestion of C-type BSE (Bruce *et al.,* 1997). It is not known whether atypical cases are causally linked to forms of human prion diseases. Recommended safety precautions for handling the agent are based on the assumption that all forms of BSE are zoonotic. Biocontainment for necropsies and tissue handling should be risk-based and compliant with relevant national regulations; any procedure creating aerosols must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*), and the laboratory must comply with national biocontainment and biosafety regulations to protect staff from exposure to the pathogen. Recommended physical inactivation is by porous load autoclaving at 134°C–138°C for 18 minutes at 30 lb/in^2 (208 kPa or 2.2 bar). However, total inactivation may not be achieved under certain conditions, such as when the test material is in the form of a macerate, of high titre or when the agent is protected within dried organic matter. Disinfection of potential fomites is carried out using sodium hypochlorite containing 2% available chlorine, or 2 N sodium hydroxide, applied for more than 1 hour at 20°C for surfaces, or overnight for equipment.

4. Differential diagnosis

For differential diagnosis all types of neurological diseases in cattle should be taken into account, including infectious encephalitis, metabolic disorders (ketosis, hypomagnesaemia), toxicoses, neoplasia and trauma.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of bovine spongiform encephalopathy and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Immunohisto-chemistry	–	–	++	+++	++	–
Western immunoblot	–	–	++	+++	++	–
Rapid screening tests	–	–	+++	+	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

There is no method capable of confirming the presence of BSE in the live animal. Identification of the 'agent' begins with clinical suspicion of disease, the post-mortem demonstration of histopathological changes or PrP^{Sc} accumulation in a suspect animal, or in a non-suspect animal through active surveillance. The nature of the 'agent' itself remains hypothetical, and it cannot therefore be isolated for diagnostic purposes. However, PrP^{Sc} is widely accepted as a consistent disease marker, and, with the exception of clinical examination and histopathology, all current diagnostic methods are based on the demonstration of this protein.

In the absence of *in-vitro* methods for isolation of the causative agent, disease can be confirmed by demonstrating characteristic TSE-specific vacuolation by histopathology at several different levels of the brain. An absence of histopathological lesions does not exclude a PrP^{Sc} accumulation. Histopathological diagnosis based on examination of a single section of medulla oblongata (at the level of the obex) has been validated against more extensive examination of the brainstem. This simplified approach enables the sampling of brainstem removed via the *foramen magnum*, using customised instrumentation, instead of whole brain removal. However, immunochemical methods of disease-specific PrP detection, including IHC techniques and Western immunoblot, are recommended to confirm the diagnosis and improve diagnostic sensitivity in early or autolysed cases. The more rapidly performed *in-vitro* methods, such as enzyme-linked immunosorbent assay (ELISA), for the detection of PrP^{Sc} has led to a variety of 'rapid' tests, which are now the principal screening tools for active surveillance. Such tests provide a preliminary diagnosis from which positive or inconclusive results are subject to examinations by IHC or Western immunoblot confirmatory methods. Rapid tests are currently the main approach by which cases are detected and their wider use as part of the confirmatory process has been agreed, providing that one of the two tests used is a Western immunoblot[3]. All currently recognised forms of BSE are detectable by these methods although a full sensitivity and specificity evaluation for atypical forms (H- and L-types) has not been carried out.

The choice of any particular method will depend on the context of its use. Contexts will extend from confirmation of clinical suspects to the screening of healthy populations for evidence of covert or preclinical disease. The case definition adopted will also differ according to whether the diagnostic method is to be applied for confirmation or for screening. Care should be taken in the interpretation of diagnostic data using methodologies that do not enable careful cross-referencing with the standards for confirmatory diagnosis that are defined here. Without appropriate comparison with previously published criteria defining the BSE phenotype, and in the absence of transmission

3 https://science.vla.gov.uk/tseglobalnet/documents/OIE%20rules%20for%20BSE%20using%20a%20second%20rapid%20test_Rev_Jan2019.pdf

studies, diagnostic results that claim the identification of a new strain may be premature. Quality control (QC) and quality assurance (QA) are essential parts of the testing procedures and advice can be supplied by the WOAH Reference Laboratories. Whether BSE-infected animals are identified by passive or active surveillance, it is good practice to detect and confirm disease by a combination of at least two test methods. The primary test can be one of the confirmatory test methods described below or a rapid test, but it is important to apply a secondary test to confirm a positive or inconclusive primary test result. Where there is a conflict between primary and secondary test results, further tests using immunohistochemistry or Western immunoblot should be applied or samples should be submitted to a WOAH Reference Laboratory for resolution.

1.1. Sample preparation

The BSE status of a country, the relative implementation of passive and active surveillance programmes and the diagnostic methods applied, will all influence sampling strategy.

In all circumstances of passive surveillance of neurological disease in adult cattle **where the occurrence of BSE within a country or state has not been established or is of low incidence**, it is recommended that clinically suspect cases are subjected to a standard neuropathological approach in which the whole brain is sampled, and a range of representative areas examined. Cattle suspected of having the disease should be killed with an intravenous injection of a concentrated barbiturate solution preceded, if necessary, by sedation. The brain should be removed as soon as possible after death by standard methods. There are no gross lesions associated with BSE, so if any are observed when the brain is removed, these should be specifically sampled to facilitate differential diagnosis.

Care must be taken to preserve suitable fixed and fresh brain samples for the immunohistochemical and immunochemical detection of PrP^{Sc}. Departure from this approach of collecting and retaining the entire brain may prevent appropriate characterisation of the case, to confirm whether or not it is typical of BSE.

Histopathology and IHC examinations are carried out initially on a single block (0.5–1.0 cm in width) cut at the obex of the medulla oblongata (Figure 1a and b, level A–A representing the centre of the block for examination), which should be fixed for a minimum of 3–5 days (dependent on block thickness) in 4% formaldehyde solution (i.e. 10% formal saline or 10% normal buffered formalin [NBF]). To decrease infectivity, fixed tissues may be immersed in 98% formic acid for one hour to reduce the prion infectivity, then washed for 30 minutes in tap water. It should be noted that this may reduce the range of further tests that could be applied for classification unless fresh frozen material is also available. Subsequent histological processing should be by conventional paraffin wax embedding methods for neural tissue. (An example of appropriate processing methods can be found on TSEglobalNet[4]).

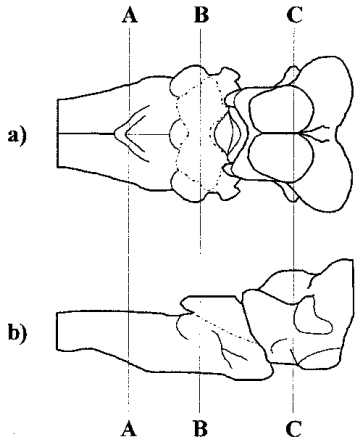

Fig 1. Brainstem after the removal of the cerebellum, from a) dorsal, and b) lateral aspects. Recommended levels at which sections should be taken: A–A = medulla, at the obex; B–B = medulla through caudal cerebellar peduncles; C–C = midbrain through rostral colliculi.

4 https://science.vla.gov.uk/tseglobalnet/confirmatory-diagnosis.html

Fresh material for the immuno-detection of PrP^{Sc} should be taken initially as a hemisection of the medulla at the level of the obex, or as a complete coronal section (2–4 g), immediately rostral, or caudal, to the obex block taken for fixation. All other brain areas should be subdivided by a sagittal paramedian cut (3–5 mm off the median). The smaller portion is reserved for the detection of PrP^{Sc} by immunochemical methods (e.g. immunoblot) and is stored frozen prior to testing (if testing is not done immediately after sampling). After sampling of the obex region for fixation and sampling of fresh tissue, the larger portion of the brain tissue is placed, intact, in approximately 4–6 litres of 10% formalin fixative, which should be changed twice weekly. After fixation for 2 weeks, if further investigation is necessary, the brain can be cut into coronal slices. The fixation time may be shortened by cutting the fresh brainstem (detached from the rest of the brain) into smaller coronal pieces, similarly to the initial removal of the obex region, but leaving intact the remaining diagnostically important cross-sectional areas at the levels of the cerebellar peduncles and the rostral colliculi (Figure 1a and b, levels B–B and C–C, respectively). Depending on some other factors (temperature, agitation, thickness of tissue block, use of microwave) the fixation time for these smaller pieces of brain may be reduced. However, evaluation of the effects of these kinds of accelerated fixation processes on subsequent IHC protocols needs to satisfy proficiency testing standards. The other formol-fixed parts of the brain may be used for differential diagnosis after completing the standard 2 weeks of fixation.

When the occurrence of BSE in a particular country has been established in the indigenous cattle population, and there is evidence that the distribution of lesions and other aspects of disease phenotype are consistent with those described for classical BSE, it is adequate, although not ideal, for disease confirmation and monitoring purposes, to remove the brainstem alone.

This can be achieved via the *foramen magnum* without removal of the calvarium (Figure 2). This will reduce the amount of fixative required and the time and equipment needed, thereby lowering costs and improving safety. The major target areas for histological examination can still be maintained. This method allows for collecting and examining a large number of samples for passive surveillance or for an active surveillance programme in abattoirs and on fallen stock animals. The brainstem is dissected through the *foramen magnum,* without opening the skull, by means of a specially designed spoon-shaped instrument with sharp edges around the shallow bowl. Such instruments are available commercially. It is possible that variations in technique, including orientation, are required with different forms of the instrument, and it is important to train operators once there is agreement on equipment to be used. This training should include information on the cross-sectional distribution of PrP^{Sc} in the brainstem, and the need for the accurate sampling and preservation of the diagnostic target areas (see below).

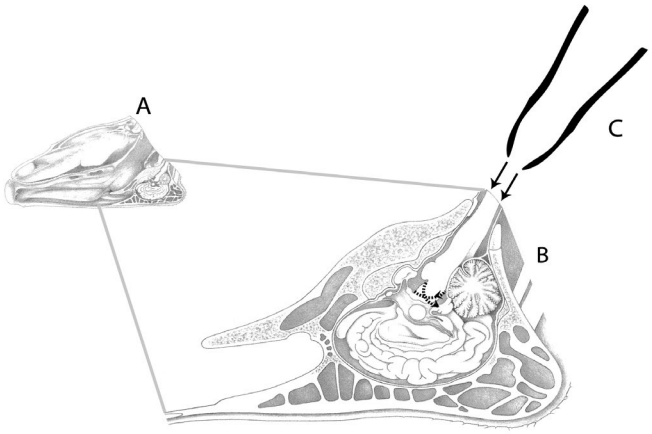

Fig. 2. After the head has been removed from the body by cutting between the atlas vertebra and the occipital condyles of the skull, it is placed on a support, ventral surface uppermost (A), with the caudal end of the brainstem (medulla oblongata) visible at the foramen magnum (see B, expanded drawing of cranium). The instrument (C) is inserted through the foramen magnum between the dura mater and the ventral/dorsal aspect (depending upon the specific approach) of the medulla and advanced rostrally, keeping the convexity of the bowl of the instrument applied to the bone of the skull and moving with a side-to-side rotational action. This severs the cranial nerve roots without damaging the brain tissue. The instrument is passed rostrally for approximately 7 cm in this way and then angled sharply (i.e. toward the dorsal/ventral aspect of the brainstem, depending on the approach) to cut and separate the brainstem (with some fragments of cerebellum) from the rest of the brain. The instrument, kept in the angled position, is then withdrawn from the skull to eject the tissue through the foramen magnum.

Where the index case is identified through active surveillance, the necessary brain areas for full phenotypic characterisation are unlikely to be available. In most countries, brainstem alone is collected, even before the first confirmation of BSE. Provision should be made for heads that have been sampled in the course of active surveillance to be retained until the outcome of initial testing is available. This would enable comprehensive sampling of the brain of positive animals in retrospect for the characterisation of cases. This is particularly important if in-house tests that are not subject to external quality assurance are used and where, in the absence of direct comparison with the methods described here, claims are made that new phenotypes have been identified. Where rapid immunoassays are used as the primary surveillance tool it is necessary to make material available for further immunohistochemistry (including morphological) and molecular examination that would allow identification of disease phenotype in the absence of a diagnosis of BSE having ever been made in that country.

1.1.1. Sampling of brainstem in active surveillance with use of rapid tests

The sampling and processing of brain tissue for use in any rapid test should be carried out precisely as specified by the supplier or manufacturer of the test method or kit. Details of this procedure vary from method to method and should not be changed without supportive validation data from the manufacturer for the variant methodology. The preferred sample for immunoassay should be at, or within 1.0 cm rostral, or caudal to, the obex, based on the caudo-rostral extent of the key target sites (Figure 3) for demonstration of PrP^{Sc} accumulations and the evaluation of sampling for rapid tests. The choice of target site has to take into account the subsequent method of confirmation. At least a hemi-section of the medulla at the level of the obex should be kept intact for fixation for immunohistochemistry/histology (as described above) should a positive result require confirmation. Sampling the medulla rostral or caudal to the obex for rapid testing does not compromise examination by histological or IHC means. However, to obtain comparable samples for rapid *and* confirmatory testing, sampling by hemi-section of the medulla at the level of the obex is preferable. While there is resultant loss of the ability to assess the symmetry of any histopathological lesions (notably vacuolation), this approach is less likely to compromise the more important IHC examination. If hemi-sectioning is adopted however, it becomes critical to ensure that the target sites are not compromised in either sample. For example, the nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve (target areas for lesions in cattle with BSE) are small, and lie relatively close to the midline (Figure 3). If sampled tissue is autolysed to the point that anatomical orientation is not possible, an unidentified aliquot can still be taken and tested. A positive result in such cases is still a valid result, but a negative test result cannot be taken to indicate a negative animal, and it should be interpreted with caution and reported with appropriate qualification.

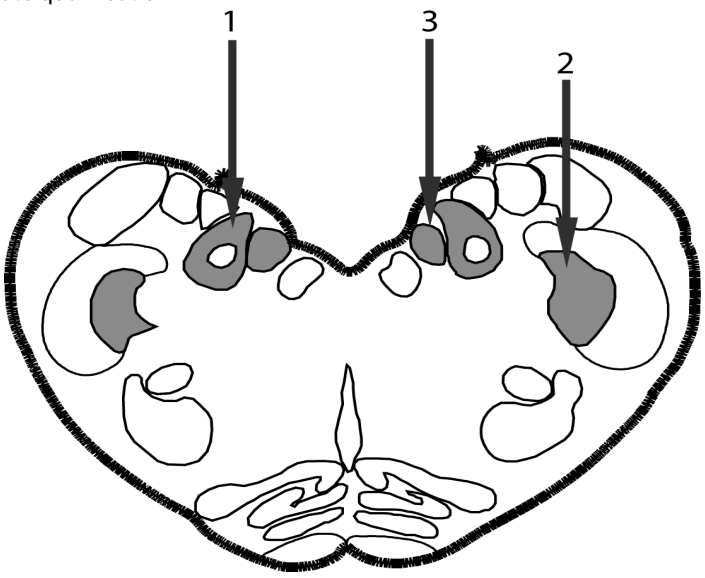

Fig 3. Cross section of the bovine brainstem at the level of the obex identifying the key target sites for diagnosis by histopathology and immunohistochemistry in BSE. These are principally the nucleus of the solitary tract [1] and the nucleus of the spinal tract of the trigeminal nerve [2]; but also the dorsal motor nucleus of the vagus nerve [3]. It follows that material taken for application of a rapid test must also include representation of these areas.

Inaccurate hemi-sectioning could result in the complete loss of a target area for confirmatory testing, and could compromise a surveillance programme. Failure to accurately sample target areas may also arise through inappropriate placement of proprietary sampling tools. Such approaches therefore need to be implemented with a very clear policy and monitoring programme for training and quality assurance of sampling procedures, including anatomical positioning, and not just sample weight. Because of the specifically targeted distribution of PrP^{Sc}, sample size and location should be as described in the diagnostic kit or, if not specified, at least 0.5 g taken from the diagnostic target areas for all confirmatory tests as detailed in Figure 3. Performance characteristics of the tests may be compromised by autolysis, particularly due to loss of the ability to ensure inclusion of target areas in the sample taken for diagnosis.

1.2. Diagnostic examination

1.2.1. Histological examination

Histopathology is mentioned in the current chapter because of its historical importance as it was the first method for TSE diagnosis and it still allows identification of TSE lesions, namely vacuoles and plaques. However, these lesions appear after the formation of disease-specific forms of PrP during the course of disease. As a result, histopathology has lower sensitivity compared with agent identification methods and it has been superseded by them. In addition, this diagnostic method requires good sample preservation, while diagnostic methods for disease-specific PrP are affected less by autolysis.

Histopathology alone is no longer the diagnostic method of choice for investigation of suspect animals, or the screening of healthy populations. However, an awareness of the histopathological changes is important, to facilitate detection of cases when conducting routine diagnostic histological examinations of cattle brains sampled for reasons other than BSE testing. For differential diagnosis, sections of medulla–obex are cut at 5 μm thickness and stained with haematoxylin and eosin (H&E). If tissue quality permits, the histopathological examination of H&E sections allows confirmation of the characteristic neuropathological changes of BSE (Simmons *et al.,* 1996; Wells & Wilesmith, 1995) by which the disease was first detected as a spongiform encephalopathy. These changes comprise mainly spongiform change and neuronal vacuolation and are closely similar to those of all other animal TSEs, but in BSE the frequent occurrence of neuroparenchymal vacuolation in certain anatomic nuclei of the medulla oblongata at the level of the obex can provide a satisfactory means of establishing a histopathological diagnosis on a single section of the medulla. As in other species, vacuolar changes in the brains of cattle, particularly vacuoles within neuronal perikarya of the red and oculomotor nuclei of the midbrain are an incidental finding (Gavier-Widen *et al.,* 2001). The histopathological diagnosis of BSE must therefore not rely on the presence of vacuolated neurons alone, particularly in these anatomical locations.

Irrespective of the histopathological diagnosis, it is recommended that immunohistochemistry is routinely employed in addition, as unpublished evidence suggests that as many as 5% of clinical suspects (which are negative on H&E section examination for vacuolar changes at the obex) can be diagnosed by IHC examination. Clearly, examination of the medulla–obex does not allow a full neuropathological examination for differential diagnoses, nor does it allow a comprehensive phenotypic characterisation of any TSE. It is for this reason that it is recommended to remove whole brains from all clinical suspects. There are still insufficient data available to describe specific histopathological features of H- or L-type BSE. There are some histological data from Italian researchers on BASE (L-type BSE) (Casalone *et al.,* 2004). Few atypical BSE cases have been found in passive surveillance and it is not possible to obtain whole brains through active surveillance programmes to increase our knowledge in this respect. The poor condition of the brain of fallen stock, where most atypical cases have been identified, also rules out a complete histological examination because of the effects of autolysis. The end-stage pathology of experimental H-type and L-type BSE in a small number of animals following intracerebral inoculation has been described (Konold *et al.,* 2014; Okada *et al.,* 2011).

Although histopathological examination has been superseded by other diagnostic methods, either as a primary or confirmatory test it is still a valuable research application.

1.2.2. Detection of disease-specific forms of PrP

The universal use of PrP detection methods provides a disease specific means of diagnosis independent of the morphological changes defined by histopathology. The detection of accumulations of PrP^{Sc} is the approach of choice for surveillance programmes and confirmatory diagnosis. It is possible (but not desirable) to undertake immunohistochemistry for PrP on material that has been frozen prior to fixation (Debeer *et al.,* 2002). Freezing prior to fixation will not compromise the immunoreactivity of a sample, but it may compromise the proper identification of target sites. A positive case will have disease-specific immunolabelling in at least one of the diagnostic target areas (Casalone *et al.,* 2006). For a case to be diagnosed as negative it must be possible to identify the presence of the target areas and to demonstrate that the IHC 'run' was technically successful through appropriate controls. If there is no disease-specific immunolabelling, and target areas cannot be identified, the case should be classified as 'unconfirmed' as opposed to negative. Both H- and L-type variants demonstrate accumulation of PrP^{Sc} in the medulla at the level of the obex (Casalone, 2006; Konold *et al.,* 2012). The range and morphological appearance of immunolabelling throughout the neuraxis differ from classical BSE, with multiple small plaque-like deposits being a common feature in variant forms. Differences in the brainstem (obex) are not always pronounced, and cannot be relied on to effectively differentiate or classify cases.

1.2.2.1. Immunohistochemical (IHC) methods

IHC examination for PrP^{Sc} accumulation is performed on the same formalin-fixed paraffin-embedded material used for the histopathological diagnosis. Different protocols have been applied successfully to the IHC detection of PrP^{Sc} for the diagnosis of BSE and although a standardised IHC method would seem desirable, it might be more important to recognise robust methods that achieve a standardised output, as monitored by participation in proficiency testing exercises, and by comparison with the results of a standardised model method in a Reference Laboratory. The generic technique established for histopathology still applies and it works well in autolysed tissues in which morphological evaluation is no longer possible (Monleon *et al.,* 2003). However, it is imperative to recognise the anatomy of the sample to determine whether or not target areas are represented. This is essential for a negative diagnosis, and may also be pivotal in accurately interpreting equivocal immunolabelling. IHC detection of PrP^{Sc} accumulations approximates to the sensitivity of the Western immunoblotting approach for detection of PrP^{Sc} (Schaller *et al.,* 1999). In combination with good histological preparations, immunohistochemistry allows detection of PrP^{Sc} accumulations and, as this, like the vacuolar pathology, exhibits a typical distribution pattern and appearance. This provides simultaneous evaluation or confirmation of this aspect of the disease phenotype. Current methods are available by reference to the WOAH Reference Laboratories.

In contrast to the diagnosis of scrapie of sheep, the limited detection of PrP^{Sc} in lymphoid tissues in BSE does not provide any scope for utilising such tissues for preclinical diagnosis by biopsy techniques.

1.2.2.2. Western immunoblot methods

Immunoblotting techniques are carried out on fresh (unfixed) tissue, and can be applied successfully even when tissue is autolysed (Hayashi *et al.,* 2004). The SAF-immunoblot (Stack, 2004) was the first such method for use in BSE diagnosis. Western immunoblot has similar diagnostic sensitivity to the IHC techniques, and remains the method of choice, along with immunohistochemistry, for the confirmation of BSE. It is a highly sensitive method using a large mass (ideally 2–4 g) of starting material and several steps to concentrate PrP^{Sc}. Alternative less time-consuming and less costly methods are now available. These use less material and are more practical. A range of Western immunoblotting methods are available on TSEglobalNet[5] or from the other WOAH BSE Reference Laboratories.

While Western immunoblot methodology is now in general use around the world, analytical sensitivity when used to detect PrP^{Sc} varies significantly between methods and laboratories.

5 https://science.vla.gov.uk/tseglobalnet/index.html

Where in-house methods are preferred to published methods for confirmatory purposes, it is important that they are evaluated as being fit for purpose and validated in consultation with a WOAH Reference Laboratory.

WOAH and national reference laboratories have established Western immunoblot methods to discriminate H- and L-type variants from classical BSE. Discrimination is based on distinct N-terminal proteinase K cleavage, antibody reactivity and glycosylation pattern of PrP^{res} (Jacob *et al.,* 2007). A detailed protocol including diagnostic criteria has been released by the European Union Reference Laboratory and is available online at TSEglobalNet[6].

1.2.2.3. Rapid test methods

Rapid Western immunoblot, lateral flow assays and ELISA techniques have been developed that allow screening of large numbers of brain samples and are commercially available. Such techniques can be performed in a few hours (see EC evaluations of rapid tests for the detection of BSE on known IHC positive and negative sample groups[7]). Tests that have been evaluated and approved for BSE surveillance within the EU are listed in point 4 of Chapter C of Annex X to the TSE Regulation (EC No 999/2001 and subsequent amendments[8]). An algorithm of how these tests may be used is available on the website of the WOAH Reference Laboratory in the UK[9].

While many countries, and a WOAH *ad hoc* Group on BSE tests, accept EU approval as an indicator of test performance, others have established their own evaluation mechanisms, most notably the United States of America, Canada and Japan (Canada's protocols for BSE surveillance[10]; National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, Outline of Regulation System of Veterinary Drugs in Japan[11]). WOAH also has an approval process and protocols for such evaluations are posted on the WOAH web site: Validation and certification of Diagnostic Assays[12], and the EU approval process has been accepted as the gold standard for future evaluations in terms of acceptable sensitivity and specificity.

The relative sensitivities of rapid tests, immunohistochemistry and other confirmatory methods have not been fully determined as, by definition, the tests cannot all be applied to identical samples, and the PrP^{Sc} distribution is anatomically variable. (As a compromise, tissue homogenates or mixtures of finely chopped tissue may be used and provide some information, for certain types of tests.) Acknowledgement of this is particularly important in evaluating rapid test performance when testing animals that are not presenting clinical signs of BSE and are likely to have smaller, more restricted depositions of PrP^{Sc} than cattle with more advanced disease. Rapid tests provide a means of initial screening for animals in the last few months of the incubation period (Arnold *et al.,* 2007), for example in surveys of post-mortem material collected from routinely slaughtered cattle. In countries conducting surveillance for the detection of the novel occurrence of BSE and in those countries in which a means of assessing the prevalence of BSE is considered necessary independent of the system of notification of suspect cases, these screening tests offer an efficient approach. Since their introduction for active screening in Europe from January 2001, such tests have been responsible for the identification of the majority of BSE-infected animals and are the preferred primary test. However, confirmation of a diagnosis of BSE ideally requires either the examination of fixed brain by immunohistochemistry or the application of an appropriate Western immunoblot protocol. In 2006, WOAH accepted that through their use in active surveillance programmes, commercial rapid tests have proved themselves to be very effective and consistent, provided they are performed by appropriately trained personnel. Indeed, at times they may out-perform the acknowledged standard of comparison if training and experience in the latter are deficient. Under such circumstances, it is now considered

6 https://science.vla.gov.uk/tseglobalnet/documents/tse-rl-blot.pdf
7 https://science.vla.gov.uk/tseglobalnet/test.html
8 https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1591112973636&uri=CELEX:02001R0999-20191214
9 https://science.vla.gov.uk/tseglobalnet/documents/tse-oie-guide.pdf
10 http://www.inspection.gc.ca/english/anima/disemala/bseesb/surv/protoce.shtml
11 http://www.maff.go.jp/nval/english/pdf/outline130325.pdf
12 https://www.woah.org/en/what-we-offer/veterinary-products/diagnostic-kits/

acceptable for diagnosis, even if not ideal for characterisation, for rapid tests to be used in combination for both primary screening in active or passive surveillance programmes and subsequent confirmation. It is essential however to ensure that the choice of primary and secondary test are compatible, and do not present a danger of generating false positive results through shared reagents. Consequently, an algorithm of preferred test combinations will be maintained on TSEglobalNet to assist those who wish to use this approach instead of immunohistochemistry or Western immunoblotting for confirmation. The combination of tests should include a Western blot method to generate useful complementary data that will assist in phenotypic characterisation of the sample in the absence of examination of fixed tissue. The confirmation should be carried out in a national reference laboratory.

The combination of the two rapid tests can only be used for the confirmation of a BSE case. A negative result by the secondary test is insufficient to define a case as negative following a primary positive result. BSE suspect cases with discordant rapid test results must therefore be investigated further using either the Western immunoblotting or IHC for the demonstration of PrP^{Sc}, or if these methods are not available, samples should be submitted to a WOAH Reference laboratory for further examination.

Although the test evaluation programmes conducted in Europe were in support of legislation on surveillance for BSE, the consequences are of relevance to other countries. The consequences of false-positive or false-negative results are so great that the introduction of new tests should be supported by thorough evaluation of test performance. Claims by test manufacturers should always be supported by data, ideally evaluated independently. It must be stressed that the process of full validation of all of these diagnostic methods for BSE has been restrained by the lack of a true reference method and the consequent need to apply standards of comparison based on relatively small studies. There is therefore a continuing need for the publication of larger scale studies of assay performance, and none of the data published so far equate with recognised procedures for test validation for other diseases.

1.3. Other diagnostic tests

BSE infectivity can be shown by inoculating mice with brain tissue from terminally affected cattle, but bioassay is impractical for routine diagnosis because of the long incubation period. It is, however, the nearest approach to a reference method for the characterisation of isolates, which has to be based on secondary biological properties in a standardised host, in the absence of an isolatable physical agent. Transgenic mice, such as those over-expressing the bovine PrP gene, offer bioassays with reduced incubation periods for BSE, but none as yet represent practical diagnostic tools.

Methods using *in-vitro* protein amplification are proving very sensitive for the detection of some prion diseases (Castilla *et al.,* 2006; Orru *et al.,* 2012), including C-type BSE (Murayama *et al*., 2010), but have not yet been formally evaluated for application within statutory surveillance systems, although some have been successfully piloted for surveillance applications in humans (Lacroux *et al.,* 2014; Orru *et al.,* 2014).

1.4. Availability of diagnostic reagents and kits

As discussed previously (Section B.1.2.2.3 above), diagnostic kits have been licensed for use in many countries and reagents are available commercially and from WOAH reference and other laboratories with a TSE surveillance programme. Laboratories should preferably use kits listed on the WOAH Register (see footnote 12) or else kits that have been fully evaluated and validated by the relevant regulatory bodies.

2. Serological tests

The infectious agents of prion diseases do not induce a significant immune response in the host so serological methods are not applicable.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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NB: There are WOAH Reference Laboratories for bovine spongiform encephalopathy
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for bovine spongiform encephalopathy

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.4.6.

BOVINE TUBERCULOSIS

See Chapter 3.1.13. Mammalian tuberculosis (*infection with* Mycobacterium tuberculosis *complex*).

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CHAPTER 3.4.7.

BOVINE VIRAL DIARRHOEA

SUMMARY

Cattle of all ages are susceptible to infection with bovine viral diarrhoea viruses (BVDV). Distribution is world-wide although some countries have recently eradicated the virus. BVDV infection results in a wide variety of clinical manifestations, including enteric and respiratory disease in any class of cattle or reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease. Animals that survive in-utero *infection in the first trimester of gestation are almost always persistently infected (PI). PI animals provide the main reservoir of the virus in a population and excrete large amounts of virus in urine, faeces, discharges, milk and semen. Identification of such PI cattle is a key element in controlling the infection. It is important to avoid the trade of such animals. They may appear clinically healthy, or weak and unthrifty. Many PI animals die before reaching maturity. They may infrequently develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, invariably leading to death. Mucosal disease can arise only in PI animals. Latent infections generally do not occur following recovery from acute infection. However bulls may rarely have a persistent testicular infection and excrete virus in semen for prolonged periods.*

Detection of the agent: *BVDV is a pestivirus in the family* Flaviviridae *and is closely related to classical swine fever and ovine border disease viruses. The two genotypes (types 1 and 2) are classified as separate species in the genus* Pestivirus*. A third putative genotype, BVDV type 3, has also recently been proposed. Although both cytopathic and non-cytopathic biotypes of BVDV type 1 and type 2 exist, non-cytopathic strains are usually encountered in field infections and are the main focus of diagnostic virus isolation in cell cultures. PI animals can be readily identified by a variety of methods aimed to detect viral antigens or viral RNA directly in blood and tissues. Virus can also be isolated by inoculation of specimens onto susceptible cell cultures followed by immune-labelling methods to detect the replication of the virus in the cultures. Persistence of virus infection should be confirmed by resampling after an interval of at least 3 weeks, when virus will again be detected. PI animals are usually seronegative. Viraemia in acute cases is transient and difficult to detect. Virus isolation in semen from bulls that are acutely or persistently infected requires special attention to specimen transport and testing. RNA detection assays are particularly useful because they are rapid, have very high sensitivity and do not depend on the use of cell cultures.*

Serological tests: *Acute infection with BVDV is best confirmed by demonstrating seroconversion using sequential paired samples, ideally from several animals in the group. The testing of paired (acute and convalescent samples) should be done a minimum of 21 days apart and samples should be tested concurrently in the same assay. Enzyme-linked immunosorbent assays and the virus neutralisation test are the most widely used.*

Requirements for vaccines: *There is no standard vaccine for BVD, but a number of commercial preparations are available. An ideal vaccine should be able to prevent transplacental infection in pregnant cows. Modified live virus vaccine should not be administered to pregnant cattle (or to their sucking calves) due to the risk of transplacental infection. Live vaccines that contain cytopathic strains of BVDV present a risk of inducing mucosal disease in PI animals. Inactivated viral vaccines are safe and can be given to any class of animal but generally require booster vaccinations. BVDV is a particularly important hazard to the manufacture of vaccines and biological products for other diseases due to the high frequency of contamination of batches of fetal calf serum used as a culture medium supplement.*

A. INTRODUCTION

1. Impact of the disease

Cattle of all ages are susceptible to infection with bovine viral diarrhoea viruses (BVDV). Distribution of the virus is world-wide although some countries have recently eradicated the virus. BVDV infection results in a wide variety of clinical manifestations, including enteric and respiratory disease in any class of cattle or reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease. Clinical presentations and severity of disease may vary with different strains of virus. BVDV viruses also cause immune suppression which can render infected animals more susceptible to infection with other viruses and bacteria. The clinical impact may be more apparent in intensively managed livestock. Animals that survive *in-utero* infection in the first trimester of gestation are almost always persistently infected (PI). PI animals provide the main reservoir of the virus in a population and excrete large amounts of virus in urine, faeces, discharges, milk and semen. The virus spreads mainly by close contact between PI animals and other cattle. Virus shedding by acutely infected animals is usually less important. This virus may also persist in the environment for short periods or be transmitted with contaminated reproductive materials. Vertical transmission plays an important role in its epidemiology and pathogenesis.

Infections of the breeding female may result in conception failure or embryonic and fetal infection which results in abortions, stillbirths, teratogenic abnormalities or the birth of PI calves. Persistently viraemic animals may be born as weak, unthrifty calves or may appear as normal healthy calves and be unrecognised clinically for a long time. However, PI animals have a markedly reduced life expectancy, with a high proportion dying before reaching maturity. Infrequently, some of these animals may later develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, invariably leading to death. Mucosal disease can arise only in PI animals. It is important to avoid the trade of viraemic animals. It is generally considered that serologically positive, non-viraemic cattle are 'safe', providing that they are not pregnant. However, a small proportion of persistently viraemic animals may produce antibodies to some of the viral proteins if they are exposed to another strain of BVDV that is antigenically different to the persisting virus. Consequently, seropositivity cannot be completely equated with 'safety'. Detection of PI animals must be specifically directed at detection of the virus or its components (RNA or antigens). Latent infections generally do not occur following recovery from acute infection. However, semen collected from bulls during an acute infection is likely to contain virus during the viraemic period and often for a short time afterwards. Although extremely rare, some recovered bulls may have a persistent testicular infection and excrete virus in semen, perhaps indefinitely.

While BVDV strains are predominantly pathogens of cattle, interspecies transmission can occur following close contact with sheep, goats or pigs. Infection of pregnant small ruminants or pigs with BVDV can result in reproductive loss and the birth of PI animals. BVDV infections have been reported in both New World and Old World camelids. Additionally, strains of border disease virus (BDV) have infected cattle, resulting in clinical presentations indistinguishable from BVDV infection. The birth of cattle PI with BDV and the subsequent development of mucosal disease have also been described. Whilst BVDV and BDV have been reported as natural infections in pigs, the related virus of classical swine fever does not naturally infect ruminants.

Although ubiquitous, control of BVDV can be achieved at the herd level, and even at the national level, as evidenced by the progress towards eradication made in many European countries (Moennig *et al*., 2005).

2. The causal agent

Bovine viral diarrhoea virus (BVDV) is a single linear positive-stranded RNA virus in the genus *Pestivirus* of the family *Flaviviridae.* The genus contains a number of species including the two genotypes of bovine viral diarrhoea virus (BVDV) (types 1 and 2) and the closely related classical swine fever and ovine border disease viruses. Viruses in these genotypes show considerable antigenic difference from each other and, within the type 1 and type 2 species, BVDV isolates exhibit considerable biological and antigenic diversity. Within the two BVDV genotypes, further subdivisions are discernible by genetic analysis (Vilcek *et al*., 2001). The two genotypes may be differentiated from each other, and from other pestiviruses, by monoclonal antibodies (MAbs) directed against the major glycoproteins E2 and ERNS or by genetic analysis. Reverse-transcription polymerase chain reaction (RT-PCR) assays enable virus typing direct from blood samples (Letellier & Kerhofs, 2003; McGoldrick *et al*., 1999). Type 1 viruses are generally more common although the prevalence of type 2 strains can be high in North America. BVDV of both genotypes may occur in non-cytopathic and cytopathic forms (biotypes), classified according to whether or not microscopically apparent cytopathology is induced during infection of cell cultures. Usually, it is the non-cytopathic biotype that circulates freely in cattle populations. Non-cytopathic strains are most frequently responsible for disease in cattle and are associated with enteric and respiratory disease in any

class of cattle or reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease (Brownlie, 1985). Cytopathic viruses are encountered in cases of mucosal disease, a clinical syndrome that is relatively uncommon and involves the 'super-infection' of an animal that is PI with a non-cytopathic virus by a closely related cytopathic strain. The two virus biotypes found in a mucosal disease case are usually antigenically closely related if not identical. Type 2 viruses are usually non-cytopathic and have been associated with outbreaks of severe acute infection and a haemorrhagic syndrome. However some type 2 viruses have also been associated with a disease indistinguishable from that seen with the more frequently isolated type 1 viruses. Further, some type 1 isolates have been associated with particularly severe and fatal disease outbreaks in adult cattle. Clinically mild and inapparent infections are common following infection of non-pregnant animals with either genotype.

There is an increasing awareness of an "atypical" or "HoBi-like" pestivirus – a putative BVDV type 3, in cattle, also associated with clinical disease (Bauermann *et al*., 2013), but its distribution is presently unclear. These viruses are readily detected by proven pan-reactive assays such as real-time RT-PCR. Some commercial antigen ELISAs (enzyme-linked immunosorbent assays) have been shown to detect these strains (Bauermann *et al*., 2012); generally virus isolation, etc., follows the same principles as for BVDV 1 and 2. It should be noted however, that antibody ELISAs vary in their ability to detect antibody to BVDV 3 and vaccines designed to protect against BVDV 1 and 2 may not confer full protection against infection with these novel pestiviruses (Bauermann *et al.,* 2012*;* 2013).

3. Pathogenesis

3.1. Acute infections

Acute infections with BVDV are encountered more frequently in young animals, and may be clinically inapparent or associated with fever, diarrhoea (Baker 1995), respiratory disease and sometimes sudden death. The severity of disease may vary with virus strain and the involvement of other pathogens (Brownlie, 1990). In particular, outbreaks of a severe form of acute disease with haemorrhagic lesions, thrombocytopenia and high mortality have been reported sporadically from some countries (Baker, 1995; Bolin & Ridpath, 1992). Infection with type 2 viruses in particular has been demonstrated to cause altered platelet function. During acute infections there is a brief viraemia for 7–10 days and shedding of virus can be detected in nasal and ocular discharges. There may also be a transient leukopenia, thrombocytopenia or temperature response, but these can vary greatly among animals. Affected animals may be predisposed to secondary infections with other viruses and bacteria. Although BVDV may cause a primary respiratory disease on its own, the immunosuppressive effects of the virus exacerbate the impact of this virus. BVDV is one of the major pathogens of the bovine respiratory disease complex in feedlot cattle and in other intensive management systems such as calf raising units.

Infection of breeding females immediately prior to ovulation and in the first few days after insemination can result in conception failure and early embryonic loss (McGowan & Kirkland, 1995). Cows may also suffer from infertility, associated with changes in ovarian function and secretions of gonadotropin and progesterone (Fray *et al*., 2002). Bulls may excrete virus in semen for a short period during and immediately after infection and may suffer a temporary reduction of fertility. Although the virus level in this semen is generally low it can result in reduced conception rates and be a potential source of introduction of virus into a naive herd (McGowan & Kirkland, 1995).

3.2. *In-utero* infections

Infection of a breeding female can result in a range of different outcomes, depending on the stage of gestation at which infection occurred. Before about 25 days of gestation, infection of the developing conceptus will usually result in embryo-fetal death, although abortion may be delayed for a considerable time (McGowan & Kirkland, 1995). Surviving fetuses are normal and uninfected. However, infection of the female between about 30–90 days will invariably result in fetal infection, with all surviving progeny PI and sero-negative. Infection at later stages and up to about day 150 can result in a range of congenital defects including hydranencephaly, cerebellar hypoplasia, optic defects, skeletal defects such as arthrogryposis and hypotrichosis. Growth retardation may also occur, perhaps as a result of pituitary dysfunction. Fetal infection can result in abortion, stillbirth or the delivery of weak calves that may die soon after birth (Baker, 1995; Brownlie, 1990; Duffell & Harkness, 1985; Moennig & Liess, 1995). Some PI calves may appear to be normal at birth but fail to grow normally. They remain PI for life and are usually sero-negative. The onset of the fetal immune response and production of antibodies occurs between approximately day 90–120, with an increasing proportion of infected calves

having detectable antibodies while the proportion in which virus may be detected declines rapidly. Infection of the bovine fetus after day 180 usually results in the birth of a normal seropositive calf.

3.3. Persistent infections

Persistently viraemic animals are a continual source of infective virus to other cattle and are the main reservoir of BVDV in a population. In a population without a rigorous BVDV control programme, approximately 1–2% of cattle are PI. During outbreaks in a naive herd or breeding group, if exposure has occurred in the first trimester of pregnancy, a very high proportion of surviving calves will be PI. If a PI animal dies, there are no pathognomonic lesions due to BVDV and the pathology is often complicated by secondary infections with other agents. Some PI animals will survive to sexual maturity and may breed successfully but their progeny will also always be PI. Animals being traded or used for artificial breeding should first be screened to ensure that they are not PI.

3.4. Mucosal disease

Persistently viraemic animals may later succumb to mucosal disease (Brownlie, 1985). However, cases are rare. This syndrome has been shown to be the outcome of the infection of a PI animal with an antigenically similar cytopathic virus, which can arise either through superinfection, recombination between non-cytopathic biotypes, or mutation of the persistent biotype (Brownlie, 1990). There is usually little need to specifically confirm that a PI animal has succumbed to mucosal disease as this is largely a scientific curiosity and of little practical significance, other than that the animal is PI with BVDV. However, cases of mucosal disease may be the first indication in a herd that BVDV infection is present, and should lead to more in depth investigation and intervention.

3.5. Semen and embryos

Bulls that are PI usually have poor quality, highly infective semen and reduced fertility (McGowan & Kirkland, 1995). All bulls used for natural or artificial insemination should be screened for both acute and persistent BVDV infection. A rare event, possibly brought about by acute infection during pubescence, can result in persistent infection of the testes and thus strongly seropositive bulls that intermittently excrete virus in semen (Voges *et al*., 1998). This phenomenon has also been observed following vaccination with an attenuated virus (Givens *et al*., 2007). Embryo donor cows that are PI with BVDV also represent a potential source of infection, particularly as there are extremely high concentrations of BVDV in uterine and vaginal fluids. While oocysts without an intact zona pellucida have been shown to be susceptible to infection *in vitro*, the majority of oocysts remain uninfected with BVDV. Normal uninfected progeny have also been 'rescued' from PI animals by the use of extensive washing of embryos and in vitro fertilisation. Female cattle used as embryo recipients should always be screened to confirm that they are not PI, and ideally, are sero-positive or were vaccinated at least 4 weeks before first use.

Biological materials used for *in-vitro* fertilisation techniques (bovine serum, bovine cell cultures) have a high risk of contamination and should be screened for BVDV. Incidents of apparent introduction of virus via such techniques have highlighted this risk. It is considered essential that serum supplements used in media should be free of contaminants as detailed in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use,* using techniques described in Section B.3.1 of this chapter.

4. Approaches to diagnosis and sample collection

The diagnosis of BVDV infection can sometimes be complex because of the delay between infection and clinical expression. While detection of PI animals should be readily accomplished using current diagnostic methods, the recognition of acute infections and detection of BVDV in reproductive materials can be more difficult.

4.1. Acute infections

Unlike PI animals, acutely infected animals excrete relatively low levels of virus and for a short period of time (usually about 7–10 days) but the clinical signs may occur during the later stages of viraemia, reducing the time to detect the virus even further. In cases of respiratory or enteric disease, samples should be collected from a number of affected animals, preferentially selecting the most recently affected. Swabs should be collected from the nares and conjunctiva of animals with respiratory disease

or from rectum and faeces if there are enteric signs. Lung and spleen are preferred from dead animals. Viral RNA may be detected by real-time RT-PCR assays and have the advantages of high sensitivity and being able to detect genome from non-infectious virus. As the virus levels are very low, it is not usually practical to undertake virus isolation unless there is a need to characterise the strain of BVDV involved. Serology undertaken on paired acute and convalescent sera (collected at least 21 days after the acute sample and from 8–10 animals) is worthwhile and gives a high probability of incriminating or excluding BVDV infection.

Confirmation that an abortion, stillbirth or perinatal death is caused by BVDV is often difficult to establish because there can be a long delay between initial infection and death or expulsion of the fetus. Sampling should take into consideration the need to detect either viral components or antibodies. Spleen and lung are preferred samples for virus detection while pericardial or pleural fluids are ideal samples for serology. The stomach of newborn calves should be checked to confirm that sucking has not occurred. While virus may be isolated from fetal tissue in some cases, emphasis should be placed on the detection of viral antigen by ELISA or RNA by real-time RT-PCR. For serology, both ELISAs and virus neutralisation test (VNT) are suitable though sample quality and bacterial contamination may compromise the ability to detect antibodies by VNT. Maternal serology, especially on a group of animals, can be of value, with the aim of determining whether there has been recent infection in the group. A high antibody titre (>1/1000) to BVDV in maternal serum is suggestive of fetal infection and is probably due to the fetus providing the dam with an extended exposure to virus.

4.2. Persistent infections

In the past, identification of PI animals relied heavily on the use of virus isolation in cell cultures. However, antigen detection ELISAs and real-time RT-PCR assays, each with relatively high sensitivity, are widely used for the detection of viral antigens or RNA in both live and dead animals. Virus isolation aimed at the detection of non-cytopathic BVDV in blood is also used, while in some countries, the virus has been identified by immunohistochemistry (IHC). Skin samples have been collected from live animals while a wide range of tissues from dead animals are suitable. Both virus isolation and IHC are labour intensive and costly and can be technically demanding. Virus isolation from blood can be confounded by the presence of maternal antibody to BVDV in calves less than 4–5 months of age. In older animals with persistent viraemia infection, low levels of antibody may be present due to their ability to seroconvert to strains of BVDV (including vaccines) antigenically different to the persisting virus (Brownlie, 1990). Bulk (tank) or individual milk samples have been used to monitor dairy herds for the presence of a PI animal. Antigen ELISA, real-time PCR and virus isolation have all been used. To confirm a diagnosis of persistent infection, animals should be retested after an interval of at least 3 weeks by testing of blood samples for the presence of the virus and for evidence of seroconversion. Care should be taken with retesting of skin samples as it has been shown that, in some acute cases, viral antigen may persist for many weeks in skin (Cornish *et al*., 2005).

4.3. Mucosal disease

Although not undertaken for routine diagnostic purposes, for laboratory confirmation of a diagnosis of mucosal disease it is necessary to isolate the cytopathic virus. This biotype may sometimes be isolated from blood, but it can be recovered more consistently from a variety of other tissues, in particular spleen, intestine and Peyer's patch tissue. Virus isolation is readily accomplished from spleen which is easy to collect and is seldom toxic for cell culture.

4.4. Reproductive materials

Semen donor bulls should be sampled for testing for freedom from BVDV infection prior to collection of semen, in accordance with the *Terrestrial Animal Health Code.* It is necessary to confirm that these bulls are not PI, are not undergoing an acute infection and to establish their serological status. This initial testing should be carried out on whole blood or serum samples. To establish that a seropositive bull does not have a persistent testicular infection (PTI), samples of semen should be collected on at least three separate occasions at intervals of not less than 7 days due to the possibility of intermittent low level virus excretion, especially during the early stages of infection. There is also a need to submit a number of straws from each collection, or an appropriate volume of raw semen. Particular care should be taken to ensure that sample transport recommendations are adhered to and that the laboratory documents the condition of the samples on arrival at the laboratory. Further details of collection, transport and test requirements are provided in sections that follow.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of bovine viral diarrhoea and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Virus isolation	+	+++	++	+++	–	–
Antigen detection by ELISA	++	+++	+++	+++	+++	–
Antigen detection by IHC	–	–	–	++	–	–
NA detection by real-time RT-PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
ELISA	+++	++	+++	–	+++	+++
VN	+	+++	++	–	+	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry method; NA = nucleic acid; RT-PCR = reverse-transcription polymerase chain reaction; ISH = *in-situ* hybridisation; VN = virus neutralisation.
[(a)]A combination of agent detection methods applied on the same clinical sample is recommended.

1. Detection of the agent

To prevent the shipment of either animals or animal derivatives (especially semen and embryos) that are infected with BVDV, it is necessary to test for the presence of the infectious virus (virus isolation), viral antigens (antigen detection ELISA) or RNA (real-time RT-PCR) in the blood of the animal being shipped, or the donor of the germplasm (semen or embryos). The exception is for seropositive bulls where semen must be tested rather than the donor bull. Serology only plays a role for establishing that sero-negative animals are not undergoing an acute infection or, to establish the serological status of donor bulls. Due to their variable sensitivity without prior virus amplification, procedures such as IHC or *in-situ* hybridisation (ISH) directly on tissues are not considered to be suitable for certification for freedom from BVDV for international trade purposes. In contrast, immune-staining is an essential component of virus isolation in cell culture to detect the presence of non-cytopathic strains of BVDV which predominate in field infections.

All test methods must be extensively validated by testing on known uninfected and infected populations of cattle, including animals with low- and high-titre viraemias. Methods based on polyclonal or MAb-binding assays (ELISA or IHC), immune labelling (VI) or on nucleic acid recognition (PCR) must be shown to detect the full range of antigenic and genetic diversity found among BVD viruses. There are three designated WOAH Reference Laboratories for BVDV that can assist with relevant information[1]; the reference laboratories for classical swine fever could also be approached to offer some advice.

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3.

1.1. Virus isolation

When performed to a high standard, BVDV isolation is very reliable. However, it does have very exacting requirements to ensure that the cell cultures and medium components give a system that is very sensitive and are not compromised by the presence of either low levels of BVDV specific antibody or virus. Virus isolation only has the capacity to detect infectious virus which imposes certain limits on sample quality. Further, to detect low levels of virus that may be present in some samples, particularly semen, it may be necessary to examine larger volumes of specimen than is usual. Some of these limitations can be overcome by the use of antigen detection ELISAs with proven high analytical sensitivity, or the use of real-time RT-PCR.

The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung, testis or turbinate). In some instances, ovine cells are also suitable. Primary or secondary cultures can be frozen as cell suspensions in liquid nitrogen. These can then be tested over a series of passages, or seeded to other susceptible cells and checked for freedom from contaminants and to evaluate their sensitivity compared to an approved batch of cells before routine use. Such problems may be reduced by the use of continuous cell lines, which can be obtained BVD-free, however, their BVDV-free status and susceptibility must be monitored regularly. Continuous cells should be used under a 'seed lot' system where they are only used over a limited passage range, within which they have been shown to have acceptable sensitivity to BVDV infection. Although particular continuous cell lines are considered to be appropriate for use for BVDV isolation, there can be significant variation in batches of cells from different sources due to differing passage histories so their suitability must still be confirmed before routine use.

Non-cytopathic BVDV is a common contaminant of bovine tissues, and cell cultures must be checked for freedom from adventitious virus by regular testing. Cells must be grown in proven cell culture medium components and a large area of cells must be examined. It is not appropriate to screen a few wells of a 96 well plate – examining all wells of a 96 well plate will be more convincing evidence of freedom. The fetal bovine serum that is selected for use in cell culture must also be free not only from virus, but also and of equal or perhaps even greater importance, from BVDV neutralising antibody. Heat treatment (56°C for 30–45 minutes) is inadequate for the destruction of BVDV in contaminated serum; irradiation with a dose of at least 25 kiloGrays (2.5 Mrad) is more certain. Commercial batches of fetal bovine serum mostly test positive by real-time RT-PCR even after the virus has been inactivated by irradiation. Further, most commercially collected batches of fetal bovine serum contain antibodies to pestiviruses, sometimes at levels that are barely detectable but sufficient to inhibit virus isolation. To overcome this, serum can be obtained from BVD virus and antibody free donor animals and used with confidence. Testing of donors for both virus and antibody occurs on an individual animal basis. Although horse serum has been substituted for bovine fetal serum, it is often found to have poorer cell-growth-promoting characteristics. Further there has sometimes been cross contamination with fetal bovine serum during processing, negating the objective of obtaining a BVDV-free product.

Buffy coat cells, whole blood, washed leukocytes or serum are suitable for isolation of the virus from live animals. Maternal antibody may interfere with isolation from serum in young calves. Tissue suspensions from post-mortem cases should be prepared by standard methods. Confirmation that a bull is not PI with BVDV is most readily achieved by testing of a blood sample. However, persistent testicular infections (PTI) have been detected in some bulls that have recovered from acute infection, are no longer viraemic and are now seropositive (Voges *et al*., 1998). Virus may be detected in most but not all collections of semen from these bulls. Although still considered to be uncommon, to exclude the potential for a PTI it is essential to screen semen from all seropositive bulls. To be confident that a bull does not have a PTI, batches of semen collected over several weeks should be screened. Once a series of collections have been screened, further testing of semen from a seropositive bull is not warranted. Raw semen, and occasionally extended semen, is cytotoxic and must be diluted in culture medium. For these reasons, it is important to monitor the health of the cells by microscopic examination at intervals during the incubation. These problems are largely overcome by the use of real-time RT-PCR which has several advantages over virus isolation, including higher sensitivity and the potential to be completed within a few hours rather than weeks for virus isolation.

There are many variations of procedure in use for virus isolation. All should be optimised to give maximum sensitivity of detection of a standard virus preparation. All biological components used for cell culture should be screened and shown to be free of both BVDV and antibodies to BVDV. Cell

cultures (whether primary or continuous lines) should be regularly checked to confirm that they maintain maximum susceptibility to virus infection. Depending on the specimen type and purpose for testing, virus isolation is likely to require one or more passages in cell cultures. While PI animals can be readily identified by screening blood or serum with one passage, semen should be routinely cultured for three passages and biological products such as fetal bovine serum up to five times (original inoculation plus four passages). Conventional methods for virus isolation are used, with the addition of a final immune-staining step (immunofluorescence or, more frequently, peroxidase staining) to detect growth of non-cytopathic virus. Thus tube cultures should include flying cover-slips, while microplate cultures can be fixed and labelled directly in the plate. Examples are given below. Alternatively, culture supernatant from the final passage can be screened by real-time RT-PCR (see below).

1.1.1. Microplate immunoperoxidase method for mass screening for virus detection in serum samples (Meyling, 1984)

i) 10–25 µl of the serum sample is placed into each of four wells of a 96-well tissue-culture grade microplate. This is repeated for each sample. Known positive and negative controls are included.

ii) 100 µl of a cell suspension at the appropriate concentration (usually about 150,000 cells/ml) in medium without fetal calf serum (FCS) is added to all wells. *Note:* the sample itself acts as the cell-growth supplement. If testing samples other than serum, use medium with 10% FCS that is free of antibodies to ruminant pestiviruses.

iii) The plate is incubated at 37°C for 4 days, either in a 5% CO_2 atmosphere or with the plate sealed.

iv) Each well is examined microscopically for evidence of cytopathology (cytopathic effect or CPE), or signs of cytotoxicity.

v) The cultures are frozen briefly at approximately –80°C and 50 µl of the culture supernatant is passaged to new cell cultures, repeating steps 3.1.1.i to iv above.

vi) The cells are then fixed and stained by one of two methods:

- **Paraformaldehyde**

a) Add 200 µl of a 1/10 dilution of formaldehyde solution (approximately 3% concentration) to the plate and leave at room temperature for 10 minutes.

b) The contents of the plate are then discarded and the plate is washed.

c) Wash plates 5 times with 0.05%Tween 20 in water (an automatic microplate washer can be used with a low pressure and speed setting).

d) To each well add 50ul of an antiviral antibody at the appropriate dilution (prepared in phosphate buffered saline/ PBS containing 1% gelatin) and incubate for 60–90 minutes at 37°C in a humidified chamber.

e) Wash plates five times as in step c).

f) Dilute the appropriate peroxidase conjugated antiserum to the optimum dilution in 1% gelatin/PBS (e.g. peroxidase conjugated rabbit anti-mouse immunoglobulin when the antiviral antibody is a mouse monoclonal). The optimum concentration should be determined for each batch of conjugate by "checkerboard" titration against reference positive and negative controls.

g) To each well of the microplate add 50ul of the diluted peroxidase conjugate and incubate for 90 minutes at 37°C in a humidified chamber.

h) Wash plates five times as in step c).

i) "Develop" the plate by adding 3-amino-9-ethyl carbazole (AEC) substrate (100 µl/well) and allowing to react for 30 minutes at room temperature.

j) Add 100 µl of PBS to each well and add a lid to each plate.

k) Examine the wells by light microscopy, starting with the negative and positive control wells. There should be no or minimal staining apparent in the cells that were uninfected

(negative control). The infected (positive control) cells should show a reddish- brown colour in the cytoplasm.

- **Acetone**

a) The plate is emptied by gentle inversion and rinsed in PBS.

b) The cells are fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied immediately and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The plate is drained thoroughly and as much fluid as possible is removed by tapping and blotting. The plate is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using radiant heat from a bench lamp). *Note:* the drying is part of the fixation process.

c) The fixed cells are rinsed by adding PBS to all wells.

d) The wells are drained and the BVD antibody (50 µl) is added to all wells at a predetermined dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum or 1% gelatin. (Horse serum or gelatin may be added to reduce nonspecific staining.)

e) Incubate at 37°C for 15 minutes.

f) Empty the plate and wash three times in PBST.

g) Drain and add the appropriate anti-species serum conjugated to peroxidase at a predetermined dilution in PBST (50 µl per well) for 15 minutes at 37°C.

h) Empty the plate and wash three times in PBST.

i) Rinse the plate in distilled water. All fluid is tapped out from the plate.

j) Add freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g. 3-amino-9-ethyl carbazole (AEC).

An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of PBS. Though the staining is not quite so intense, these chemicals have the advantage that they can be shipped by air.

k) The plate is examined microscopically. Virus-positive cells show red-brown cytoplasmic staining.

Alternative methods for fixation of the cells may be used and include the use of heat (see Chapter 3.9.3 *Classical swine fever*, Section B.2.2.1.viii). These should be first evaluated to ensure that the capacity to detect viral antigen is not compromised.

1.1.2. Tube method for tissue or buffy coat suspensions

Note: this method can also be conveniently adapted to 24-well plastic dishes. *Note:* a minimum of 2 and preferably 3 passages (including primary inoculation) is required.

i) Tissue samples are ground up and a 10% suspension in culture medium is made. This is then centrifuged to remove the debris.

ii) Test tube cultures with newly confluent or subconfluent monolayers of susceptible bovine cells are inoculated with 0.1 ml of the sample. The culture is left to adsorb for 1 hour at 37°C.

iii) The culture is washed with 1 ml of medium; this is then discarded and 1 ml of culture maintenance medium is added.

iv) The culture is incubated for 4–5 days at 37°C, and examined microscopically for evidence of CPE or signs of cytotoxicity.

v) The culture should then be frozen and thawed for passage to fresh cultures for one or preferably two more passages (including the culture inoculated for the final immunostaining). At the final passage, after freeze–thaw the tissue culture fluid is harvested and passaged on to microtitre plates for culture and staining by the immunoperoxidase method (see section B.3.1.1 above) or by the immunofluorescent method. For immunofluorescence, cover-slips are included in the tubes and used to

support cultured cells. At the end of the culture period, the cover slips are removed, fixed in 100% acetone and stained with an immunofluorescent conjugate to BVDV. Examine the cover slips under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of pestiviruses. Alternatively, culture supernatant from the final passage can be screened by real-time RT-PCR (see below).

1.1.3. Virus isolation from semen

The samples used for the test are, typically, extended bovine semen or occasionally raw semen. Semen samples should be transported to the laboratory in liquid nitrogen, or on dry ice. The samples should be stored in liquid nitrogen or at lower than –70°C (for long-term storage) or 4°C (for short-term storage of not more than 1–2 days). The receiving laboratory should document the condition under which samples are received. Raw semen is generally cytotoxic and should be prediluted (e.g. 1/10 in BVDV free bovine serum) before being added to cell cultures. At least 0.1 ml of raw semen should be tested with three passages in cell culture. Toxicity may also be encountered with extended semen. For extended semen, an approximation should be made to ensure that the equivalent of a minimum of 0.1 ml raw semen is examined (e.g. a minimum of 1.0 ml extended semen). If toxicity is encountered, multiple diluted samples may need to be tested to reach a volume equivalent to 0.1 ml raw semen (e.g. 5 × 1 ml of a sample of extended semen that has been diluted 1/5 to reduce toxicity). A suggested method is as follows:

i) Dilute 200 µl fresh semen in 1.8 ml bovine serum containing antibiotics. This can be the same serum as is being used for supplementing the cell cultures, and must be shown to be free from antibodies against BVDV.

ii) Mix vigorously and leave for 30 minutes at room temperature.

iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus isolation from tissue above) in cell culture tubes or a six-well tissue culture plate.

iv) Incubate the cultures for 1 hour at 37°C.

v) Remove the mixture, wash the monolayer several times with maintenance medium and then add new maintenance medium to the cultures.

vi) Include BVDV negative and positive controls in the test. Special caution must be taken to avoid accidental contamination of test wells by the positive control, for example always handling the positive control last.

vii) Observe plates microscopically to ensure freedom from contamination and cytotoxicity. No cytopathology is expected as a result of BVDV infection but other viruses such as BHV-1 could be inadvertently isolated.

viii) After 5–7 days, the cultures are frozen at or below approximately –70°C and thawed, clarified by centrifugation, and the supernatant used to inoculate fresh monolayers.

ix) At the end of the second passage, the supernatant from the freeze-thaw preparation should be passaged onto cultures in a suitable system for immunoperoxidase staining or other antigen detection or by real-time RT-PCR after 5 days of culture. This is most readily achieved in 96 well microplates. The sample is considered to be negative, if there is no evidence of viral antigen or BVDV RNA detected.

1.2. Nucleic acid detection

Conventional gel based RT-PCR has in the past been used for the detection of BVD viral RNA for diagnostic purposes. A multiplex RT-PCR has been used for the simultaneous amplification and typing of virus from cell culture, or direct from blood samples. However, gel based RT-PCR has the disadvantage that it is relatively labour intensive, expensive and prone to cross contamination. These problems had been markedly reduced following the introduction of probe-based real-time or quantitative RT-PCR methods . Nevertheless, stringent precautions should still be taken to avoid nucleic acid contamination in the test system and general laboratory areas where samples are handled and prepared (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* and Chapter 2.2.3 *Development and optimisation of nucleic acid detection assays*). These assays have even higher sensitivity than gel based RT-PCR and can be completed in a few hours. They

are in widespread use for the diagnosis of infectious diseases, allowing the direct detection of viral RNA from a wide range of specimens including serum, whole blood, tissues, milk and semen. The high analytical sensitivity allows the adoption of strategies to screen pools of individual samples or testing of bulk tank milk. By using this approach the presence of one or more PI animals can be identified in herds containing several hundred cows. Although slightly more expensive than immunostaining methods, real-time RT-PCR is a quick and reliable method that can also be used to screen culture supernatant from the final passage of cell cultures. While real-time RT-PCR has very high sensitivity and can be applied to the screening of biological materials used for vaccine manufacture, caution is needed in the interpretation of results, as the detection of viral RNA does not imply *per se* that infective virus is present. Real-time RT-PCR assays based on fluorescent-labelled DNA probes can also be used to differentiate pestiviruses (e.g. McGoldrick *et al*., 1999).

Primers for the assay should be selected in highly conserved regions of the genome, ideally the 5'-noncoding region, or the NS3 (p80 gene). There are published assays that are broadly reactive across the pestivirus genus, detecting all BVDV types, CSFV and most of the 'atypical' pestiviruses (e.g. Hoffman *et al*., 2006). A sensitive broadly reactive assay is recommended for diagnostic applications because interspecies transfer of different pestiviruses is occasionally encountered. When further identification of the specific virus is required, pestivirus species-specific assays can be applied to further type the virus. It is important to thoroughly optimise all aspects of the real-time RT-PCR assay, including the nucleic acid extraction and purification. Optimal concentrations of Mg^{2+}, primers, probe and polymerase, and the cycling parameters need to be determined. However, fully formulated and optimised 'ready to use' 'mastermixes' are now available commercially and only require addition of optimised concentrations of primers and probe. Optimised cycling conditions are often recommended for a particular mastermix.

A variety of commercially available nucleic acid purification systems are available in kit form and several can be semi-automated. Systems based on the capture and purification of RNA using magnetic beads are in widespread use and allow rapid processing of large numbers of samples. Specific products should be evaluated to determine the optimal kit for a particular sample type and whether any preliminary sample processing is required. For whole blood samples, the type of anticoagulant and volume of blood in a specimen tube is important. More problems with inhibitors of the PCR reaction are encountered with samples collected into heparin treated blood than EDTA. These differences are also exacerbated if the tube does not contain the recommended volume of blood, thereby increasing the concentration of anticoagulant in the sample. To identify possible false-negative results, it is recommended to spike an exogenous ('internal control') RNA template into the specimen prior to RNA extraction (e.g. Hoffman *et al*., 2006). By the inclusion of PCR primers and probe specific to the exogenous sequence, the efficiency of both the RNA extraction and also the presence of any PCR inhibitors can be monitored. While valuable for all sample types, the inclusion of an internal control is particularly desirable when testing semen and whole blood. When using an internal control, extensive testing is necessary to ensure that PCR amplification of the internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity (see also chapter 1.1.6).

When it is suspected that a sample may contain substances that are adversely affecting either the efficiency of RNA extraction or the real-time RT-PCR assay, modest dilution of the sample in saline, cell culture medium or a buffer solution (e.g. PBGS) will usually overcome the problem. Dilution of a semen sample by 1/4 and whole unclotted blood at 1/10 is usually adequate. As the real-time RT-PCR has extremely high analytical sensitivity, dilution of the sample rarely has a significant impact on the capacity of the assay to detect viral RNA when present.

1.2.1. Real-time polymerase chain reaction for BVDV detection in semen

Real-time RT-PCR has been shown to be extremely useful to screen semen samples to demonstrate freedom from BVDV and, apart from speed, often gives superior results to virus isolation in cell culture, especially when low virus levels are present, such as may be found in bulls with a PTI. The real-time RT-PCR described here uses a pair of sequence-specific primers for amplification of target DNA and a 5'-nuclease oligoprobe for the detection of amplified products. The oligoprobe is a single, sequence-specific oligonucleotide, labelled with two different fluorophores. The primers and probe are available commercially and several different fluorophores options are available. This pan-pestivirus real-time RT-PCR assay is designed to detect viral DNA of all strains of BVDV1 and BVDV2 as well as BDV, CSFV and most atypical pestivirurses. The assay selectively amplifies a 208 base pair sequence of the 5' non-translated

region (5' NTR) of the pestivirus genome. Details of the primers and probes are given in the protocol outlined below.

i) Sample preparation, equipment and reagents

a) The samples used for the test are, typically, extended bovine semen or occasionally raw semen. If the samples are only being tested by real-time RT-PCR, it is acceptable for them to be submitted chilled but they must still be cold when they reach the laboratory. Otherwise, if a cold chain cannot be assured or if virus isolation is being undertaken, the semen samples should be transported to the laboratory in liquid nitrogen or on dry ice. At the laboratory, the samples should be stored in liquid nitrogen or at lower than –70°C (for long-term storage) or 4°C (for short-term storage of up to 7 days). *Note:* samples for virus isolation should not be stored at 4°C for more than 1–2 days.

b) Due to the very high analytical sensitivity of real-time RT-PCR, much smaller volumes of semen may be used. However, at least three straws (minimum 250 µl each) from each collection batch of semen should be processed. The semen in the three straws should be pooled and mixed thoroughly before taking a sample for nucleic acid extraction.

c) A real-time PCR detection system, and the associated data analysis software, is required to perform the assay. A number of real-time PCR detection systems are available from various manufacturers. Other equipment required for the test includes a micro-centrifuge, a chilling block, a micro-vortex, and micropipettes. As real-time RT-PCR assays are able to detect very small amounts of target nucleic acid molecules, appropriate measures are required to avoid contamination, including dedicated and physically separated 'clean' areas for reagent preparation (where no samples or materials used for PCR are handled), a dedicated sample processing area and an isolated area for the PCR thermocycler and associated equipment. Each area should have dedicated reagents and equipment. Furthermore, a minimum of one negative sample should be processed in parallel to monitor the possibility of low level contamination. Sources of contamination may include product carry-over from positive samples or, more commonly, from cross contamination by PCR products from earlier work.

d) The real-time RT-PCR assay involves two separate procedures.

1) Firstly, BVDV RNA is extracted from semen using an appropriate validated nucleic acid extraction method. Systems using magnetic beads for the capture and purification of the nucleic acid are recommended. It is also preferable that the beads are handled by a semi-automated magnetic particle handling system.

2) The second procedure is the RT-PCR analysis of the extracted RNA template in a real-time RT-PCR system.

ii) Extraction of RNA

RNA or total nucleic acid is extracted from the pooled (three straws collected at the same time from the same animal) semen sample. Use of a commercially available magnetic bead based extraction kit is recommended. However, the preferred kit should be one that has been evaluated to ensure optimal extraction of difficult samples (semen and whole blood). Some systems and kit protocols are sufficiently refined that it is not necessary to remove cells from the semen sample. Prior to extraction dilute the pooled semen sample 1/4 in phosphate buffered gelatin saline (PBGS) or a similar buffered solution. Complete the RNA extraction by taking 50 µl of the diluted, pooled sample and add it to the sample lysis buffer. Some commercial extraction kits may require the use of a larger volume. It has also been found that satisfactory results are obtained by adding 25 µl of undiluted pooled sample to sample lysis buffer. Complete the extraction by following the kit manufacturer's instructions.

iii) Real-time RT-PCR assay procedure

a) Reaction mixture: There are a number of commercial real-time PCR amplification kits available from various sources and the particular kits selected need to be compatible

with the real-time PCR platform selected. The required primers and probes can be synthesised by various commercial companies. The WOAH Reference Laboratories for BVDV can provide information on suitable suppliers.

b) Supply and storage of reagents: The real-time PCR reaction mixture is normally provided as a 2 × concentration ready for use. The manufacturer's instructions should be followed for application and storage. Working stock solutions for primers and probe are made with nuclease-free water at the concentration of 20 µM and 3 µM, respectively. The stock solutions are stored at –20°C and the probe solution should be kept in the dark. Single-use or limited use aliquots can be prepared to limit freeze–thawing of primers and probes and extend their shelf life.

c) Primers and probe sequences

Selection of the primers and probe are outlined in Hoffmann *et al*. (2006) and summarised below.

Forward: BVD 190-F 5'-GRA-GTC-GTC-ART-GGT-TCG-AC

Reverse: V326 5'-TCA-ACT-CCA-TGT-GCC-ATG-TAC

Probe: TQ-pesti 5'-FAM-TGC-YAY-GTG-GAC-GAG-GGC-ATG-C-TAMRA-3'

d) Preparation of reaction mixtures

The PCR reaction mixtures are prepared in a separate room that is isolated from other PCR activities and sample handling. For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC), appropriate negative control (NC) two positive controls (PC1, PC2) should be included. The positive and negative controls are included in all steps of the assay from extraction onwards while the NTC is added after completion of the extraction. The PCR amplifications are carried out in a volume of 25 µl. The protocol described is based on use of a 96 well microplate based system but other options using microtubes are also suitable. Each well of the PCR plate should contain 20 µl of reaction mix and 5 µl of sample as follows:

12.5 µl	2× RT buffer – from a commercial kit.
1 µl	BVD 190-F Forward primer (20 µM)
1 µl	V326 Reverse primer (20 µM)
1 µl	TQ-pesti Probe (3 µM)
2 µl	tRNA (40 ng/µl)
1.5 µl	water
1 µl	25× enzyme mix
5 µl	sample (or controls – NTC, NC, PC1, PC2)

e) Selection of controls

NTC: usually consists of tRNA in nuclease free water that is added in place of a sample when the PCR reaction is set up.

NC: In practice, many laboratories use PBGS or a similar buffer. Ideally the controls for testing of semen samples should be negative semen, from sero-negative bulls. However, as a minimum, the assay in use should have been extensively validated with negative and positive samples to confirm that it gives reliable extraction and amplification with semen.

PCs: There are two positive controls (PC1=moderate – [Ct 29-32] and PC2=weak [Ct 32–35] positive). Positive semen from naturally infected bulls is preferable as a positive control. However, this is likely to be difficult to obtain. Further, semen from a PI bull is not considered suitable because the virus loads are usually very high and would not give a reliable indication of any moderate reduction in extraction or assay performance. Negative semen spiked with defined quantities of BVDV virus could be used as an alternative. If other samples are used as a routine PC, as a minimum the

entire extraction process and PCR assay in use must have been extensively validated using known positive semen from bulls with a PTI or from bulls undergoing an acute infection. If these samples are not available and spiked samples are used for validation purposes, a number of samples spiked with very low levels of virus should be included. On a day to day basis, the inclusion of an exogenous control with each test sample will largely compensate for not using positive semen as a control and will give additional benefits by monitoring the efficiency of the assay on each individual sample. Positive control samples should be prepared carefully to avoid cross contamination from high titred virus stocks and should be prepared in advance and frozen at a 'ready to use' concentration and ideally 'single use' volume.

f) Extracted samples are added to the PCR mix in a separate room. The controls should be added last, in a consistent sequence in the following order: NTC, negative and then the two positive controls.

g) Real-time polymerase chain reaction

The PCR plate or tubes are placed in the real-time PCR detection system in a separate, designated PCR room. Some mastermixes have uniform reaction conditions that are suitable for many different assays. As an example, the PCR detection system is programmed for the test as follows:

1× 48°C 10 minutes

1× 95°C 10 minutes

45 × (95°C 15 seconds, 60°C 1 minute)

h) Analysis of real-time PCR data

The software program is usually set to automatically adjust results by compensating for any background signal and the threshold level is usually set according to the manufacturer's instructions for the selected analysis software used. In this instance, a threshold is set at 0.05.

i) Interpretation of results

a) Test controls – all controls should give the expected results with positive controls PC1 and PC2 falling within the designated range and both the negative control NC and no template control NTC should have no Ct values.

b) Test samples

1) Positive result: Any sample that has a cycle threshold (Ct) value less than 40 is regarded as positive.

2) Negative result: Any sample that shows no Ct value is regarded as negative. However, before reporting a negative result for a sample, the performance of the exogenous internal control should be checked and shown to give a result within the accepted range for that control (for example, a Ct value no more than 2–3 Ct units higher than the NTC).

1.3. Enzyme-linked immunosorbent assay for antigen detection

Antigen detection by ELISA has become a widely adopted method for the detection of individual PI animals. These assays are not intended for the detection of acutely infected animals (though from to time this may be achieved). Importantly, these assays are not designed for screening of semen or biological materials used in assays or vaccine manufacture. Several methods for the ELISA for antigen detection have been published and a number of commercial kits are available. Most are based on the sandwich ELISA principle, with a capture antibody bound to the solid phase, and a detector antibody conjugated to a signal system, such as peroxidase. Amplification steps such as the use of biotin and streptavidin in the detection system are sometimes used to increase assay sensitivity. Both monoclonal- and polyclonal-based systems are described. The test measures BVD antigen (NS2-3 or ERNS) in lysates of peripheral blood leukocytes; the new generation of antigen-capture ELISAs (ERNS capture ELISAs) are able to detect BVD antigen in blood as well as in plasma or serum samples. The best of the methods gives a sensitivity similar to virus isolation, and may be preferred in those rare

cases where persistent infection is combined with sero-positivity. Due to transient viraemia, the antigen ELISA is less useful for virus detection in acute BVD infections.

The NS2-3 ELISA may be less effective in young calves that have had colostrum due to the presence of BVDV maternal antibodies. The real-time RT-PCR is probably the most sensitive detection method for this circumstance, but the ERNS ELISA has also been shown to be a sensitive and reliable test, particularly when used with skin biopsy (ear-notch) samples (Cornish *et al*., 2005).

1.4. Immunohistochemistry

Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections, particularly where suitable MAbs are available. However, these assays are not appropriate to certify animals for international trade and use should be limited to diagnostic investigations. It is important that the reagents and procedures used be fully validated, and that nonspecific reactivity be eliminated. For PI cattle almost any tissue can be used, but particularly good success has been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta. Skin biopsies, such as ear-notch samples, have shown to be useful for *in-vivo* diagnosis of persistent BDV infection.

2. Serological tests

Antibody to BVDV can be detected in cattle sera by a standard VNT or by ELISA, using one of several published methods or with commercial kits (e.g. Edwards, 1990). Serology is used to identify levels of herd immunity, for the detection of the presence of PI animals in a herd, to assist with investigation of reproductive disease and possible involvement of BVDV and to establish the serological status of bulls being used for semen collection and to identify whether there has been a recent infection. ELISA for antibody in bulk milk samples can give a useful indication of the BVD status of a herd (Niskanen, 1993). A high ELISA value (0.8 or more absorbance units) in an unvaccinated herd indicates a high probability of the herd having been exposed to BVDV in the recent past, most likely through one or more persistently viraemic animals being present. In contrast, a very low or negative value (≤0.2) indicates that it is unlikely that persistently viraemic animals are present. However, ELISA values are not always a reliable indicator of the presence of PI animals on farms, due to differing husbandry (Zimmer *et al*., 2002), recent administration of vaccine and also due to the presence of viral antigen in bulk milk, which may interfere with the antibody assay itself. Determination of the antibody status of a small number of young stock (9–18 months) has also been utilised as an indicator of recent transmission of BVDV in the herd (Houe *et al*., 1995), but this approach is also dependent on the degree of contact between different groups of animals in the herd and the potential for exposure from neighbouring herds. VN tests are more frequently used for regulatory purposes (e.g. testing of semen donors) while ELISAs (usually in the form of commercially prepared kits) are commonly used for diagnostic applications. Whether ELISA or VNT, control positive and negative standard sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. In the VNT, a 'serum control' to monitor sample toxicity should also be included for each test sample.

2.1. Virus neutralisation test

Selection of the virus strain to include in a VNT is very important. No single strain is likely to be ideal for all circumstances, but in practice one should be selected that detects the highest proportion of serological reactions in the local cattle population. Low levels of antibody to BVD type 2 virus may not be detectable by a neutralisation test that uses type 1 strain of the virus, and vice versa (Fulton *et al*., 1997). It is important that BVD type 1 and BVD type 2 be used in the test and not just the one that the diagnostician thinks is present, as this can lead to under reporting. Because it makes the test easier to read, most laboratories use highly cytopathic, laboratory-adapted strains of BVDV for VN tests. Two widely used cytopathic strains are 'Oregon C24V' and 'NADL'. However immune-labelling techniques are now available that allow simple detection of the growth or neutralisation of non-cytopathic strains where this is considered desirable, especially to support the inclusion of a locally relevant virus strain. An outline protocol for a microtitre VN test is given below (Edwards, 1990):

2.1.1. Test procedure

i) The test sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, three or four wells are used at each dilution depending on the degree of

precision required. At each dilution of serum, for each sample one well is left without virus to monitor for evidence of sample toxicity that could mimic viral cytopathology or interfere with virus replication. Control positive and negative sera should also be included in each batch of tests.

iii) An equal volume (e.g. 50 µl) of a stock of cytopathic strain of BVDV containing 100 $TCID_{50}$ (50%) tissue culture infective dose is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30–300 $TCID_{50}$).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the cell concentration is adjusted to 1.5×10^5/ml. 100 µl of the cell suspension is added to each well of the microtitre plate.

vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO_2 atmosphere or with the plate sealed.

vii) The wells are examined microscopically for CPE or fixed and stained by immunoperoxidase staining using an appropriate monoclonal antibody. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber or Reed Muench methods. A sero-negative animal will show no neutralisation at the lowest dilution (1/4), equivalent to a final dilution of 1/8. For accurate comparison of antibody titres, and particularly to demonstrate significant (more than fourfold) changes in titre, samples should be tested in parallel in the same test.

2.2. Enzyme-linked immunosorbent assay

Both indirect and blocking types of test can be used. A number of commercial kits are available. As with the virus neutralisation test, ELISAs configured using antigen from one genotype of BVD may not efficiently detect antibody induced by another genotype. Tests should therefore be selected for their ability to detect antibody to the spectrum of genotypes and strains circulating in the country where the test is to be performed.

The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The virus must be grown under optimal culture conditions using a highly permissive cell type. Any serum used in the medium must not inhibit growth of BVDV. The optimal time for harvest should be determined experimentally for the individual culture system. The virus can be concentrated and purified by density gradient centrifugation. Alternatively, a potent antigen can be prepared by treatment of infected cell cultures with detergents, such as Nonidet P40, N-decanoyl-N-methylglucamine (Mega 10), Triton X-100 or 1-octylbeta-D-glucopyranoside (OGP). Some workers have used fixed, infected whole cells as antigen. In the future, increasing use may be made of artificial antigens manufactured by expressing specific viral genes in bacterial or eukaryotic systems. Such systems should be validated by testing sera specific to a wide range of different virus strains. In the future, this technology should enable the production of serological tests complementary to subunit or marker vaccines, thus enabling differentiation between vaccinated and naturally infected cattle. An example outline protocol for an indirect ELISA is given below (Edwards, 1990).

2.2.1. Test procedure

i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for 24 hours at 37°C.

ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove the cell debris. The supernatant antigen is stored in small aliquots at –70°C, or freeze-dried. Non-infected cells are processed in parallel to make a control antigen.

iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. Alternate rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at 4°C. The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use in the test.

iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in PBST.

v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum diluent) for 1 hour at 37°C, then the plates are again washed five times in PBST.

vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After colour development, the reaction is stopped with sulphuric acid and the absorbance is read on an ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to give a net absorbance value for each serum.

vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage positivity) by dividing net absorbance by the net absorbance on that test of a standard positive serum that has a net absorbance of about 1.0. This normalisation procedure leads to more consistent and reproducible results.

C. REQUIREMENTS FOR VACCINES

1. Background

BVDV vaccines are used primarily for disease control purposes although they can convey production advantages especially in intensively managed cattle such as in feedlots. In some countries where BVDV eradication is being undertaken, PI animals are removed and remaining cattle are vaccinated to maintain a high level of infection and prevent the generation of further PI animals. Vaccination to control BVDV infections can be challenging due in part to the antigenic variability of the virus and the occurrence of persistent infections that arise as a result of fetal infection. on-going maintenance of the virus in nature is predominantly sustained by PI animals that are the product of *in-utero* infection. The goal for a vaccine should be to prevent systemic viraemia and the virus crossing the placenta, If this is successfully achieved it is likely that the vaccine will prevent the wide range of other clinical manifestations, including reproductive, respiratory and enteric diseases and immunosuppression with its secondary sequelae. There are many different vaccines available in different countries. Traditionally, BVD vaccines fall into two classes: modified live virus or inactivated vaccines. Experimental recombinant subunit vaccines based on BVD viral glycoprotein E2 expressed with baculovirus or transgenic plants and BVDV E2 DNA vaccines have been described but few if any are in commercial production. They offer a future prospect of 'marker vaccines' when used in connection with a complementary serological test.

1.1. Characteristics of a target product profile

Traditionally, BVD vaccines fall into two classes: modified live or inactivated virus vaccines. The essential requirement for both types is to afford a high level of fetal infection. Many of the live vaccines have been based on a cytopathic strain of the virus which is considered to be unable to cross the placenta. However, it is important to ensure that the vaccine virus does not cause fetal infection. In general vaccination of breeding animals should be completed well before insemination to ensure optimal protection and avoid any risk of fetal infection. Live virus vaccine may also be immunosuppressive and precipitate other infections. On the other hand, modified live virus vaccines may only require a single dose. Use of a live product containing a cytopathic strain of BVDV may precipitate mucosal disease by superinfection of persistently viraemic animals. Properly formulated inactivated vaccines are very safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. A combined vaccination protocol using inactivated followed by live vaccine may reduce the risk of adverse reaction to the live strain. Whether live or inactivated, because of the propensity for antigenic variability, the vaccine should contain strains of BVDV that are closely matched to viruses found in the area in which they are used. For example, in countries where strains of BVDV type 2 are found, it is important for the vaccine to contain a suitable type 2 strain. For optimal immunity against type 1 strains, antigens from the dominant subtypes (e.g. 1a and 1b) should be included. Due to the need to customise vaccines for the most commonly encountered strains within a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon globally.

Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

For optimal efficacy, it is considered that there should be a close antigenic match between viruses included in a vaccine and those circulating in the target population. BVDV type 2 strains should be included as appropriate. Due to the regional variations in genotypes and subtypes of BVDV, many vaccines contain more than one strain of BVDV to give acceptable protection. A good appreciation of the antigenic characteristics of individual strains can be obtained by screening with panels of MAbs (Paton *et al*., 1995).

2.1.1. Biological characteristics of the master seed

Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The separation and purification of the two biotypes from an initial mixed culture is important to maintain the expected characteristics of the seen and depends on several cycles of a limiting dilution technique for the noncytopathic virus, or plaque selection for the cytopathic virus. Purity of the cytopathic virus should be confirmed by at least one additional passage at limiting dilution. When isolates have been cloned, their identity and key antigenic characteristics should be confirmed. The identity of the seed virus should be confirmed by sequencing. Where there are multiple isolates included in the vaccine, each has to be prepared separately.

While retaining the desirable antigenic characteristics, the strains selected for the seed should not show any signs of disease when susceptible animals are vaccinated. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact' animals and should not be able to infect the fetus. Ideally seeds prepared for the production of inactivated vaccines should grow to high titre to minimise the need to concentrate the antigens and there should be a minimal amount of protein from the cell cultures incorporated into the final product. Master stocks for either live or inactivated vaccines should be prepared under a seed lot system involving master and working stocks that can be used for production in such a manner that the number of passages can be limited and minimise antigenic drift. While there are no absolute criteria for this purpose, as a general guide, the seed used for production should not be passaged more than 20 times beyond the master seed and the master seed should be of the lowest passage from the original isolate as is practical.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

It is crucial to ensure that all materials used in the preparation of the bulk antigens have been extensively screened to ensure freedom from extraneous agents. This should include master and working seeds, the cell cultures and all medium supplements such as bovine serum. It is particularly important to ensure that any serum used that is of bovine origin is free of both adventitious BVDV of all genotypes and antibodies against BVDV strains because low levels of either virus or antibody can mask the presence of the other. Materials and vaccine seeds should be tested for sterility and freedom from contamination with other agents, especially viruses as described in the chapter 1.1.8 and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

2.1.3. Validation as a vaccine strain

All vaccines should pass standard tests for efficacy. Tests should include as a minimum the demonstration of a neutralising antibody response following vaccination, a reduction in virus shedding after challenge in vaccinated cattle and ideally a prevention of viraemia. Efficacy tests of BVD vaccines by assessing clinical parameters in non-pregnant cattle can be limited by the difficulty of consistently establishing clinical signs but, when employed, clinical parameters such as a reduction in the rectal temperature response and leukopenia should be monitored. Although it can be difficult by using virus isolation in cell culture to consistently demonstrate the

low levels of viraemia associated with an acute infection, real-time PCR could be considered as an alternative method to establish the levels of circulating virus.

If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the capacity to prevent transplacental transmission. If there is a substantial reduction and ideally complete prevention of fetal infection, a vaccine would be expected to be highly effective in other situations (for example prevention of respiratory disease). A suitable challenge system can be established by intranasal inoculation of noncytopathic virus into pregnant cows between 60 and 90 days of gestation (Brownlie *et al*., 1995). Usually this system will reliably produce persistently viraemic offspring in non-immune cows. In countries where BVDV type 2 viruses are commonly encountered, efficacy in protecting against BVDV2 infections should be measured.

2.2. Method of manufacture

2.2.1. Procedure

Both cytopathic and noncytopathic biotypes will grow in a variety of cell cultures of bovine origin. Standard procedures may be used, with the expectation for harvesting noncytopathic virus on days 4–7 and cytopathic virus on days 2–4. The optimal yield of infectious virus will depend on several factors, including the cell culture, isolate used and the initial seeding rate of virus. These factors should be taken into consideration and virus replication kinetics investigated to establish the optimal conditions for large scale virus production. Whether a live or inactivated vaccine, the essential aim will be to produce a high-titred virus stock. This bulk antigen preparation can subsequently be prepared according to the type of vaccine being considered.

2.2.2. Requirements for ingredients

Most BVDV vaccines are grown in cell cultures of bovine origin that are frequently supplemented with medium components of animal origin. The material of greatest concern is bovine serum due to the potential for contamination with BVD viruses and antibodies to these viruses. These adventitious contaminants not only affect the efficiency of production but also may mask the presence of low levels of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all materials should be tested for sterility and freedom from contamination with other agents, especially viruses as described in chapters 1.1.8 and 1.1.9. Further, materials of bovine or ovine origin should originate from a country with negligible risk for transmissible spongiform encephalopathies [TSEs] (see chapter 1.1.9).

2.2.3. In-process controls

In-process controls are part of the manufacturing process. Cultures should be inspected regularly to ensure that they remain free from contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate. While the basic requirement for efficacy is the capacity to induce an acceptable neutralising antibody response, during production, target concentrations of antigen required to achieve an acceptable response may be monitored indirectly by assessment of the quantity of infectious virus or antigen mass that is produced. Rapid diagnostic assays such as the ELISA are useful to monitor BVDV antigen production. Alternatively, the quality of a batch of antigen may be determined by titration of the quantity of infectious virus present, although this may underestimate the quantity of antigen. For inactivated vaccines, infectivity is evaluated before inactivation. For inactivated vaccines the inactivation kinetics should be established so that a suitable safety margin can be determined and incorporated into the routine production processes. At the end of production, in vitro cell culture assays should be undertaken to confirm that inactivation has been complete. These innocuity tests should include a sufficient number of passages and volume of inoculum to ensure that very low levels of infectious virus would be detected if present.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9.

ii) Identity

Identity tests should demonstrate that no other strain of BVDV is present when several strains are propagated in a facility producing multivalent vaccines.

iii) Safety

Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is demonstrated and APPROVED in the registration dossier and production is consistent with that described in chapter 1.1.8.

The safety test is different to the inocuity test (see above).

Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals. Live vaccines containing cytopathic strains should have an appropriate warning of the risk of inducing mucosal disease in PI cattle.

iv) Batch potency

BVD vaccines must be demonstrated to produce adequate immune responses, when used in their final formulation according to the manufacturer's published instructions. The minimum quantity of infectious virus and/or antigen required to produce an acceptable immune response should be determined. *In-vitro* assays should be used to monitor individual batches during production.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the relevant authorities. Unless otherwise specified by the authorities, information should be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

There is no standard method for the manufacture of a BVD vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine or beta-propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

2.3.2. Safety requirements

In-vivo tests should be undertaken using a single dose, overdose (for live vaccines only) and repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) and contain the maximum permitted antigen load and, depending on the formulation of the vaccine, the maximum number of vaccine strains.

i) Target and non-target animal safety

The safety of the final product formulation of both live and inactivated vaccines should be assessed in susceptible young calves that are free of maternally derived antibodies and in pregnant cattle. They should be checked for any local reactions following administration, and, in pregnant cattle, for any effects on the unborn calf. Live attenuated vaccines may contribute to immunosuppression that might increase mortality. It may also contribute to the development of mucosal disease in PI animals that is an animal welfare concern. Therefore vaccination of PI animals with live attenuated vaccines containing cytopathic BVDV should be avoided. Live attenuated vaccines must not be capable of being transmitted to other unvaccinated animals that are in close contact.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Virus seeds that have been passaged at least up to and preferably beyond the passage limit specified for the seed should be inoculated into young calves to confirm that there is no evidence of disease. If a live attenuated vaccine has been registered for use in pregnant animals, reversion to virulence tests should also include pregnant animals. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact' animals.

iii) Precautions (hazards)

BVDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory. A live virus vaccine should be identified as harmless for people administering the product however adjuvants included in either live or inactivated vaccines may cause injury to people. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

The potency of the vaccine should be determined by inoculation into seronegative and virus negative calves, followed by monitoring of the antibody response. Antigen content can be assayed by ELISA and adjusted as required to a standard level for the particular vaccine. Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration. Each production batch of vaccine should undergo potency and safety testing as batch release criteria. BVD vaccines must be demonstrated to produce adequate immune responses, as outlined above, when used in their final formulation according to the manufacturer's published instructions.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

To date, there are no commercially available vaccines for BVDV that support use of a true DIVA strategy. Experimental subunit vaccines based on baculovirus-expressed BVD viral glycoprotein E2 have been described but are not available commercially. They offer a future prospect of 'marker vaccines' when used in connection with a complementary serological test. Experimental BVDV E2 DNA vaccines and BVDV E2 subunit vaccines expressed using transgenic plants and alphavirus replicon have also been described.

2.3.5. Duration of immunity

There are few published data on the duration of antibody following vaccination with a commercial product. Protocols for their use usually recommend a primary course of two inoculations and boosters at yearly intervals. Only limited data are available on the antibody levels that correlate with protection against respiratory infections (Bolin & Ridpath, 1995; Howard *et al*., 1989) or *in-utero* infection (Brownlie *et al*., 1995). However, there are many different commercial formulations and these involve a range of adjuvants that may support different periods of efficacy. Consequently, duration of immunity data must be generated separately for each commercially available product by undertaking challenge tests at the end of the period for which immunity has been claimed.

2.3.6. Stability

There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that attenuated virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. Inactivated virus vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong shelf life for either type, but adjuvants in killed vaccine may preclude this. Bulk antigens that have not been formulated into finished vaccine can be reliably stored frozen at low temperatures but the antigen quality should be monitored with in vitro assays prior to incorporation into a batch of vaccine.

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NB: There are WOAH Reference Laboratories for bovine viral diarrhoea (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine viral diarrhoea

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2015.

CHAPTER 3.4.8.

CONTAGIOUS BOVINE PLEUROPNEUMONIA (INFECTION WITH *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES*)[1]

SUMMARY

Contagious bovine pleuropneumonia (*CBPP*) *is a disease of ruminants* (Bos *and* Bubalus *genuses*) *caused by* Mycoplasma mycoides *subsp.* mycoides (Mmm)*. It is manifested by anorexia, fever and respiratory signs such as dyspnoea, polypnoea, cough and nasal discharges in bovines. Definite diagnosis requires the isolation or detection of the aetiological agent. The main problems for control or eradication are the frequent occurrence of subacute or subclinical infections, the persistence of chronic carriers after the clinical phase and the lack of extensive vaccine coverage.*

Detection of the agent: *Samples to be taken from live animals are nasal swabs and/or broncho-alveolar washings or pleural fluid obtained by puncture. Samples to be taken at necropsy are lung lesions, lymph nodes, pleural fluid and synovial fluid from those animals with arthritis.*

For cultivation of the pathogen, the tissues are ground in buffered solution and inoculated into selective broth and solid media with antibiotics or other inhibitors to prevent the growth of cell-walled bacteria. The growth of Mmm *can take up to 10 days, depending upon the type of sample and the mycoplasma titre.*

In broth, growth is visible as a homogeneous cloudiness which forms swirls when shaken; on agar, small colonies develop, 1 mm in diameter, with the classical 'fried-egg' appearance. The biochemical characteristics of Mmm *are the following: sensitivity to digitonin, reduction of tetrazolium salts, fermentation of glucose, absence of arginine hydrolysis, and the absence of or very slight phosphatase and proteolytic activities. Special media have been described that are recommended for these tests. Diagnosis may be confirmed by immunological tests, such as the growth inhibition and immunofluorescence tests* (*both use hyperimmune sera*)*. The polymerase chain reaction is a rapid, specific, sensitive and easy-to-use test.*

Serological tests: *For diagnosis, the modified Campbell & Turner complement fixation test remains a suitable test for certifying individual animals prior to movement, including for international trade. The competitive enzyme-linked immunosorbent assay is also a suitable test for certifying individual animals prior to movement. An immunoblotting test has undergone evaluation and is highly specific and sensitive.*

Requirements for vaccines: *The attenuated strains T1/44 and T1sr are now recommended for vaccine production. The minimal required titre is 10^7 mycoplasmas per vaccine dose, but higher titres of at least 10^8 are recommended.*

A. INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is an infectious and contagious respiratory disease of *Bovidae* caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) with a major impact on livestock production and a potential for rapid spread. As a result, CBPP-infected countries are excluded from international trade of live animals.

1 After taxonomic revision of the *Mycoplasma mycoides* cluster by Manso-Silvan *et al*. (2009) the designations Small Colony (SC) and Large Colony (LC) are no longer used.

Mmm is a mycoplasma, i.e. a wall-less bacteria (mollicute), belonging to the so-called "mycoides cluster" that groups five mycoplasma species that are ruminant pathogens (Manso-Silvan *et al*., 2009). These five mycoplasmas share phenotypic and genotypic characteristics that cause cross-reactions in conventional diagnostic techniques. The closest relative to *Mmm* is *M. mycoides* subsp *capri* (*Mmc*), which is usually found in goats.

In natural conditions, *Mmm* affects only the ruminants of the *Bos* genus, i.e. mainly bovine and zebu cattle but also the yak (*Bos grunniens*) and water buffaloes (*Bubalus bubalis*) (Santini *et al*., 1992). *Mmm* has been isolated from sheep and goats in Africa, in Portugal and in India (Srivastava *et al*., 2000). Among wild animals, one single case has been reported in American buffaloes (*Bison bison*) and none in African buffaloes (*Syncerus caffer*) or other wild ruminants. Small ruminants and wild animals do not play a role in the epidemiology of the disease, and CBPP is not a zoonotic agent.

The incubation period for naturally infected animals can range from 3 weeks to 6 months. The clinical manifestations in cattle range from hyperacute through acute, subacute and chronic forms.

CBPP is manifested by anorexia, fever and respiratory signs, such as dyspnoea, polypnoea, cough and nasal discharges during the acute stage of the disease when the causative agent can spread rapidly; in the chronic stage there may be long-term persistence of the agent. Typical lesions include a unilateral pneumonia associated with pleurisy. During the chronic stage of the disease, clinical signs wane and infected animals are more difficult to identify. In these cases, lungs may contain typical encapsulated lesions called sequestra. These 'silent' carriers may be infectious and thus responsible for unnoticed persistence of the infection in a herd; they play an important role in the maintenance and in the epidemiology of the disease.

CBPP has been unequivocally identified in Europe since the 18th century and it gained a world-wide distribution during the second half of the 19th century through cattle trade. CBPP was eradicated from many countries at the beginning of the 20th century, mostly through stamping-out strategies (UK, USA) or by vaccination campaigns followed by stamping-out strategies (Australia). Today, CBPP remains enzootic in many Sub-Saharan African countries, while in Europe the last CBPP cases were observed in Portugal in 1999. The situation in some Asian countries is unclear. Please consult WOAH WAHIS interface[2] for latest disease situation.

In the field, CBPP might be confused with other diseases causing respiratory problems such as pasteurellosis or other mycoplasmosis. The absence of confirmatory diagnosis may lead to antibiotic treatments being used in the case of CBPP outbreaks.

There is no known risk of human infection with *Mmm*. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.* In CBPP-free areas, it is advisable to manipulate *Mmm* in biosafety level (BSL) 2 laboratories, while BSL1 would be sufficient in enzootic zones. Good laboratory practices should be used for all procedures.

B. DIAGNOSTIC TECHNIQUES

Clinical diagnosis of CBPP is unreliable as initial signs may be slight or non-existent and may be indistinguishable from any severe pneumonia. Therefore, CBPP should be investigated by pathological, microbiological, molecular or serological diagnostic methods. As the pathological lesions of CBPP are distinctive, and pathognomonic, abattoir surveillance for CBPP involving lung examination is a practical method for disease monitoring.

It is recommended to isolate and identify the causative organism in order to confirm an outbreak. Table 1 lists the laboratory methods used for the diagnosis of CBPP.

2 http://www.woah.org/en/animal-health-in-the-world/the-world-animal-health-information-system/the-world-animal-health-information-system/

Table 1. Laboratory methods currently used for diagnosis of CBPP and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination*
Detection of the agent[(a)]						
***In-vitro* culture isolation (followed by species identification tests)**	+	–	–	+++	–	–
Direct molecular test (PCR)	–	–	–	++	–	–
Detection of immune response						
CFT	+++	++	+++	++	+++	–
Immunoblotting	++	++	++	++	++	–
C-ELISA	+++	++	+++	++	+++	–

*NB: at present, there is no test described in the table that allows evaluation of the immune status of an animal after vaccination, with the current T1 strains.

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose.

PCR = polymerase chain reaction; CFT = complement fixation test; C-ELISA = competitive enzyme-linked immunosorbent assay.

[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

1.1. Samples

A key to isolation success lies in collecting good quality samples. *Mmm* can be isolated from samples taken either from live animals or at necropsy. Samples taken from live animals are: nasal swabs or nasal discharges, broncho-alveolar lavage or transtracheal washing and pleural fluid collected aseptically by puncture made in the lower part of the thoracic cavity between the seventh and eighth ribs. Samples taken at necropsy are: lungs with lesions, pleural fluid ('lymph'), lymph nodes of the broncho-pulmonary tract, and synovial fluid from those animals with arthritis. The lung samples should be collected from lesions at the interface between diseased and normal tissue.

When collecting nasal swab samples, a transport medium should be used to protect the mycoplasmas and prevent proliferation of cell-walled bacteria and fungi (heart-infusion broth without peptone and glucose, 10% yeast extract, 20% heat-treated serum (horse or pig), 0.3% agar, 500 International Units [IU]/ml penicillin, 0.2 g/litre thallium acetate).

After collection, all samples must be kept refrigerated at 4°C and sent to the laboratory within 24 hours. For longer periods they should be frozen at or below –20°C.

1.2. *In-vitro* culture

The presence of the pathogen varies greatly with the stage of development of the lesions and a negative result is not conclusive, particularly if the animal was treated with an antibiotic.

Mmm needs selective media to grow, but it is not considered a fastidious mycoplasma. There are several media compositions used in different reference laboratories but, essentially, they should contain: a basic medium such as heart-infusion broth or PPLO broth (pleuropneumonia-like organisms), 1–2.5% yeast extract, 10–20% inactivated horse serum, 0.1% glucose, 1% tryptose, and 0.0024% DNA. To avoid growth of other bacteria, the media can also contain an antibiotic of the penicillin family (for example, 500 IU/ml penicillin G) as mycoplasmas are naturally resistant. The media should be used both as broth and solid. All culture media prepared should be subjected to quality control and must support growth of low passage *Mycoplasma* spp. from small inocula. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are performed correctly.

After grinding with broth, the tissue samples are diluted tenfold to minimise contaminating bacteria and are inoculated into five tubes of broth and onto five solid media plates. To avoid contaminating bacteria and to reduce the number of tubes and plates per sample, the supernatant of the ground sample may be filtered through a 25-mm filter with 0.45 pore size. The pleural fluid can be inoculated directly without prior dilution or filtration as, when infected, it is almost a pure culture of *Mmm*. To ensure the best conditions for mycoplasma growth, the tubes and plates are incubated at 37°C in a 5% CO_2 atmosphere and should be inspected daily for up to 10 days. After this time if there is no growth, the sample is considered negative. Positive samples in liquid medium show a homogeneous cloudiness, usually within 2–4 days. They frequently present a silky, fragile filament called a 'comet'. During the following days a uniform opacity develops which forms swirls when shaken. On agar media, the colonies are small (1 mm in diameter) and have the classical appearance of 'fried eggs' with a dense centre. At this stage, biochemical tests, the indirect fluorescent antibody (IFA) test or polymerase chain reaction (PCR) can be performed to identify the colonies.

1.3. Biochemical and immunological identification tests

Biochemical tests were used routinely in the past but have now been superseded by other tests, namely the PCR. Biochemical tests alone do not allow identification of a precise *Mycoplasma* species because of overlapping of the few phenotypic traits that can be evaluated. Therefore, molecular tests such as PCR are recommended for identification of isolates. The biochemical methods are given below for historic reasons.

Following subculture, antibiotics should be omitted from the medium to check if the isolate is a mycoplasma or an L-form of a bacterium that will regain its original form in the medium without inhibitors. Once this test is done, the organism can be identified using biochemical tests (Freundt *et al*., 1979).

Mmm needs cholesterol to grow and is, therefore, sensitive to digitonin (like all members of the order Mycoplasmatales), does not produce 'film and spots', ferments glucose, reduces tetrazolium salts (aerobically and anaerobically), does not hydrolyse arginine, has no phosphatase activity, and has no or weak proteolytic activity.

For these tests, special media have been developed that include the same basic ingredients (heart-infusion broth or Bacto PPLO broth, horse serum, 25% yeast extract solution, 0.2% DNA solution), to which is added 1% of a 50% glucose solution for glucose hydrolysis, 4% of a 38% arginine HCl solution for arginine hydrolysis, and 1% of a 2% triphenyl tetrazolium chloride solution for tetrazolium reduction, as well as a pH indicator (e.g. phenol red). (NOTE: a pH indicator should not be added to a medium containing triphenyl tetrazolium chloride.) For demonstration of proteolysis, growth is carried out on casein agar and/or coagulated serum agar.

Once the biochemical characteristics have been checked, one of the following immunological tests can be performed to confirm the identification: disk growth inhibition test (DGIT) (Freundt *et al*., 1979), fluorescent antibody test (FAT), IFA, agar gel immunodiffusion test (AGID) (Provost, 1972), or dot immunobinding on a membrane filter (MF-dot) test (Brocchi *et al*., 1993).

At present, few laboratories use immunochemical tests on a routine basis for identification of *Mmm* due to the development of PCR-based tests that are more specific, sensitive, rapid and easy to perform and standardise.

1.4. Molecular identification and typing methods – polymerase chain reaction (PCR)-based tests

See Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* for further details of implementation and validation of PCR-based tests.

PCR has become the method of choice for the rapid and specific identification of *Mmm* when the organism is isolated from a clinical sample. To avoid cross-contamination and carryover-contamination, strict separation of laboratory rooms, used for PCR preparation and handling of reactions, is needed. Various authors have developed a PCR system for *Mmm* identification and there is no preferred one, though the more sensitive, nested PCR should be avoided because of the higher risk of PCR product carryover, resulting in false positives. As the DNA target is not from a sample, where the number of cells vary and PCR inhibitors can be present, but from an isolate the sensitivity is not a critical point. Primers complementary to DNA regions CAP-21, *lppA* gene and 16S rRNA gene of the genome of *Mmm* have been designed by different authors and used in PCR systems, followed by restriction endonuclease analysis of the amplified product (amplicon) (PCR-REA) or by a second amplification (nested-PCR) (Table 2).

Table 2. Conventional PCR systems used for* Mmm *identification

Target DNA region*	Amplicon (bp)	Restriction enzyme	Hydrolyses products (bp)	Specificity	Reference
CAP-21 (position: 181628)	574	*Asn*I	379, 178 220, 178, 153	*Mmm* *Mmc*	Bashiruddin *et al.*,1994
(position: 443115)	275		43	*Mmm*	Dedieu *et al.*, 1994**
Gene *lppA* (nested)	717	–		*Mmm*	Miserez *et al.*, 1997***
(position: 20061)	503				

**Mmm* PG1 sequence Gene Bank accession number: NC005364
**Primers sequence: MSC1: ATA-CTT-CTG-TTC-TAG-TAA-TAT-G; MSC2: CTG-ATT-ATG-ATG-ACA-GTG-GTC-A
***Primers sequence: SC3NEST1-L: ACA-AAA-AGA-AGA-TAT-GGT-GTT-GG and SC3NEST1-R: ATC-AGG-TTT-ATC-CAT-TGG-TTG-G; SC3VII: ATT-AGG-ATT-AGC-TGG-TGG-AGG-AAC and SC3IV-S: TCT-GGG-TTA-TTC-GAA-CCA-TTA-T

As an example of one PCR system that is used in routine for *Mmm* identification, the PCR-REA procedure adapted from Bashiruddin *et al.,*1994, is provided below.

1.4.1. DNA extraction

Any accepted method for DNA extraction would be appropriate. A simple and effective method is to select a single colony, resuspend in 100 µl of PCR-grade water, boil for 15 minutes to lyse the cells and release the DNA, and centrifuge at 8000 ***g*** for 1 minute. The supernatant with the DNA will be used in the PCR reaction after diluting 1/10 in PCR-grade water. Alternatively 500 µl of a 4-day broth culture, from a single colony, is centrifuged at 8000 ***g*** for 1 minute, the pellet is resuspended in 100 µl of PCR-grade water and boiled and centrifuged as above. After diluting 1/10, 1 µl of the supernatant is used as template in the PCR.

1.4.2. PCR amplification

i) Preparation of the master mix

Synthetic oligonucleotides should be dissolved in TE buffer (Tris-EDTA [ethylene diamine tetra-acetic acid]) to a 100 µM concentration. This stock solution is stable at –20°C for at least 4 years. A working solution is prepared from the stock solution by a 1/2 dilution, to obtain a final concentration of 50 µM. In a final volume of 25 µl the PCR reaction should contain the following:

	Concentration	Volume in one reaction (total volume 25 µl)	Final concentration in the reaction
H_2O PCR-grade	–	16 µl	–
Reaction buffer without $MgCl_2$	10 ×	2.5 µl	1×
$MgCl_2$	25 mM	3 µl	3 mM
dNTPs	10 mM	1 µl	400 µM
Primer MM450*	50 µM	0.5 µl	1 µM
Primer MM451**	50 µM	0.5 µl	1 µM
Taq pol.	5U/µl	0.5 µl	2.5 U
template DNA	1 µl		

*Sequence: 5'-GTA-TTT-TCC-TTT-CTA-ATT-TG-3'
**Sequence: 5'-AAA-TCA-AAT-TAA-TAA-GTT-TG-3'

ii) Amplification conditions

One µl of template DNA of tested sample, or positive control (DNA from *Mmm*-type strain PG1) or negative control (water) is added to the mix. The amplification is performed under the following conditions: 30 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; and hold at 4°C indefinitely.

iii) Detection of amplified products

After amplification, the reactions should be opened in a separate room where the PCR products are detected by 1.5% agarose gel electrophoresis for 2 hours at 90 V. Positive reactions and the positive control, and not the negative control, should present an amplicon of 574 bp visible under UV light after staining with a suitable fluorescent dye.

1.4.3. Restriction endonuclease analysis (REA)

An enzymatic restriction of the PCR product is carried out in 10 µl reaction volume containing: 2 µl of PCR reaction, 5 U *Asn*I (2 µl), 1× buffer, at 37°C for 1 hour. The result is analysed in a 2% agarose gel electrophoresis for 1 hour at 100 V and the products visualised as above.

Identification is based on the sizes of the restriction products as presented in Table 2.

NB: Direct detection and identification of *Mmm* by PCR in clinical samples has not yet been fully evaluated and so the conventional PCR may be inadequate because of the presence of PCR inhibitors, fewer microorganisms in the sample and the presence of other bacteria, with a concomitant reduction in sensitivity and specificity. These problems should be avoided through the use of real-time PCR assays for *Mmm* detection, as fluorescence resulting from specific genomic amplification is detected and measured as the amplicon is being synthesised, and a 2–3 log increase in sensitivity, compared with conventional PCR, is obtained (Lorenzon *et al.,* 2008). When using samples such as pleural fluid, the PCR can be performed after boiling the sample and centrifuging to recover DNA in the supernatant. For lung fragments, the PCR is applied after DNA extraction procedures using an extraction kit or a phenol/chloroform extraction.

1.4.4. *Mmm* strain typing

Mmm strain typing has greatly benefited from advances in DNA sequencing. There are now a number of *Mmm* genomes that are available online[3]. However, the comparison of full *Mmm* genomes is not straightforward because of multiple genome rearrangements or DNA insertions. To overcome this problem while keeping the very fine differentiation of strains, an extended

3 https://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=656088

multilocus sequence typing (MLST) approach has been developed that allows unique identification of strains, phylogenetic analysis as well as molecular dating (Dupuy *et al.,* 2012).

2. Serological tests

Serological test results for CBPP should not be analysed and interpreted individually but in groups of animals from the same herd or region because false positive or false negative results may occur in individual animals. Tests on single animals can be misleading, either because the animal is in the early stage of disease, which may last for several months, before specific antibodies are produced, or it may be in the chronic stage of the disease when very few animals are seropositive. False-positive results can occur (2%), of which an important cause is serological cross-reactions with other mycoplasmas, particularly other members of the *M. mycoides* cluster. The validity of the results has to be confirmed by post-mortem and bacteriological examination, and serological tests on blood taken at the time of slaughter.

The complement fixation test (CFT) and enzyme-linked immunosorbent assays (ELISAs) are recommended for screening and eradication programmes. The highly specific immunoblotting test is useful as a confirmatory test but is not fit for mass screening.

2.1. Complement fixation (recommended for determining disease free status)

The modified Campbell & Turner CFT remains the recommended procedure and it is widely used in countries where infection occurs (Provost *et al*., 1987). The CFT is most conveniently carried out in a microtitre format and has been harmonised within countries of the EU (European Commission, 2001).

With a sensitivity of 63.8% and a specificity of 98% (Bellini *et al*., 1998), the CFT can detect nearly all sick animals with acute lesions, but a rather smaller proportion of animals in the early stages of the disease or of animals with chronic lesions.

2.1.1. Reagents

For CFT validation and accreditation, the quality control and standardisation of all the reagents is a critical issue as well as the pipettes and tips that are used to dispense them. To facilitate the accreditation procedure (ISO/IEC[4] 17025) appropriate antigen and sera controls can be obtained from WOAH Reference Laboratories for CBPP.

The features of the following reagents, should be taken into account, as they have an impact in the final result.

i) Ultra-pure sterilised water. The quality of the water is critical for the development of the reaction.

ii) *Positive reference sera:* for harmonisation purposes a positive bovine reference standard serum (PRS) is available from the WOAH Reference Laboratories in Portugal and Italy, and should be used in the diagnostic laboratory for routine use and antigen titration. The PRS has been obtained from a naturally CBPP-infected bovine, and is negative against *Brucella*, *Mycobacterium paratuberculosis*, *Chlamydia*, *Coxiella burnetii*, *Leptospira*, bovine viral diarrhoea virus, respiratory syncytial virus, infectious bovine rhinotracheitis virus, adenovirus, bovine herpes virus 4, foot and mouth disease viruses, bovine leukosis virus, and parainfluenza 3 virus, and should also be negative for adventitious viruses. The PRS presents 0% haemolysis in a dilution of 1/160 and 50% haemolysis in a dilution of 1/320. The PRS should be stored at –20°C in aliquots, this would prevent repetitive freeze–thawing, which causes deterioration of the serum.

iii) *Negative reference sera:* the negative control serum (NRS), also available from WOAH Reference Laboratories in Portugal and in Italy, is a serum from a healthy bovine from a bovine spongiform encephalopathy (BSE)-free source. It is CBPP free and should be negative against the above microorganisms. The NRS should be stored in aliquots at –20°C.

4 ISO/IEC: International Organisation for Standardisation/International Electrotechnical Commission.

iv) *Antigen:* the test must use an antigen that has been prepared from a suspension of a selected *Mmm* strain essayed previously and presenting the five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa. The antigen is previously checkerboard titrated and used at a dose of 2 complement fixing units (CFU) and should be standardised to give 50% fixation, at a dilution of 1/320 of PRS. It must be stored at 4°C ± 3°C and should not be frozen. It is produced, standardised and delivered by WOAH Reference Laboratories. The use of an antigen standardised against the WOAH reference sera promotes international harmonisation of diagnostic testing.

v) *Buffer:* veronal buffered saline (VB), pH 7.2 ± 1, is the standard diluent for the CFT. The VB can be prepared from tablets commercially available or it may be prepared from a stock solution of sodium chloride (42.5 g), barbituric acid (2.875 g), sodium diethyl barbiturate (1.875 g), magnesium chloride ($MgCl_2 \times 6\ H_2O$ – 0.501 g), and calcium chloride ($CaCl_2 \times 6\ H_2O$ – 0.18 g) in 1 litre of distilled water. A concentrated stock solution is used diluted 1/5 in double-distilled water, before use.

vi) *Haemolysin* (*amboceptor*): the haemolysin is a hyperimmune rabbit serum to SRBC (sheep red blood cells). The quantity used is 6 haemolytic doses read at 50% end-point (HD_{50} [50% haemolysing dose]). The SRBC are obtained by aseptic puncture of the jugular vein. They can be preserved in Alsever's solution or in sodium citrate. They are used in a 6% suspension. Haemolysin is also available commercially.

vii) *Haemolytic system* (*HS*): HS is prepared by diluting haemolysin in VB to give a dose of 12 HD_{50}. An equal volume of 6% SRBC antibody suspension is added, and the system is sensitised in a water bath at 37°C for 30 minutes with periodic shaking.

viii) *Complement* (*C'*): C' is obtained from normal guinea-pig serum. It is freeze-dried and reconstituted with double-distilled water. It must be kept at –20°C after reconstitution. A commercially produced complement can be used, according to the manufacturer's instructions. It is titrated by making a close dilution series in VB containing an appropriate quantity of the antigen to be used in the test. After incubation at 37°C for 30 minutes, an appropriate quantity of sensitised SRBC is added to each dilution. The titration is read after incubation for a further 30 minutes. The highest dilution giving complete haemolysis of the SRBC equals 1 C' unit, from which can be calculated the dilution required for 2.5 units in 25 µl.

2.1.2. Test procedure (using microplates)

The most critical factors for CFT performance are the control of the reagents and that it is carried out by trained and qualified personnel. Temperature and incubation times should also be carefully controlled. The entire procedure should be performed in a temperature-controlled room at 21°C ± 3°C.

Contaminated or haemolysed sera should not be tested, as this will interfere with the reaction. Undiluted test sera samples and appropriate working standards should be inactivated for 30 minutes in water bath at 56°C ± 2°C. This will destroy the complement of the sera and reduce or eliminate the anti-complementary activity (ACA).

Usually, only one serum dilution (1/10 in VB) is tested routinely but serial dilutions must be done for the positive control (PRS). Using standard 96-well microtitre plates with round (U) bottoms, the technique is performed as follows:

i) Dispense 25 µl of test serum inactivated and diluted 1/10 in VB in the wells of the first and second rows. The first row is an anti-complementary control for each serum.

ii) Volumes of 25 µl of antigen at a dose of 2 CFU are added to each well except the anti-complementary controls, to which 25 µl of VB should be added to compensate for lack of antigen.

iii) Add 25 µl of C' at a dose of 2.5 units. Shake gently and incubate at 37°C for 30 minutes with periodic (at least twice during the incubation period) or continuous shaking.

iv) Add 25 µl of HS. Shake gently and incubate at 37°C for 30 minutes. Microplates must be shaken twice during the incubation period.

v) It is necessary to set up the following controls:

a) C': 0.5 units, 1 unit and 2.5 units.

b) HS: 75 µl of VB + 25 µl of HS.

c) Antigen: 25 µl of 2 CFU of antigen + 25 µl of C' at 2.5 units + 25 µl of HS = 25 µl of VB.

These controls, along with the positive control serum (PRS) and the negative control serum (NRS), should always be used in each microplate or in a series of microplates where the same batches of reagents are used.

vi) *Interpretation of results*

After centrifugation of the microplates at 125 ***g*** for 5 minutes, the analysis is carried out based on the percentage of complement fixation observed.

Positive result: 100% fixation at 1/10;

Doubtful results: 25, 50 or 75% fixation at 1/10.

vii) *Validation of results*

Expected results for the plate controls are as follows:

a) PRS: expected titre.

b) NRS: 100% haemolysis

c) Anticomplementary control of serum samples: 100% haemolysis

d) Antigen control: 100% haemolysis

e) Complement units' control: 100% haemolysis for 2.5 units

f) HS control in absence of complement: 0% haemolysis

It is recommended that any CFT result, even partial (25, 50 or 75%), at a serum dilution of 1/10 be confirmed by additional investigations, such as immunoblotting or post-mortem examination and bacteriological tests according to contingency.

2.2. Competitive enzyme-linked immunosorbent assay

A competitive ELISA (C-ELISA) developed by the WOAH Collaborating Centre for the diagnosis and control of animal diseases in tropical countries[5] (Le Goff & Thiaucourt, 1998), has been validated internationally in accordance with WOAH standards (Amanfu *et al*., 1998). The performance of this C-ELISA method has also been validated by the French Committee for Accreditation in 2009.

Compared with the CFT, the C-ELISA has equal sensitivity and greater specificity. Advice on standard protocols and the availability of reagents can be obtained from the WOAH Reference Laboratories for CBPP, or the WOAH Collaborating Centre for ELISA and Molecular Techniques in Animal Disease Diagnosis (see Table given in Part 4 of this *Terrestrial Manual*).

Validation tests (Amanfu *et al.,* 1998; Le Goff & Thiaucourt, 1998) that have been carried out in several African and European countries indicated: i) that the true specificity of the C-ELISA has been reported to be at least 99.9%; ii) that the sensitivity of the C-ELISA and the CFT are similar; and iii) antibodies are detected by the C-ELISA in an infected herd very soon after they can be detected by the CFT, and C-ELISA antibody persists for a longer period of time (Niang *et al.,* 2006).

To enhance its repeatability and the robustness, this C-ELISA is now provided as a ready-made kit that contains all the necessary reagents, including precoated plates kept in sealed bags. This kit can be obtained commercially and availability can be checked through the WOAH Reference Laboratory in France. The kit has been especially designed to be robust and offers a good repeatability. Sera are analysed in single wells. The chromogen is TMB (tetramethyl benzidine) in a liquid buffer and the reading

5 See online list for contact details: https://www.woah.org/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-3

is at 450 nm. The colour turns from pale green to blue in the first place and becomes yellow once the stopping solution has been added. MAb controls exhibit a darker colour while strong positive serum controls are very pale. The cut-off has been set at 50% and should be valid in every country. However, each laboratory should establish its own measurement uncertainty in agreement with ISO 17025 requirements; see chapter 2.2.4 *Measurement uncertainty.*

2.3. Immunoblotting test

An immunoblotting test (IBT) is an immunoenzymatic test that has been developed to confirm doubtful CFT or C-ELISA results. A field evaluation indicated a higher specificity than the CFT enabling the detection of CFT false positives (Gonçalves *et al.* 1998).

2.3.1. Reagents

i) Antigen strips (see Section B.2.3.2 below).

ii) *Positive control serum:* serum from a naturally CBPP infected animal, used at a dilution of 1/100 in the IBT. This control presents 50% fixation at 1/80 dilution in the CFT and shows an immunoblotting profile with five specific *Mmm* antigenic bands of 110, 98, 95, 62/60 and 48 kDa. This serum is available from the WOAH Reference Laboratory in Portugal.

iii) *Dilution buffer:* phosphate-buffer saline **(**PBS), pH 7.2, containing 0.1% skim milk and 0.1% egg albumin. Skim milk quality is a critical issue, as it will influence the sensitivity of the reaction. Standardised skim milk should be used instead of common non-fat dry milks (Gaurivaud & Poumarat, 2012).

iv) *Peroxidase-conjugated anti-bovine IgG* (*H + L chains*): this is commercially available and should be previously checkerboard titrated against the positive control serum, to select the appropriate dilution at which the five specific bands are clearly visible (Gaurivaud & Poumarat, 2012).

v) *Substrate:* substrate is prepared by adding 30 mg 4-chloro-1-naphthol, dissolved in 10 ml methanol, to 50 ml PBS (pH 7.2) and 30 µl H_2O_2. Alternative substrates, such as BCIP/NTB (5-bromo-4-chloro-3-indolyl phosphate combined with nitrotetrazolium blue chloride), are available.

2.3.2. Preparation of antigen strips

i) The strain used to prepare the antigen is critical. Antigen should be prepared from a tested *Mmm* strain that must present the five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa. The antigen is a suspension of *Mmm* cells in PBS, pH 7.2, obtained from a 48-hour culture.

ii) The 5–15% polyacrylamide gel gradient used historically (Gonçalves *et al.* 1998) is not optimal for the repeatability of western blotting. The use of 7.5% polyacrylamide gel as recommended by Schubert *et al.* (2011) and Gaurivaud & Poumarat (2012), allows a good separation of all the five antigenic proteins and also a good repeatability of the pattern between electrophoresis runs.

iii) The separated proteins are transferred to a 14 × 14 cm 0.45 µm nitrocellulose membrane at 70 V in transfer buffer (20% methanol, 193 mM glycine, 25 mM Tris/HCl, pH 8.3). The effectiveness and homogeneity of the transfer have to be checked. This can be easily carried on with commercial staining solutions or kits that allow reversible staining and imaging of the blot prior to use.

iv) The membrane is dried and labelled on the side on which the proteins were electrophoresed. The nitrocellulose membrane is incubated in blocking buffer (PBS containing 5% skim milk, 1 M glycine and 1% egg albumin) for 2 hours at room temperature. After washing at room temperature three times for 15 minutes in 0.1% (v/v) Tween 20 in PBS, the nitrocellulose membrane is further washed in PBS. The membrane is then dried and one strip cut and tested for the presence of the five specific bands identified at 110, 98, 95, 62/60 and 48 kDa.

v) The nitrocellulose membrane is cut into strips, 0.4 cm wide and used for antibody testing. Each batch of strips should be tested, with a positive and negative reference serum, in order

to evaluate the clear presence of the five specific antigenic proteins and the absence of background. The resolution of the 95 and 98 kDa proteins should be carefully checked.

2.3.3. Test procedure

NB: The strips must be kept with the antigen side up during the procedure.

i) Sera samples to be tested are diluted 1/3 in dilution buffer (see Section B.2.3.1.iii).

ii) Each test sample together with the positive and negative control sera is placed in contact with the antigens strips and incubated at 37°C for 2 hours with continuous agitation. Strips are then washed at room temperature three times for 15 minutes each in 0.1% (v/v) Tween-20 in PBS, and further washed once in PBS.

iii) An appropriate dilution of peroxidase-conjugated anti-bovine IgG (H + L chains) is incubated with the strips for 1 hour at room temperature with continuous agitation. Wash as above.

iv) Substrate (see Section B.2.3.1.v) is added to the strips, which are then left in the dark with continuous agitation and examined periodically until the protein bands are visible (maximum 30–40 minutes). The reaction is stopped with distilled water.

v) *Results analysis:* The strips are dried and examined for the presence of the core IgG immunoblot profile of the five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa that should be seen in the positive control serum. Samples presenting a similar immunobloting profile are considered to be CBPP positive.

NB: The IBT is rather difficult to standardise as many factors can influence the final banding pattern (Gaurivaud & Poumarat, 2012). Of major concern is the *Mmm* culture stage and the strain that has been chosen. Recent *Mmm* strains of European origin lack the 98 kDa band. This could lead to dubious results in animals infected by such strains. The CBPP WOAH Reference Laboratory in Portugal can provide antigen strips as well as the positive and negative control sera, upon request.

C. REQUIREMENTS FOR VACCINES

1. Background

Since the beginning of the 20th century, many vaccines against CBPP have been described (e.g. killed vaccines, and heterologous vaccines), but none of them has proven to be completely satisfactory and cost effective. Today, the only vaccines commonly used are produced with attenuated *Mmm* strains.

Various attenuated *Mmm* strains have been used in the past and have been abandoned, such as the KH3J or V5 strains. Two strains are now used for preparing CBPP vaccines: strain T1/44, a naturally mild strain isolated in 1951 by Sheriff & Piercy in Tanzania (Sheriff & Piercy, 1952), and its derivative strain T1sr (Wesonga & Thiaucourt, 2000; Yaya *et al*., 1999). The 44th egg-passage of strain T1, called T1/44, was sufficiently attenuated to protect cattle without post-vaccinal severe reactions, however such reactions may still occur in the field although rarely. Their frequency is unpredictable. Cattle breeds should be assessed for their sensitivity before mass vaccination. It should be noted that when given by intubation, the vaccine can produce CBPP lesions (Mbulu *et al*., 2004); however, as the vaccine is to be injected subcutaneously, this should not create a serious risk (Hubschle *et al*., 2002).

T1/44 and T1sr vaccines can effectively protect herds when vaccinations are regularly performed (i.e. once a year for T1/44 and twice a year for T1sr). They can be used for CBPP control on a wider scale (national or regional), but they cannot lead to CBPP eradication when used alone.

The target species are the susceptible hosts of the *Bos* and *Bubalus* genuses.

New attenuated vaccines should have less residual virulence than T1/44 while maintaining or improving the same duration of immunity (1 year) and immunogenicity.

New inactivated vaccines should induce a significantly longer immunity (>1 year), should not hamper the detection of CBPP outbreaks and, ideally, should be compatible with other antigens to produce multivalent vaccines.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature as manufacturers may be obliged to meet either European Pharmacopeia, United States (of America) Department of Agriculture or other national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

At present, only two *Mmm* attenuated strains are recommended for CBPP vaccination: T1/44 and T1sr. T1/44 was attenuated by passaging a mild field strain 44 times in embryonated eggs. This ensured an attenuation of the strain while keeping its immunogenic properties. However some residual virulence has been observed in this strain and the percentage of reactors varies greatly from one region to another. These reactions are usually observed in primo-vaccinated animals. It has been observed that immunogenicity declines as attenuation increases. Strain T1sr is a direct derivative of T1/44, adapted to streptomycin resistance by four serial passages in growth medium with increasing concentrations of streptomycin. T1sr has no residual virulence but induces shorter immunity (6 months)

The master seed used for vaccine production should be as close as possible to the original vaccinal strains. Grand parental stock of these strains are kept at the WOAH Reference Laboratory in France and at the WOAH Collaborating Centre for Quality Control of Veterinary Vaccines in Ethiopia.

The genome sequence of strain T1/44 was published in 2016 (Gourgues *et al.,* 2016).

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

It is recommended to cultivate the master seed in a suitable medium that does not contain any preservative such as antibiotics so as to allow it to be shown that the master seed stock is pure. The freedom from extraneous agents should be tested according to international or national guidelines. Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

The purity and identity of the master seed is difficult to establish by conventional microbiological techniques. Notably, the morphological aspects of mycoplasma colonies on solid medium is not typical and may vary according to the composition of the medium (percentage of serum or agar for example). In addition it has been noted that T1/44 master seed could lead to colonies of different aspects maybe in relation to hypervariable antigens. Hence, the purity of the master seed should be established after a filter-cloning procedure and specific identification of at least 10 individual clones. T1 strains can be identified by a specific PCR (Lorenzon *et al.,* 2000). T1sr clones can be differentiated from T1/44 by their ability to grow in the presence of streptomycin.

2.1.3. Validation as a vaccine strain

Although *Mmm* strains can now be subtyped by exquisite molecular markers, there is as yet no evidence that a single vaccine strain could not protect against all circulating *Mmm* strains.

i) Innocuity

As noted above, strain T1/44 has a known residual virulence that may vary according to local conditions. Post-vaccinal reactions are characterised by a localised inflammatory reaction that develops at the site of injection (Willems' reaction). It may be noticed as early as 1 week post-injection. In many cases this local reaction will wane naturally but in some instances it may become extensive and lead to the death of the animal if no suitable antibiotic treatment is administered. The percentage of reactors being unpredictable, preliminary trials with a few animals is not a complete guarantee of inocuity.

After reconstitution, the master seed is inoculated subcutaneously into two mice, intraperitoneally into two mice and intraperitoneally into two male guinea-pigs. None of the

animals should die within the following month, and the guinea-pigs should not show signs of orchitis. Inocuity tests should be carried out on (at least two) cattle or zebu cattle. These are inoculated with ten vaccinal doses each, and observed for adverse effects for at least 4 weeks.

ii) Immunogenicity

For CBPP there is no susceptible laboratory animal allowing an easy control of potency. There is also no strict correlation between antibody titres after vaccination and actual protection. The only way to control the potency of a vaccine is to perform a natural challenge in the susceptible host by the 'in-contact' method. The potency of the grand parental stock has been assessed. Primo-vaccination with the minimum required dose gave a 40–60% protection rate. Higher protection rates have been obtained after repeated vaccinations.

2.2. Method of manufacture

2.2.1. Procedure

Vaccine bulk cultures must be obtained with a maximum of three successive passages of the master seed. A passage is defined here by a 1/100 dilution of a culture in the exponential phase of growth. For example, 0.5 ml of culture from the seed are transferred to 50 ml of fresh medium and, when turbidity is observed, these 50 ml are used to seed 5000 ml of medium, which represents the final product when the optimum titre has been reached. Each vaccine producer should then evaluate the speed of growth of the vaccine strain in the medium that is used to optimise the harvest time.

A stabiliser can be added to final cultures before freeze drying. The manufacturer should ensure an homogeneous distribution in the vials and use of a proper freeze dryer to have similar titres in all the vials when the freeze drying process is finished.

2.2.2. Requirements for ingredients

A suitable sterile liquid medium allowing the growth of the vaccine strain at the requested titre is needed. It is usually composed of a 'base' that can be autoclaved, which is then supplemented to allow for mycoplasma growth. The base consists of a meat digest. It can be prepared in-house or bought in powder form (for example, brain–heart infusion, PPLO broth, etc.). The supplement usually consists of animal serum (often horse serum at 10% final concentration), fresh yeast extract (5–10%) and other ingredients such as glucose, glycerol, DNA, and fatty acids. However there is no specific requirement regarding the composition of the medium but rather regarding the guarantee that all ingredients comply with quality assurance as mentioned in chapter 1.1.8, with special focus on products of biological origin as well as any national requirement.

It is usually recommended that no preservative, such as penicillin or similar antibiotic, be added to the medium. However, strain T1sr has to be grown in a medium containing 1 mg/ml streptomycin so as to prevent reversion to streptomycin-sensitivity.

2.2.3. In-process controls

It is recommended to assess the purity of the product during the production process. For example, purity can be assessed rapidly, before freeze-drying by observation under the microscope at ×40 with phase-contrast trans-illumination. Mycoplasmas will appear as very small grey dots, sometimes forming chains, having a Brownian agitation. Classical bacteria will appear as much bigger and bright, sometimes with a noticeable mobility.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use have to be performed according to recommendations of this *Terrestrial Manual* (chapter 1.1.9) or according to National regulations.

ii) Identity

See Section C.2.1.2.

iv) Stability

Freeze-dried products are considered stable when stored at –20°C (i.e. several years).

Stability may be shorter when freeze dried products are stored at 4°C. This stability may vary for a number of reasons, such as the quality of the vials, the stoppers and the freeze-drying process. It remains the responsibility of the vaccine producer to prove that its product still has the requested titre at the expiry date.

To this end it is recommended that the residual moisture of the product does not exceed 3%.

v) Batch potency

NB: Potency tests are not performed routinely with production batches as there is no laboratory animal that would allow this test to be performed at low cost. Potency tests in cattle are also not performed because of the cost. Getting statistically significant protection rates would involve using at least 50 naïve animals.

Potency of the final product is ensured by using a master seed lot of well known origin for which the potency test has already been performed, by strictly following the production standard protocols (avoiding multiple passages) and by ensuring that the final titres are correct.

The minimum titre is 10^7 live mycoplasmas per vaccine dose, but higher titres are recommended because of the loss of titre between production plant and actual injection into animals. Titration is performed after reconstitution of the freeze-dried vaccine in the diluent recommended for vaccination and, preferably, with the diluent provided by the vaccine manufacturer. Titrations should be performed on at least three vials per batch. This titre must be evaluated with a titration technique that allows a precision of ±0.25 logs. A batch passes the test if three vials chosen randomly have titres of at least of 10^7 live mycoplasmas per vaccine dose. Manufacturers must ensure that their production processes are able to yield homogeneous batches with minimal variations from one vial to another. In this case, three vials are enough to assess the batch titre. Otherwise, sampling frames must be put in place to ensure that the sample number is sufficient to validly represent the vaccine batch. The manufacturer must also ensure that the minimum titre is retained until the expiry date if the product is kept at the correct temperature.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.2.1 and C.2.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

2.3.2. Safety requirements

i) Target and non-target animal safety

Safety has to be ascertained for target animals solely (i.e. the *Bos* genus).

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

T1/44 strains are known to induce post-vaccinal reactions in some animals vaccinated for the first time. Adequate warnings must be included in the leaflet describing this vaccine, including the recommended antibiotic treatment to be put in place. Local subcutaneous

reactions should not be considered as a hazard to naïve cattle as there is normally no shedding, however, in the absence of reliable data, surveillance should be put in place in cattle herds where post-vaccinal reactions have been observed.

iii) Precautions (hazards)

CBPP vaccines based on T1/44 or T1sr strains and satisfying quality controls are harmless to humans.

2.3.3. Efficacy requirements

As a result of the very high cost of efficacy testing in cattle for CBPP only indirect assurance will be sought. Vaccine producers must ensure that each batch has been issued from a reference grand-parental stock of known origin and characteristics, that it has been produced according to good manufacturing practices and, more specifically, that the final product has not been obtained with procedures that may have induced a drift from the original master seed.

It has also to be reminded that complete protection of cattle herds are obtained only after repeated vaccinations.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable to actual CBPP vaccines.

2.3.5. Duration of immunity

Not required to actual CBPP vaccines.

2.3.6. Stability

As part of the approval procedure, the manufacturer should be required to demonstrate the stability of the vaccine's titre at the end of the claimed shelf-life period. Storage temperature shall be indicated for the freeze-dried product and also for the final product once reconstituted in the appropriate diluents. Warnings should be given if product is damaged by improper vaccine temperatures.

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NB: There are WOAH Reference Laboratories for contagious bovine pleuropneumonia (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for contagious bovine pleuropneumonia

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.4.9.

ENZOOTIC BOVINE LEUKOSIS

SUMMARY

Description and importance of the disease: *Enzootic bovine leukosis (EBL) is a disease of cattle caused by the bovine leukaemia virus (BLV), a member of the family* Retroviridae. *Cattle may be infected at any age, including the embryonic stage. Most infections are subclinical, but a proportion of cattle (~30%) over 3 years old develop persistent lymphocytosis, and a smaller proportion develop lymphosarcomas (tumours) in various internal organs. Natural infection has also been recorded in water buffaloes and capybaras. Clinical signs, if present, depend on the organs affected. Cattle with lymphosarcomas may die suddenly, or weeks or months after the onset of clinical signs dependent on the location and number of tumours and the tumour's growth characteristics.*

Identification of the agent: *Virus can be detected in the culture supernatant following* in-vitro *culture of peripheral blood mononuclear cells (PBMC) from infected animals, by BLV antigen detection, by polymerase chain reaction (PCR) or by electron microscopy. Proviral DNA can also be detected in PBMC or tumours of infected animals by PCR.*

Serological tests: *The antibody detection methods widely used are the agar gel immunodiffusion (AGID) assay using serum and the enzyme-linked immunosorbent assay (ELISA) using serum or milk. These tests have formed the basis for successful eradication policies in many countries. Other tests, such as radio-immunoassay, can also be used. A number of AGID and ELISA kits are commercially available.*

Requirements for vaccines: *No vaccine against BLV is available.*

A. INTRODUCTION

There may be several causes of lymposarcomas in cattle, but the only definitely known cause is the retrovirus, bovine leukaemia virus (BLV), which causes enzootic bovine leukosis (EBL). The term sporadic bovine leukosis (SBL) is usually reserved for young animals (calves) as well as cutaneous and thymic types of lymphoma, which is defined by the age of the animal affected and the distribution of the tumours. The cause of SBL is not known. There may also be lymphosarcomatous conditions that do not fall into either the SBL or EBL categories, i.e. adult multicentric lymphoma with sporadic occurrence of unknown aetiology. Only lymphomas caused by BLV infection should be termed leukosis or enzootic bovine leukosis (Gillet *et al.*, 2007).

Although animals can become infected with BLV at any age, tumours (lymphosarcomas) are seen typically in animals over 3 years of age. Infections are usually subclinical; only 30–70% of infected cattle develop persistent lymphocytosis, and 0.1–10% of the infected animals develop tumours. Clinical signs will depend on the site of the tumours and may include digestive disturbances, inappetence, weight loss, weakness or general debility and sometimes neurological manifestations. Superficial lymph nodes may be obviously enlarged and may be palpable under the skin and by rectal examination. At necropsy, lymph nodes and a wide range of tissues are found to be infiltrated by neoplastic cells. Organs most frequently involved are the abomasum, right auricle of the heart, spleen, intestine, liver, kidney, omasum, lung, and uterus. The susceptibility of cattle to persistent lymphocytosis, and perhaps also to tumour development, is genetically determined.

There is growing evidence of the role of the virus in immunological dysfunctions and in increased culling rates. Two large-scale investigations estimated the mean decline in milk production per cow among test-positive BLV herds compared with test-negative herds as very similar at 2.5% and 2.7%, respectively (Emanuelsson *et al.*, 1992; Ott *et al.*, 2003). Such findings have been again confirmed in recent studies (Nekouei *et al.*, 2016; Norby *et al.*, 2016). In addition, a 7% lower conception rate in BLV test-positive cows compared with test-negative cows has

been reported. Increased culling rates and a greater susceptibility to other diseases with infectious aetiology, e.g. mastitis, diarrhoea and pneumonia were also demonstrated among test-positive BLV herds (Emanuelsson *et al.*, 1992). Reduced protective immunity following vaccination in BLV infected cattle has also been reported (Frie *et al.,* 2016; Puentes *et al.,* 2016). Therefore despite no obvious clinical signs during the long subclinical infection period, economic losses caused by persistent BLV infections are relevant.

Virus can be detected by *in-vitro* cultivation of peripheral blood mononuclear cells (PBMC). The virus is present in PBMC and in tumour cells as provirus integrates into the DNA of infected cells. Virus is also found in the cellular fraction of various body fluids (nasal and bronchial fluids, saliva, milk). Natural transmission depends on the transfer of infected cells, for example during parturition. Artificial transmission occurs, e.g. by blood-contaminated needles, surgical equipment, gloves used for rectal examinations. Lateral transmission in the absence of these contributory factors is usually slow (Monti *et al.*, 2005). In regions where blood-sucking insects occur in large numbers, especially tabanids, these may transmit the virus mechanically. Viral antigens and proviral DNA can be identified in semen, milk and colostrum of infected animals (Dus Santos *et al.*, 2007; Romero *et al.*, 1983). Natural transmission through these secretions, however, has not clearly been demonstrated.

Although several species can be infected by inoculation of the virus, natural infection occurs only in cattle (*Bos taurus* and *Bos indicus*), water buffaloes, and capybaras. Sheep are very susceptible to experimental inoculation and develop tumours more often at a younger age than cattle. A persistent antibody response can also be detected after experimental infection in deer, rabbits, rats, guinea-pigs, cats, dogs, sheep, rhesus monkeys, chimpanzees, antelopes, pigs, goats and buffaloes.

BLV was probably present in Europe during the 19th century, from where it spread to the American continent in the first half of the 20th century. It may then have spread back into Europe and been introduced into other countries for the first time by the import of cattle from North America (Johnson & Kaneene, 1992). Despite the global BLV presence, a number of countries, particularly in Western Europe, are recognised as officially free from BLV infection due to continued surveillance programmes.

Several studies have been carried out in an attempt to determine whether BLV causes disease in humans, especially through the consumption of milk from infected cows (Burmeister *et al*., 2007; Perzova *et al*., 2000). There was speculation about the involvement in BLV in human breast cancers (Buehring *et al.,* 2015), however such findings were not confirmed by other researchers (Gillet & Willems, 2016; Zhang *et al.,* 2016). Hence with no conclusive evidence of zoonotic transmission, it is now generally thought that BLV is not a hazard to humans.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of enzootic bovine leukosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	–	+	–	+	–	–
PCR	+	++	+	++	+	–
Detection of immune response						
AGID	+++	+++	+++	+++	+++	–
ELISA	+++	+++	+++	+++	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; ELISA = enzyme-linked immunosorbent assay.
[(a)] A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

BLV is an exogenous retrovirus and belongs to the genus *Deltaretrovirus* within the subfamily *Orthoretrovirinae* and the family *Retroviridae.* It is structurally and functionally related to the primate T-lymphotropic viruses 1, 2 and 3 (STLV-1, -2, -3) and human T-lymphotropic virus[es] 1 and 2 (HTLV-1 and -2). The major target cells of BLV are B lymphocytes (Beyer *et al.*, 2002; Gillet *et al.*, 2007). The virus particle consists of two positive sense single-stranded RNA that encode for the nucleoprotein p12, capsid (core) protein p24, transmembrane glycoprotein gp30, envelope glycoprotein gp51, and several enzymes, including the reverse transcriptase. Proviral DNA, which is generated after reverse transcription of the viral genome, integrates randomly into the DNA of the host cell where it persists without constant production of viral progeny. When infected cells are cultured *in-vitro*, usually by co-cultivation of PBMC with indicator cells, infectious virus is produced, most readily through stimulation with mitogens.

1.1. Virus isolation

PBMC from 1.5 ml of peripheral blood in ethylene diamine tetra-acetic acid (EDTA) are separated on a ficoll/sodium metrizoate density gradient, cultured with 2×10^6 fetal bovine lung (FBL) cells, and subsequently grown for 3–4 days in 40 ml of minimal essential medium (MEM) containing 20% fetal calf serum. Virus causes syncytia to develop in the cell monolayer of the FBL cells. Short-term cultures can be prepared by culturing PBMC in the absence of FBL cells in 24-well plates for 3 days (Miller *et al.,* 1985). The p24 and gp51 antigens can subsequently be detected in the supernatant of the cultures by radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot or agar gel immunodiffusion (AGID), and the presence of the BLV particles or BLV-provirus can be demonstrated by electron microscopy and by PCR, respectively.

1.2. Nucleic acid detection by polymerase chain reaction (PCR)

The use of the polymerase chain reaction (PCR) to detect BLV provirus has been described by various workers (Fechner *et al.*, 1996; Rola-Łuszczak *et al.,* 2013). Primers constructed to match the *gag, pol* and *env* regions of the genome have all been used with variable success. So far, real-time PCR is the most rapid and sensitive method. The methods described are conventional PCRs based on primer sequences from the *env* gene, coding for gp51 and a real-time method based on detection of the pol gene. The technique is restricted to those laboratories that have the facilities for molecular virology, and the usual precautions and control procedures must be in place to ensure validity of the test results (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

PCR is mainly used as an adjunct to serology for confirmatory testing. The detection of BLV infection in individual animals by PCR can be useful in the following circumstances:

i) Young calves with colostral antibodies;

ii) Tumour cases, for differentiation between sporadic and infectious lymphoma;

iii) Tumour tissue from suspected cases collected at slaughterhouses;

iv) New infections, before development of antibodies to BLV;

v) Cases of weak positive or uncertain results in ELISA;

vi) The systematic screening of cattle in progeny-testing stations (before introduction into artificial insemination centres);

vii) Cattle used for production of vaccines, ensuring that they are BLV free.

1.2.1. Sensitivity and reliability of the method

i) *Analytical sensitivity*

Although the PCR assay has a theoretical sensitivity of one target molecule, in practice the analytical sensitivity may be approximately five to ten target molecules of proviral DNA.

ii) *False-positive results*

The high sensitivity of the nested PCR may cause problems of false-positive results due to contamination between samples (Belak & Ballagi-Pordany, 1993). To minimise this, several special procedures are adopted throughout the protocol, such as the use of laminar air-flow hoods, separate rooms for different steps of the procedure, new gloves or the use of special tube openers for each individual assay and negative controls (e.g. water blanks).

iii) *False-negative results*

It should be noted that only a small proportion of the PBMC can be infected, thus limiting the sensitivity of the assay. The presence of inhibitory substances in some samples may cause false-negative results. To detect this, at least one positive control is used on every test run. In addition, assays can use internal controls (mimics) that are added to each sample. The mimic is a modified target molecule that is amplified with the same primers as the real target, but that generates a PCR product with different size, which can be visualised by agarose gel electrophoresis. The mimic is added at a low concentration that favours the amplification of the real target (Ballagi-Pordany & Belak, 1996). However, it is possible for the mimic to compete with the true target. It may therefore be necessary to analyse each sample with or without the mimic.

1.2.2. Sample preparation

PBMC are separated from EDTA blood samples by using a density separation centrifugation method. Alternatively buffy coat may be used, or even whole blood, e.g. where samples have been frozen.

Tumours or other tissues should be homogenised to a 10% suspension.

1.2.3. DNA extraction

Purification of total DNA is a prerequisite for achieving optimal sensitivity. Various purification methods are commercially available and suitable for the assay.

Special precautions should be taken during all steps to minimise the risk of contamination (Belak & Ballagi-Pordany, 1993).

1.2.4. Nested PCR procedure

Several PCR protocols for the detection of BLV provirus sequences have been published; as an example, an assay developed by Fechner *et al.,* 1996 is described in detail. The BLV region used as target is the env gene, encoding for gp51 protein. The sequence used for designing the primers is available from GenBank, accession No. K02120.

1.2.4.1. Method developed by Fechner *et al.* (1996)

i) Primer design and sequences

Oligo	Env-Sequence (5'–3')	Position
BLV-env-1	TCT-GTG-CCA-AGT-CTC-CCA-GAT-A	5032–5053
BLV-env-2	AAC-AAC-AAC-CTC-TGG-GAA-GGG	5629–5608
BLV-env-3	CCC-ACA-AGG-GCG-GCG-CCG-GTT-T	5099–5121
BLV-env-4	GCG-AGG-CCG-GGT-CCA-GAG-CTG-G	5542–5521

The BLV-env-1/BLV-env-2 PCR-product size is 598 bp. The BLV-env-3/BLV-env-4 PCR-product size is 444 bp

ii) Reaction mixtures

Reaction solutions are mixed (except DNA sample) before adding to the separate reaction tubes. One negative control (double distilled H_2O) per five samples, and one positive control should be added. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one.

The first PCR can be performed using a 50 µl reaction volume. For one reaction, the assay is optimised to 5 µl (10×) PCR buffer, 20 µl DNA (~1 µg of DNA), 1.25 µl each of the env-specific primers BLV-env-1 and BLV-env-2 (20 pmol/µl), 0.15 dNTP (each 25 mM), 3 µl $MgCl_2$ (25 mM), 0.25 µl Taq polymerase (1.25U), and 19.1 µl of distilled H_2O. The reaction follows the temperature profile: 2 minutes denaturation at 94°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 60 seconds at 72°C; followed by 4 minutes at 72°C.

The nested PCR can be performed using a 50 µl reaction volume. For one reaction, the assay is optimised to 3 µl PCR product of the first PCR, 5 µl (10×) PCR buffer, 1.25 µl each of the env-specific primers BLV-env-3 and BLV-env-4 (20 pmol/µl), 0.15 dNTP (each 25 mM), 0.25 µl Taq polymerase (1.25U), and 36.1 µl of distilled H_2O. The reaction follows the temperature profile: 2 minutes denaturation at 94°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 60 seconds at 72°C; followed by 4 minutes at 72°C.

iii) Laboratory procedure

Mix PCR-reagents for the first or nested PCR and use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Heat the thermoblock to 94°C. Place samples in the thermoblock and start the PCR-programmes accordingly.

iv) Agarose gel electrophoresis

Load approximately 10 µl of the nested PCR products with 20 µl loading buffer on a 2% agarose gel containing 0.01% ethidiumbromide (or alternative, safer stains for visualising PCR products). Using 0.5 × Tris/borate/EDTA (TBE) buffer, electrophoresis is performed with 90 mA for 2 hours. To control the size of the amplification products, a 100 bp ladder is recommended. Analysis of PCR products is done by UV illumination.

v) Interpretation of the results

a) Positive samples should have PCR products of the expected size (444 bp), similar to the positive control.

b) Negative samples should have no PCR products of the expected size (444 bp).

c) The assay must be repeated if the positive control remained negative, or if the negative water controls are positive.

vi) Confirmatory testing

For confirmatory identification, the PCR products can be sequenced, hybridised to a probe, or analysed by restriction fragment length polymorphism (RFLP) analysis (Fechner *et al.*, 1997).

1.2.5. Real-time PCR procedure

Several real-time PCR protocols for the detection of BLV provirus sequences have been published: the Rola-Łuszczak *et al.* (2013) method is described in detail here as an example. The BLV region used as target is the pol gene. The sequence used for designing the primers is available from GenBank, accession No. K02120.

1.2.5.1. Method developed by Rola-Łuszczak *et al.* (2013)

i) Primer design and sequences

Oligo	Pol-Sequence (5'–3')	Position
MRBLVL	CCT-CAA-TTC-CCT-TTA-AAC-TA	2321-2340
MRBLVR	GTA-CCG-GGA-AGA-CTG-GAT-TA	2421-2440
MRBLV probe	6FAM GAA-CGC-CTC-CAG-GCC-CTT-CA BHQ1	2341-2360

PCR-product size: 120 bp

ii) Reaction mixtures

Reaction solutions are mixed (except DNA sample) before adding to the separate reaction tubes. One negative control (double-distilled H_2O) per five samples and one positive control should be added. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one.

The reaction mixture for each PCR test contains 12.5 µl of 2 × PCR master mix, 0.4 µM of each of the primers and 0.2 µM of the specific BLV probe and 500 ng of extracted genomic DNA, using a final reaction volume of 25 µl. Amplification was performed according to the following conditions: initial incubation and polymerase activation at 95°C for 15 minutes, denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds through 50 cycles.

iii) Laboratory procedure

Mix PCR-reagents and use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Place samples in the thermoblock and run at appropriate parameters.

iv) Interpretation of the results

a) Positive samples are those with Ct less than or equal to 40.95.

b) Negative samples are those without a Ct value or those with a value greater than 40.96.

c) The assay must be repeated if the positive control remained negative, or if the negative water controls are positive. Samples on the borderline of cut-off (i.e. Ct of 40) should be retested and confirmed.

v) Confirmatory testing

For confirmatory identification, the PCR products can be sequenced.

2. Serological tests

Infection with the virus in cattle is lifelong and gives rise to a persistent antibody response. Antibodies can first be detected 3–16 weeks after infection. Maternally derived antibodies may take up to 6 or 7 months to disappear. There is no way of distinguishing passively transferred antibodies from those resulting from active infection. Active infection, however, can be confirmed by the detection of BLV provirus by the PCR. Passive antibody tends to protect calves against infection. During the periparturient period, cows may have serum antibody that is undetectable by AGID because of an antibody shift from the dam's circulation to her colostrum. Therefore, when using the AGID test, a negative test result on serum taken at this time (2–6 weeks pre- and 1–2 weeks post-partum) is not conclusive and the test should be repeated. However, the AGID can be performed at this stage with first-phase colostrum.

The antibodies most readily detected are those directed towards the gp51 and p24 of the virus. Most AGID tests and ELISAs in routine use detect antibodies to the glycoprotein gp51, as these appear earlier. Methods of performing these tests have been published (Dimmock *et al.,* 1987; European Commission, 2009). ELISAs are usually more sensitive than the AGID tests.

Weak positive and negative WOAH reference sera for use in ELISA are available in freeze-dried, irradiated form from the WOAH Reference Laboratory in Germany[1]. The calibration of these sera is based on the accredited WOAH reference serum, named E05, which has been validated against the former reference serum E4 by different AGID and ELISAs.

2.1. Enzyme-linked immunosorbent assay

Either an indirect or blocking ELISA may be used. Assays based on both of these are available commercially; different kits may be required for serum or milk samples. Some ELISAs are sufficiently sensitive to be used with pooled samples. ELISAs are carried out in solid-phase microplates. BLV antigen is used to coat the plates either directly or by the use of a capture polyclonal or monoclonal antibody (MAb). The antigen is prepared from the cell culture supernatant of persistently BLV-infected cell lines. Fetal lamb kidney (FLK) cells are most commonly used for commercial tests (Van der Maaten & Miller, 1976). Since 2004, a new BLV-producing cell line, PO714, which is free from other viral infections and contains a provirus of the Belgian subgroup, has been made available (Beier *et al.*, 2004). The antigen is used at a predetermined dilution (e.g. 1/10) in phosphate buffered saline. In kit form, the plates are sometimes purchased precoated. Some preservatives may be added to milk samples to prevent souring. Preserved samples will not usually deteriorate significantly if stored for up to 6 weeks at 4°C.

2.1.1. Blocking enzyme-linked immunosorbent assay – serum ELISA

The following method is suitable for antibody detection in single or pooled serum samples.

2.1.1.1. Test procedure

i) Coating the plate

All wells are coated with BLV antibody, prediluted in coating buffer (100 µl/well), the plate is sealed and incubated for 18 hours at 4°C. A wash cycle (standard wash) is performed, which is three washes filling wells to the top, with a 3-minute soak in between each wash, and then the plate is blotted. BLV antigen is added, prediluted in wash buffer (100 µl/well), the plate is sealed and incubated for 2 hours at 37°C. A standard wash cycle is performed.

ii) Preparation and addition of samples and controls

The positive and negative control sera are prediluted (1/2) in wash buffer and the solution is added to four wells per control (100 µl/well). For testing pooled samples, 80 sera may be bulked then diluted (1/2) using wash buffer and the solution is added to two wells (100 µl/well) per sample. Single samples should be diluted 1/100 using wash buffer and the solution added to two wells (100 µl/well) per sample. After plating out the samples, the plate is sealed and incubated for 18 hours at 4°C. A brief wash is performed by filling the wells and immediately emptying them.

iii) Preparation and addition of conjugates and substrate

Prediluted biotinylated antibody is added (100 µl/well) to all wells – predilute using wash buffer + 10% fetal calf serum – the plate is sealed and incubated on a rocking table for 1 hour at 37°C. A standard wash is performed as described earlier. The peroxidase-conjugated avidin is prediluted in wash buffer and the solution is added to all wells (100 µl/well). The plate is sealed and incubated on a rocking table for 30 minutes at 37°C. A standard wash is performed. 100 µl orthophenylamine diamine substrate is added to all wells, the plate is covered and left in the dark for 9 minutes. The reaction is stopped with 100 µl of 0.5 M sulphuric acid per well.

2.1.1.2. Reading and interpretation of results

The plate reader is blanked on air and the absorbance is read at 490 nm. For dual wavelength readers a reference filter between 620 nm and 650 nm is used. Results are read within 60 minutes after the addition of stop solution.

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

The absorbance of the negative control should be about 1.1 ± 0.4; if the absorbance is below 0.7, the colour development time in step iii above (preparation and addition of conjugates and substrate) should be increased. Conversely, the time should be shortened if the absorbance is above 1.5. The absorbance of the positive control should be less than the absorbance of the negative control × 0.25.

A sample is positive when the absorbance of each of the two test wells is identical with or less than the mean absorbance of the four negative wells × 0.5.

A sample is negative when the absorbance of each of the two test wells is identical with or higher than the mean absorbance of the four negative control wells × 0.65.

For samples giving values between the absorbance of the negative control × 0.5 and × 0.65, it is recommended to retest the animal, using a sample taken 1 month later.

2.1.1.3. Sensitivity of the enzyme-linked immunosorbent assay

The sensitivity of pooled milk ELISAs can be evaluated using the WOAH weak positive and reference sera. Assays should give a positive result on WOAH reference sera E05 diluted in negative milk 250 times more than the number of individual milks in the pool (EU Directive 88/406). For example, for pools of 60 milks, E05 should be diluted 1/250 × 60 = 1/15,000. For individual milk samples the positive WOAH reference sera E05 diluted 1/250 in negative milk must be positive.

Where pooled serum samples are tested, the WOAH reference serum E05 must test positive at a dilution 10 times higher than the number of individual animals in the pool. For example, for a pool of 50 individual samples, the WOAH reference serum diluted 1/500 in negative serum should give a positive result. In assays where serum samples are tested individually, WOAH reference serum E05 diluted 1/10 must be positive.

For some ELISA kits, a positive result is not recommended as the sole determinant of individual animal disease status; verification by a secondary method is recommended.

2.1.2. Indirect enzyme-linked immunosorbent assay – Milk ELISA

The following method is suitable for antibody detection in pooled milk samples.

2.1.2.1. Controls

Strong positive, weak positive, negative milk and diluent controls should be included in each assay. A strong positive control should be prepared by diluting the WOAH reference serum E05 1/25 in negative milk. A weak positive control should be prepared by diluting, in negative milk, the WOAH reference serum E05 25 times the number of individual milk samples in the pool under test. The milk used for diluting the WOAH reference serum controls should be unpasteurised, cream free and preserved.

2.1.2.2. Example test procedure

i) Milk samples must be stored, undisturbed in a refrigerator until a definite cream layer has formed (24–48 hours), or alternatively, centrifuged at 2000 rpm for 10 minutes, the cream layer should be removed prior to testing.

ii) A BLV antigen and a control negative antigen are precoated in alternate columns in the plate. 100 µl of test sample is added to 100 µl wash buffer in the plate to make a 1/2 dilution, adding to two control antigen wells and two BLV antigen wells.

iii) The plate is sealed and mixed on a shaker.

iv) The plate is incubated between 14 and 18 hours at 2–8°C.

v) 300 µl per well of wash diluent is added and discarded, and then 200 µl per well wash diluent is added, shaken for 10 seconds and discarded. Finally, 300 µl of wash diluent is added and soaked for 3 minutes and discarded.

vi) 200 µl per well of anti-bovine IgG-horseradish peroxidase affinity-purified conjugate diluted in wash diluent is added and the plate is incubated for 90 minutes at room temperature.

vii) The plate is washed by adding 300 µl of wash diluent per well; this is then discarded and a further 300 µl of wash diluent is added. This is left to soak for 3 minutes and discarded. Steps vi and vii are repeated.

viii) 200 µl of ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) substrate (prewarmed to 25°C) is added and the plate is incubated for 20 minutes at room temperature in the dark. The reaction may be stopped by adding 50 µl of stopping solution.

2.1.2.3. Reading and interpreting the results

The plate reader is blanked on air and the absorbance is read at 405 nm. All microplate wells must be read within 2 hours of addition of stopper. The absorbance readings of the wells containing negative antigen are subtracted from the readings of wells containing the positive antigen. The two net absorbance values for each test sample should be averaged. The same applies for the replicate weak positive controls. Replicates should be within 0.1 absorbance units of each other.

For the test to be considered valid, the averaged net absorbance of the weak positive (WP) controls should be 0.2–0.6 absorbance units. The net absorbance of the strong positive control should be >1.0 absorbance units. The net absorbance of the negative and diluent controls should be less than the lower limit of the inconclusive range.

Assuming that the above criteria are met:

i) Test samples are positive if their net absorbance value is greater than or equal to that of the WP control.

ii) Test samples are inconclusive if their net absorbance value is 75% or less of the net absorbance value of the WP control.

i.e. if the WP control net absorbance = 0.40

then the lower limit of the inconclusive range = 0.40 × 0.750 = 0.30

the inconclusive range in this example would be 0.30–0.39

and samples of ≥0.40 are considered positive.

iii) Test samples are negative if their net absorbance value is less than the lower limit of the 'inconclusive' range (<0.30 in the example).

2.2. Agar gel immunodiffusion

The AGID test is a specific, but not very sensitive, test for detecting antibody in serum samples from individual animals. It is, however, unsuitable for milk samples (except first colostrums) because of lack of specificity and sensitivity. The AGID is simple and easy to perform and has proven to be highly useful and efficient as a basis for eradication schemes. Reference sera are included with commercial AGID test kits.

2.2.1. Agar gel

A 0.8–1.2% solution of agar or agarose is prepared in 0.2 M Tris buffer, pH 7.2, with 8.5% NaCl. One method of preparing the agar is to dissolve 24.23 g of Tris methylamine in 1 litre of distilled water and adjust to pH 7.2 with 2.5 M HCl. Sodium chloride (85 g) is dissolved in 250 ml Tris/HCl and made up to 1 litre. Agarose (8 g) is added and the mixture is heated in a pressure cooker or autoclave at 4.55 kg/sq. cm for 10 minutes. The mixture is dispensed in 15 ml aliquots, which can be stored at 4°C for up to approximately 6 weeks.

2.2.2. Antigen

The antigen must contain specific glycoprotein gp51 of BLV. Antigen is prepared in a suitable cell culture system, such as permanently infected FLK cell monolayers. The cells used to produce the BLV antigen should be free from noncytopathic bovine viral diarrhoea virus and of bovine retroviruses, bovine immunodeficiency-like virus (lentivirus), and bovine syncytial virus (spumavirus). After 3–4 days' culture at 37°C, the growth medium is replaced with maintenance medium. The cells are harvested after 7 days using standard trypsin/versene solution. The cell suspension is centrifuged at 500 ***g*** for 10 minutes. Cells are resuspended in growth medium; 30% of the cells are returned to the culture vessel and the remainder is discarded. All culture supernatants are collected. The supernatants are concentrated 50–100-fold by available methods. This can be done by concentration in Visking tubing immersed in polyethylene glycol, or by precipitation with ammonium sulphate followed by ultrafiltration, or by precipitation in polyethylene glycol followed by desalting and size separation on a polyacrylamide bead column. The antigen contains gp51 predominantly, but may also contain p24.

The antigen may be standardised for glycoprotein gp51 by titration against the WOAH reference serum E05 as follows: a twofold dilution of the antigen preparation is made. The highest dilution that, when tested against undiluted WOAH reference serum E05, gives a precipitation line equidistant between the antigen and the serum will contain one unit. Two units of antigen are used in the test.

2.2.3. Known positive control serum

The positive control serum comes from a naturally or experimentally infected animal (cattle or sheep). The precipitation line formed should be a sharp distinct line midway between the antigen and the control serum wells. A dilution of the control positive serum that gives a weak positive result should be included in the test as an indicator of the test's sensitivity.

2.2.4. Known negative control serum

Serum from uninfected animals (cattle, sheep) is used.

2.2.5. Test sera

Sera from any species of animal are suitable.

2.2.6. Test procedure

i) The agar is melted by heating in a water bath and poured into Petri dishes (15 ml per Petri dish of diameter 8.5 cm). The poured plates are allowed to cool at 4°C for about 1 hour before holes are cut in the agar. A punch is used that cuts a hexagonal arrangement of six wells round a central well. Various dimensions of wells can be used; one satisfactory pattern has been produced using wells of 6.5 mm in diameter with 3 mm between wells. For best results, agar plates are used the same day that they are poured and cut.

ii) Antigen is placed in the central wells of the hexagonally arranged patterns. Test sera are placed alternately with positive control serum in the outer wells. There should be one control pattern per plate with positive control serum, weak positive control serum and negative control serum in the place of test sera.

iii) The test plates are kept at room temperature (20–27°C) in a closed humid chamber, and read at 24, 48 and 72 hours.

iv) *Interpretation of the results:* A test serum is positive if it forms a specific precipitation line with the antigen and forms a line of identity with the control serum. A test serum is negative if it does not form a specific line with the antigen and if it does not bend the line of the control serum. Nonspecific lines may occur; these do not merge with or deflect the lines formed by the positive control. A test serum is a weak positive if it bends the line of the control serum towards the antigen well without forming a visible precipitation line with the antigen; the reaction is inconclusive if it cannot be read either as negative or positive. A test is invalid if the controls do not give the expected results. Sera giving inconclusive or weak positive results can be concentrated and retested.

C. REQUIREMENTS FOR VACCINES

Despite advances in research on experimental vaccines, there is as yet no commercially available vaccine for the control of EBL.

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NB: There are WOAH Reference Laboratories for enzootic bovine leukosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratory for any further information on
diagnostic tests and reagents for enzootic bovine leukosis

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.4.10.

HAEMORRHAGIC SEPTICAEMIA (*PASTEURELLA MULTOCIDA* SEROTYPES 6:B AND 6:E)

SUMMARY

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes characterised by an acute, highly fatal septicaemia with high morbidity and mortality. It is caused by certain serotypes of Pasteurella multocida *that are geographically restricted to some areas of Asia, Africa, the Middle East and southern Europe.*

The diagnosis of HS depends on the isolation of the causative organism, P. multocida*, generally from the blood or bone marrow of a dead animal, by cultural and biological methods, and the identification of the organism by biochemical, serological and molecular methods.*

Isolation and detection of the agent: *Pure cultures of* P. multocida *can be obtained by streaking materials on to artificial media and the subsequent identification on the basis of the morphological, cultural, and biochemical characteristics of* P. multocida*.*

Conventionally, the identification of the specific serotype is carried out using one or more serological methods. These include rapid slide agglutination, indirect haemagglutination for 'capsular' typing using sheep red blood cells coated with bacterial extracts, 'somatic' typing by agar gel immunodiffusion tests using heat-treated cell extracts, or agglutination using acid-treated cells. Confirmation of the isolates can be made using molecular techniques.

Serology: *Serological tests for detecting specific antibodies are not normally used for diagnostic purposes.*

Requirements for vaccines: *Effective vaccines against haemorrhagic septicaemia are formalin-killed bacterins, or dense bacterins with adjuvants. The latter enhance the level and prolong the duration of immunity.*

Seed cultures for the production of vaccines should contain capsulated organisms. Vaccines are standardised as to their bacterial density on the basis of turbidity tests and dry bacterial weight. Potency tests are carried out in mice and/or rabbits.

A. INTRODUCTION

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity (Bain *et al.*, 1982; Carter & De Alwis, 1989; De Alwis, 1992; Mustafa *et al.*, 1978; Shivachandra *et al.,* 2011; Singh *et al.*, 1996). The disease has been recorded in wild mammals in several Asian and European countries (Carigan *et al.*, 1991; Rosen, 1981). In many Asian countries disease outbreaks mostly occur during the climatic conditions typical of monsoon (high humidity and high temperatures). The disease is caused by *Pasteurella multocida*, a Gram-negative coccobacillus residing mostly as a commensal in the nasopharynx of animals. The Asian serotype B:2 and the African serotype E:2 (Carter and Heddleston system), corresponding to 6:B and 6:E (Namioka-Carter system), are mainly responsible for the disease. In wild ruminants, serotype B:2,5 is predominantly present while serotype B:3,4 also has been reported from fallow deer (Aalbæk *et al.*, 2009). The association of other serotypes, namely A:1, A:3 with a HS-like condition in cattle and buffaloes in India has been recorded (Kumar *et al.*, 1996; 2004). The geographical distribution of HS includes some areas of Asia, Africa, the Middle East and southern Europe. It has never been confirmed in Mexico, Central or South America.

Clinically, HS caused by B:2 or E:2 strains is typified by fever, respiratory distress with nasal discharge, and frothing from the mouth, leading eventually to recumbency and death. Infection with serotypes A:1 and A:3 predominantly involves pneumonia and death. Septicaemia is the main characteristic feature in all forms of the disease. The incubation period varies from 3 to 5 days. In peracute cases, sudden death without clinical signs may be observed (Carter & De Alwis, 1989; De Alwis, 1992). Water buffaloes are generally more susceptible to HS than cattle and show more severe forms of disease with profound clinical signs. Severe subcutaneous oedema of the mandible, neck and brisket is a distinctive feature of the disease. In endemic areas mortality is largely confined to older calves and young adults.

At *post mortem,* most animals succumbing to HS typically show marked swelling of the neck caused by severe blood-tinged oedema. There are also abundant petechial haemorrhages in many tissues and organs, particularly in serosal membranes. The thoracic, pericardial and abdominal cavities may contain sero-sanguinolent fluid. The lungs are notably congested and oedematous, and foam is generally present in the nasal cavity, trachea and bronchi. Microscopically, there is interstitial pneumonia and pulmonary oedema as well as focal infiltrates of neutrophils and macrophages in many tissues. All these lesions are similar to those observed in severe sepsis and septic shock.

Massive epizootics may occur in endemic as well as non-endemic areas (Carter & De Alwis, 1989; De Alwis, 1992). HS has been identified as a secondary complication in cattle and water buffaloes following outbreaks of foot and mouth disease (FMD). Case fatality approaches 100% if treatment is not followed at the initial stage of infection (Carter & De Alwis, 1989; De Alwis, 1992). Appropriate antibiotics need to be selected following antimicrobial susceptibility tests for effective reduction of case fatality as multidrug resistant *P. multocida* strains are emerging (Shivachandra *et al.,* 2011).

The diagnosis of the disease is based on the clinical signs, gross lesions, morbidity and mortality patterns. Confirmation requires the isolation and characterisation of the pathogen using conventional and molecular techniques. There are no confirmed reports of human infections with *P. multocida* B:2 and E:2; however, other serotypes do cause human infections and precautions should be taken to avoid exposure. The organism should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Bacterial isolation	–	–	+	+++	+	–
Antigen detection	–	–	+	+++	+	–
Mouse pathogenicity test	–	–	+	+++	+	–
CIEP	–	–	+	+++	+	+
Rapid slide agglutination test	–	+	+	+++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
PCR	–	+	+	+++	+	–
Real-time PCR	–	+	+	+++	+	–
Detection of immune response						
IHA	–	+	+	++	+	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
CIEP = counter-immuno-electrophoresis; PCR = polymerase chain reaction; IHA = indirect haemagglutination.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Isolation and detection of the agent

1.1. Cultural and biochemical methods

True septicaemia in HS occurs at the terminal stage of the disease, therefore blood samples should be taken from sick animals immediately before death. Animals in the early stages of the disease may not contain *P. multocida* in blood. The bacteria are also not consistently present in the nasal secretions or body fluids of sick animals. Blood samples collected from ailing animals undergoing antibiotic treatment may not indicate the presence of bacteria due to their suppression in the circulation.

A blood sample or swab collected from the heart is satisfactory only if taken immediately after death. If the animal has been dead for a long time, bone marrow from a long bones (humerus, femur, tibia, etc.) can be used for bacterial isolation. If there is no facility for post-mortem examination, blood can be collected from the jugular vein by incision or aspiration. Blood samples in any standard transport medium should be dispatched on ice and well packed to avoid any leakage. If the blood samples are not transported to the laboratory within a few hours, they can be frozen. However repeated freezing and thawing may destroy the organism and is not advisable.

Blood smears from affected animals are stained with Gram, Leishman's or methylene blue stains. The organisms appear as Gram-negative, bipolar-staining short bacilli. No conclusive diagnosis can be made on the basis of direct microscopic examinations alone.

Blood samples, or swabs eluted into 2–3 ml sterile physiological saline, are cultured. Alternatively, the surface of a long bone is disinfected with alcohol and split open. The marrow is extracted aseptically and cultured. Direct culture is usually satisfactory only if the material is fresh and free from contaminants or post-mortem invaders that would otherwise overgrow any *Pasteurella* present.

For biological examinations, a small volume (0.2 ml) of eluted nasal/blood swabs or a portion of tissues/bone marrow in saline is inoculated subcutaneously or intramuscularly into mice. The mouse usually serves as a biological 'screen' for extraneous organisms. If viable *P. multocida* is present, the mice die 24–36 hours following inoculation, and a pure growth of *P. multocida* can be seen in blood smears. Pure cultures of *P. multocida* can usually be grown from blood (preferably from heart) of the mice, even when the original samples (nasal swab and tissue samples) come from relatively old carcasses. The organism can be identified by its morphological and cultural characteristics, biochemical reactions and serological tests. The mouse inoculation method may be used as a last resort in isolation of bacterial agent, particularly from heavily contaminated nasal/tissue materials.

A suitable bacteriological medium for *Pasteurella* is casein/sucrose/yeast (CSY) agar containing 5% blood. The composition of this medium is casein hydrolysate (3 g), sucrose (3 g), yeast extract (5 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (3 g), and distilled water to 1 litre. The pH is adjusted to 7.3–7.4, after which 1.5% agar is added. The medium is autoclaved at 1 bar for

15 minutes. After cooling to 45–50°C, 5% calf blood (antibody-free *P. multocida*) is added (Wijewardana *et al.*, 1986). Conventional blood agar may also be used.

Freshly isolated *P. multocida* forms smooth, greyish glistening translucent colonies, approximately 1 mm in diameter, on blood agar after 24 hours' incubation at 37°C. Colonies grown on CSY agar are larger. Old cultures, particularly those grown on media devoid of blood, may produce smaller colonies. *Pasteurella multocida* does not grow on MacConkey agar. Gram-stained blood or tissue smears show Gram-negative, short, ovoid, bipolar-staining coccobacilli. A degree of pleomorphism will be noted, particularly in old cultures, with longer rods of varying length. The bipolar staining will be more evident with methylene blue or Leishman's stain.

HS organisms produce oxidase, catalase and indole, and will reduce nitrates. They do not produce hydrogen sulphide or urease, and fail to use citrate or liquefy gelatin. Glucose and sucrose are always fermented with the production of acid only. Most strains also ferment sorbitol. Some strains ferment arabinose, xylose and maltose, whereas salicin and lactose are almost invariably not fermented.

One property of HS-causing strains of *P. multocida* is the ability to produce the enzyme hyaluronidase (Carter & Chengappa, 1980). Having identified the genus and species by cultural characteristics and biochemical tests, hyaluronidase production may then be used as a specific test for HS-causing pasteurellae. It should be noted that B serotypes other than B:2 (or 6:B), and type E, are hyaluronidase negative.

A hyaluronic-acid-producing culture is streaked across the centre of a dextrose starch agar plate. The *Pasteurella* culture to be tested for hyaluronidase production is streaked at right angles. The plates are incubated at 37°C for 18 hours. Originally, hyaluronic-acid-producing *Streptococcus equi* was used, but a convenient culture for this purpose is a capsulated mucoid *P. multocida* type A culture. At the point of intersection, the mucoid growth of the hyaluronic acid producer will diminish into a thin line of growth, indicating the production of hyaluronidase by the test culture. Use of freshly prepared plates and a humidified incubator will facilitate hyaluronic acid production and, thereby, the interpretation of the test.

1.2. Serotyping methods

Several serotyping tests are used for the identification of the HS-causing serotypes of *P. multocida*. These consist of a rapid slide agglutination test (Namioka & Murata, 1961a), an indirect haemagglutination (IHA) test for capsular typing (Carter, 1955), an agglutination test using hydrochloric-acid-treated cells for somatic typing (Namioka & Murata, 1961b), the agar gel immunodiffusion (AGID) test (Anon, 1981; Heddleston *et al.*, 1972; Wijewardana *et al.*, 1982), and the counter immunoelectrophoresis test (CIEP) (Carter & Chengappa, 1981).

Hyperimmune antisera for most of these tests are prepared against specific reference strains in rabbits. Cultures in CSY broth (6–8-hours old) are seeded on to CSY blood agar medium. After overnight incubation (18–20 hours) the growth is washed into physiological saline containing 0.3% formalin. The turbidity of the cell suspension is adjusted to that of MacFarland's tube No. 4. Rabbits are inoculated intravenously at 3–4-day intervals with 0.2, 0.5, 1.0, 1.5 and finally, 2.0 ml of this suspension. The rabbits are inoculated subcutaneously or intramuscularly 1 week after the last injection with 0.5 ml of a similar, but live, suspension. The animals are bled 10 days later. The serum is stored at –20°C, but small quantities for regular use are stored at 4°C with the addition of 1/10,000 merthiolate.

1.2.1. Rapid slide agglutination test (capsular typing)

A single colony is mixed with a drop of saline on a slide, a drop of antiserum is added, and the slide is warmed gently. A coarse, floccular agglutination appears within 30 seconds. Old cultures may give a fine, granular agglutination that takes longer to appear.

1.2.2. Indirect haemagglutination test (capsular typing)

This was originally performed using antigen-sensitised human type 'O' red blood cells (RBCs) (Carter, 1955), but more recently sheep RBCs have been used (Sawada *et al.*, 1985; Wijewardana *et al.*, 1986). The antigen is prepared as follows:

A 6–8-hour broth culture of a reference strain is seeded on to CSY blood agar plates and incubated overnight at 37°C. The growth is harvested in 3 ml physiological saline containing 0.3% formalin. This suspension is then heated at 56°C for 30 minutes, centrifuged at 3000 ***g*** for 15 minutes at 4°C, and the clear supernatant fluid is stored at –20°C. If a refrigerated centrifuge is not available, centrifugation at 1500 ***g*** for 30 minutes gives a supernatant fluid. This is used as the antigen extract. A similar procedure is followed for preparing an antigen extract from an unknown strain that is to be typed.

Sheep blood is collected aseptically into an anticoagulant and centrifuged at 500 ***g*** for 10 minutes. The packed RBCs are washed three times in sterile physiological saline. The antigen extract from an unknown strain prepared by the method described above is used to sensitise the RBCs or absorbed on to the RBCs. This is done by adding 15 volumes of the antigen extract to the RBCs and incubating the mixture for 1 hour at 37°C with frequent shaking. The sensitised RBCs are recovered by centrifugation, washed three times in sterile physiological saline, and made up to a final 1% suspension in physiological saline. The type-specific hyperimmune antiserum (three volumes) is absorbed by the addition of packed RBCs (one volume) for 30 minutes at room temperature, and then centrifuged at 500 ***g*** for 10 minutes to pellet the RBCs. The absorbed antiserum is then inactivated by heating at 56°C for 30 minutes.

The test itself can be carried out in tubes or plates, and is performed in two rows. The test described below is for standard microtitre plates.

i) The capsular extract of the unknown strain is prepared as described above and used to sensitise the sheep RBCs. The known type-specific hyperimmune sera raised in rabbits against types A, B, D and E are diluted as follows:

ii) Using four separate rows of wells, the first wells are filled with 0.72 ml saline followed by 0.4 ml in the next six wells or more.

iii) The type-specific hyperimmune sera are each separately diluted in each row by adding 0.08 ml of the serum to the first well and mixing with a pipette. From this well 0.4 ml is transferred to the next well, mixed, and the process carried on until well seven. This constitutes 1/10 dilution in the first well and a doubling dilution thereafter.

iv) All the wells are each filled with 0.4 ml of antigen-adsorbed/sensitised RBCs, shaken slightly and left at room temperature. By the addition of the sensitised blood, the serum dilutions in the wells are doubled, i.e. 1/20 in well one, 1/40 in the second, and so on. A positive, negative and saline control are included in each test run.

v) The first reading is taken after 2 hours and a final reading after 18 hours. A course agglutination of the RBCs along the sides of the concave wells is taken as a positive reading, and the formation of a button at the centre of the wells as negative. An arbitrary score of 1–4 is given depending on the size of the agglutination. An unknown strain is identified with the hyperimmune serum that has agglutination. In the absence of agglutination with all sera, the strain is considered to be untypeable.

While IHA can be used for typing unknown strains, the test itself is more efficient when dealing with serotypes B and E and is more reliable as a quantitative test against these strains.

1.2.3. Agar gel immunodiffusion tests

AGID tests are used for what is described as 'capsular' as well as 'somatic' typing, depending on the antigens and antisera used. The double-diffusion technique is employed. Wells are punched in the solid agar in a circular pattern with one centre well surrounded by six peripheral wells.

i) *Capsular typing:* The gel medium is 1.0% Noble agar, or equivalent product, in 0.2 M phosphate buffer containing merthiolate at a final concentration of 1/10,000 (Anon, 1981; Wijewardena *et al.*, 1982). Antigens and antisera are the same as for capsular typing by the IHA method (Carter, 1955). The standard antiserum is placed in the centre well, and the test antigens are placed in the peripheral wells alternately with standard homologous antigen.

ii) *Somatic typing:* The gel medium consists of special Noble agar, or equivalent product, at a concentration of 0.9% in 0.85% sodium chloride solution.

iii) For antigen preparation, the growth from each plate is harvested in 1 ml of 8.5% sodium chloride containing 0.3% formalin. The suspension is heated at 100°C for 1 hour, the cells are sedimented by centrifugation, and the supernatant fluid is used as antigen.

iv) Antisera against 16 somatic types (Heddleston *et al.*, 1972) are prepared in chickens. Oil-emulsified bacterin[1] (1 ml) is injected subcutaneously into the mid-portion of the neck of 12–16-week-old male birds. A further injection is made 3 weeks later of 1 ml intramuscularly into the breast, 0.5 ml on each side of the sternum. The birds are bled 1 week later, and the serum is separated and preserved with 0.01% thiomersal and 0.06% phenol. Sera are tested against all somatic types and sera that cross-react are discarded. Some antisera preparations against B:2 may cross react with the somatic type 5.

v) The test antigen is placed in the centre well and antisera against the different serotypes are placed in the peripheral wells. All haemorrhagic septicaemia serotypes (Asian and African) will react with type 2 antiserum. Cross-reactions may occur with type 5.

1.2.4. Counter immunoelectrophoresis

CIEP offers a rapid method for the identification of capsular types B and E cultures.

i) *Preparation of capsular substance*

Capsular substance is prepared in the same manner as described for the IHA test.

ii) *Preparation of hyperimmune antisera*

Antisera are prepared in rabbits as for the IHA test.

iii) *Medium for CIEP*

The medium for the CIEP consists of agarose (2.0 g), barbitone sodium (2.06 g), diethyl barbituric acid (0.37 g), distilled water (180 ml), and 1/1000 merthiolate (20 ml).

iv) *Veronal acetate buffer* (*barbitone buffer*)

The barbitone buffer consists of barbitone sodium (29.24 g), anhydrous sodium acetate (11.70 g), 0.1 N hydrochloric acid (180 ml), and distilled water to 3 litres. The pH should be 8.8.

v) *Preparation of slides*

The electrophoresis plates are prepared by precoating glass slides (57 mm × 70 mm) with 12 ml volumes of the medium. Seven wells, 4 mm in diameter and 7 mm apart, are cut in a row. A parallel set of wells is cut 6 mm (centre to centre) away from the other set of wells.

vi) *Test procedure*

The well on the side of the cathode is loaded with a 20 µl volume of capsular antigen, while an equal volume of type-specific antiserum is loaded on to the well on the side of the anode. Controls included in the test are 0.85% sodium chloride solution against positive antiserum, and capsular extract against negative rabbit serum as well as positive and negative control samples. The electrophoresis tank is filled with barbitone buffer, pH 8.8. The antigen and antiserum are electrophoresed for 30 minutes at 150 V (25 V/cm). The plates are then examined for precipitation lines.

vii) *Interpretation of the results*

The presence of a distinct line between the antigen and antiserum wells is considered to be a positive result.

1 The bacterial antigens in broth are covered by a light mineral oil (adjuvant) and then emulsified (stabilised) with an emulsifying agent, in this case lanolin or lanoline (wool fat). This has to be done as the watery phase with the bacteria (broth) will not mix with the oily phase (adjuvant). The proportion of oil to emulsifying agent will vary with different batches of lanolin and will have to be adjusted accordingly. The higher the percentage of lanoline, the higher the stability of the emulsion.

1.2.5. Agglutination tests (somatic antigen)

The somatic 'O' antigen is prepared by a method similar to that described previously for the IHA test (Namioka, 1978; Namioka & Murata, 1961b). A 6–8-hour test culture is seeded on to CSY blood agar and incubated overnight. The growth is harvested in 2–3 ml of physiological saline containing 0.3% formalin per plate, and centrifuged at 3000 ***g*** for 15 minutes at 4°C (or 1200–1500 ***g*** for 30–45 minutes at room temperature). The deposited bacteria are resuspended in 25 ml normal HCl saline (0.85% saline in a normal HCl solution) to give an opacity approximately equivalent to Brown's opacity tube No. 6, and incubated overnight. The suspension is again centrifuged, the supernatant fluid is discarded, and the cell residue is washed three times successively in phosphate buffered saline (PBS) at pH 5.0, 6.0 and 7.0, respectively.

Finally, a suspension of the residual cells, equivalent to Brown's opacity tube No. 6, is prepared in PBS at pH 7.0. Any suspensions that autoagglutinate should be discarded.

Antisera are prepared against whole bacterial cell suspensions of the reference strains B:2 (Asian HS), E:2 (African HS) and 11:B (Australian 989, non-HS). Agglutination tests are carried out on a slide and the test antigen is used against the three types of sera. A fine granular agglutination indicates a specific somatic agglutination. Tests carried out against the standard antigens will facilitate reading and interpretation. When nonspecific partial agglutination occurs, the tests carried out with tenfold dilutions of the serum against the test and standard antigens will help to identify somatic antigen.

1.2.6. Serotype designation

Broadly, two typing systems are adopted. One is 'capsular' typing by Carter's IHA test (Carter, 1955) or by AGID tests (Anon, 1981; Wijewardena *et al.*, 1982). The other is 'somatic' typing by the method of Namioka & Murata (Namioka, 1978; Namioka & Murata, 1961b; 1961c), and by the method of Heddleston *et al.* (1972). It is generally agreed that designation of serotypes should be based on a somatic–capsular combination. Two systems commonly in use are the Namioka–Carter and the Carter–Heddleston systems. In the former system, Asian and African HS serotypes are designated 6:B and 6:E, respectively, while in the latter system they are designated B.2 and E.2, respectively.

1.3. Nucleic acid recognition methods

1.3.1. *Pasteurella multocida*-specific PCR assay

PCR technology can be applied for rapid, sensitive and specific and/or detection of *P. multocida* (Miflin & Blackall, 2001; Townsend *et al.*, 1998a). The rapidity and high specificity of two of the *P. multocida*-specific assays (Miflin & Blackall, 2001; Townsend *et al.*, 1998a) provide optimal efficiency without the need for additional hybridisation. The *P. multocida*-specific PCRs (Miflin & Blackall, 2001; Townsend *et al.*, 1998a) identify all subspecies of *P. multocida*. The Miflin & Blackall PCR (Miflin & Blackall, 2001) was described as giving a false positive with both *P. avium* biovar 2 and *P. canis* biovar 2, while the Townsend *et al.* PCR (1998a) gave a false positive with *P. canis* biovar 2 (it has not been tested against *P. avium* biovar 2). Recently, both P. avium biovar 2 and *P. canis* biovar 2 have been re-named as *P. multocida* (Christensen *et al.*, 2004) – meaning that both the Townsend *et al.* (1998a) and the Miflin & Blackall (Miflin & Blackall, 2001) PCR assays are now regarded as being specific for *P. multocida*. Some difficulties remain as it is now known that sucrose-negative *P. multocida*-like organisms from large cat bite wounds form two groups. While both are positive in the Miflin & Blackall *P. multocida*-specific PCR (2001) only one group has been confirmed as true *P. multocida* by other genotypic methods (Christensen *et al.*, 2005). The Townsend *et al.* (1998a) PCR is described in the following paragraph.

A fraction of an isolated colony of the suspect organism is transferred directly into the PCR mixture. Alternatively, template DNA can be obtained from 2 µl of either a mixed or pure broth culture. All currently used methods for the preparation of template DNA produce reproducible results with the KMT1 primers (Townsend *et al.*, 1998a), and allow detection of ≤10 organisms per reaction. The sensitivity and specificity of the *P. multocida*-specific PCR offer the most compelling argument for the use of PCR technology in laboratory investigation of suspected HS cases. *Pasteurella multocida* can be detected regardless of the purity of the specimen, an advantage if

the specimen is from an old carcass or from tonsil or nasal swabs. In such cases, the swab should be inoculated in 2 ml CSY broth and incubated on a roller for 2–4 hours; 2 µl of the culture is then added directly to the PCR mixture prior to amplification.

Primer sequences (Townsend *et al.*, 1998a):

P.-multocida-specific PCR:	KMT1T7	5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'
	KMT1SP6	5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

PCR conditions:

Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM $MgCl_2$, 3.2 pmol of each primer and 0.5 u *Taq* DNA polymerase. Thermocycler parameters are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; with a final extension at 72°C for 7 minutes. 5 µl of each sample is electrophoresed on a 2% agarose gel in 1 × Tris-acetate running buffer (TAE) at 4 V/cm for 1 hour. The gel is stained with 1% ethidium bromide and DNA fragments are viewed by UV transillumination.

The PCR amplification yields a product of 460 bp.

1.3.2. *Pasteurella multocida* multiplex capsular PCR typing system

Identification of the genes involved in the biosynthesis of the *P. multocida* A:1 (Chung *et al.*, 1998) and B:2 (Boyce *et al.*, 2000) polysaccharide capsules provided the required information to determine the biosynthetic region of the remaining three serogroups (D, E, and F) (Boyce *et al.*, 2000). Moreover, with the use of serogroup specific multiplex PCR, conflicting results as regards to typing of some strains could be confirmed (Townsend *et al.*, 2001). With this knowledge, serogroup-specific sequences were identified for use as primers in a multiplex capsular PCR-typing system (Townsend *et al.*, 2001). The *P.-multocida*-specific primers are included as an internal control for species identification. In the multiplex capsular PCR typing system, the amplicon band giving the typing result may be unclear. In such cases, removal of the *P. multocida*-specific primers (KMT1T7, KMT1SP6) from the mixture can improve the result.

Primer sequences (Townsend *et al.*, 2001):

Multiplex capsular PCR:	CAPA-FWD	5'-TGC-CAA-AAT-CGC-AGT-CAG-3'
	CAPA-REV	5'-TTG-CCA-TCA-TTG-TCA-GTG-3'
	CAPB-FWD	5'-CAT-TTA-TCC-AAG-CTC-CAC-C-3'
	CAPB-REV	5'-GCC-CGA-GAG-TTT-CAA-TCC-3'
	CAPD-FWD	5'-TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC-3'
	CAPD-REV	5'-CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG-3'
	CAPE-FWD	5'TCC-GCA-GAA-AAT-TAT-TGA-CTC-3'
	CAPE-REV	5'-GCT-TGC-TGC-TTG-ATT-TTG-TC-3'
	CAPF-FWD	5'-AAT-CGG-AGA-ACG-CAG-AAA-TCA-G-3'
	CAPF-REV	5'-TTC-CGC-CGT-CAA-TTA-CTC-TG-3'
	KMT1T7	5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'
	KMT1SP6	5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

Size of resulting fragments:

Serogroup A	CAPA-FWD/CAPA-REV	1044 bp
Serogroup B	CAPB-FWD/CAPB-REV	760 bp
Serogroup D	CAPD-FWD/CAPD-REV	657 bp
Serogroup E	CAPE-FWD/CAPE/REV	511 bp
Serogroup F	CAPF-FWD/CAPF-REV	851 bp

PCR conditions:

Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM $MgCl_2$, 3.2 pmol of each primer and 1 u *Taq* DNA polymerase. In the original publication (Townsend *et al.*, 2001) it is suggested to use a standard cycling programme as per *P.-multocida*-specific PCR assay. However, the cycling

programme should be optimised to and validated for the model of thermocycler in use. Agarose gel electrophoresis is as described above.

1.3.3. HS-causing type-B-specific PCR assay

Presumptive identification of HS-causing type-B-specific *P. multocida* is also possible by PCR amplification (Townsend *et al.*, 1998a). Type B cultures with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of a ~620 bp fragment with the KTSP61 and KTT72 primers.

Primer sequences (Townsend *et al.*, 1998a):

HS-causing type-B-specific PCR	KTT72	5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3'
	KTSP61	5'-ATC-CGC-TAA-CAC-ACT-CTC-3'

Conditions for HS-causing type-B-specific PCR are as described for *P. multocida*-specific PCR. The usefulness of these primers has been reported for identification of serogroup B strains.

HS-causing type-B-specific PCR primers can also be used in a multiplex PCR with the *P. multocida*-specific primers, dramatically decreasing the time required for *P. multocida* detection and presumptive identification of the HS-serotype. Multiplex PCR conditions are as described above except that 3.2 pmol of each of the four primers and 1 u *Taq* DNA polymerase are used. The use of the multiplex *P.-multocida*-specific/HS-causing type-B-specific PCR on suspect organisms can confirm the identity and provide a presumptive serotype within 3–4 hours, in comparison with biochemical analysis and conventional serotyping, which can take up to 2 weeks.

1.3.4. *Pasteurella multocida* type A specific PCR

Primers useful for typing of serogroup A strains with several somatic types have been reported to be useful in specific identification of isolates (Gautam *et al.*, 2004).

Primers:

RGPMA5:	5'-AAT-GT-TTG-CGA-TAG-TCC-GTT-AGA-3'
RGPMA6:	5'-ATT-TGG-CGC-CAT-ATC-ACA-GTC-3'

PCR conditions:

Template DNA (50 ng) is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µm each dNTPs, 1.5 mM $MgCl_2$, 20 pmol of each primer and 1 unit *Taq* DNA polymerase. Standard amplification conditions are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 45 seconds, 56°C for 45 seconds, 72°C for 45 seconds, followed by final extension of 72°C for 6 minutes. Amplified products are separated by agarose gel electrophoresis (1.5% agarose gel) in 0.5 × TBE buffer at 5 v/cm for 2 hours.

The PCR amplification yields a product of 564 bp.

The test can be applied on direct culture, boiled cell lysate and infected tissues.

1.3.5 *Pasteurella multocida* multiplex LPS PCR typing system

Pasteurella multocida strains can be classified into 16 somatic or lipopolysaccharide (LPS) serovars (serotypes) using the Heddleston gel diffusion precipitin test. The genes required for LPS assembly have been identified. The multiplex PCR method was developed for LPS molecular typing based on LPS outer core structure genes (Harper *et al.,* 2015).

Primers:

BAP6119:	5'- ACA-TTC-CAG-ATA-ATA-CA-CCCG-3'
BAP6120:	5'- ATT-GGA-GCA-CCT-AGT-AAC-CC-3'
BAP6121:	5'- CTT-AAA-GTA-ACA-CTC-GCT-ATT-GC-3'
BAP6122:	5'- TTT-GAT-TTC-CCT-TGG-GAT-AGC-3'

BAP7213: 5'-TGC-AGG-CGA-GAG-TTG-ATA-AAC-CAT-C-3'

BAP7214: 5'- CAA-AGA-TTG-GTT-CCA-AAT-CTG-AAT-GGA-3'

BAP6126: 5'- CTT-TAT-TTG-GTC-TTT-ATA-TAT-ACC-3'

BAP6125: 5'- TTT-CCA-TAG-ATT-AGC-AAT-GCC-G-3'

BAP6129: 5'- AGA-TTG-CAT-GGC-GAA-ATG-GC-3'

BAP6130: 5'- CAA-TCC-TCG-TAA-GAC-CCC-C-3'

BAP7292: 5'- TCT-TTA-TAA-TTA-TAC-TCT-CCC-AAG-G-3'

BAP7293: 5'- AAT-GA-AGG-TTT-AAA-AGA-GAT-AGC-TGG-AG-3'

BAP6127: 5'- CCT-ATA-TTT-ATA-TCT-CCT-CCC-C-3'

BAP6128: 5'- CTA-ATA-TAT-AAA-CCA-TCC-AAC-GC-3'

BAP6133: 5'- GAG-AGT-TAC-AAA-AAT-GAT-CGG-C-3'

BAP6134: 5'- TCC-TGG-TTC-ATA-TAT-AGG-TAG-G-3'

Size of resulting fragments:

L1 (Heddleston Serovar 1 and14)	BAP6119/ BAP6120	1307 bp
L2 (Heddleston Serovar 2/5)	BAP6121/ BAP6122	810 bp
L3 (Heddleston Serovar 3 and 4)	BAP7213/ BAP7214	474 bp
L4 (Heddleston Serovar 6 and 7)	BAP6126/ BAP6125	550 bp
L5 (Heddleston Serovar 9)	BAP6129/ BAP6130	1175 bp
L6 (Heddleston Serovar 10, 11, 15, and 12)	BAP7292/ BAP7293	1668 bp
L7 (Heddleston Serovar 8 and 3)	BAP6127/ BAP6128	931 bp
L8 (Heddleston Serovar 16)	BAP6133/ BAP6134	255 bp

PCR conditions:

Each of the final multiplex LPS PCRs (50 µl final volume) is performed in 1×Taq polymerase buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl) containing 0.4 µM each primer, 0.2 mM deoxynucleoside triphosphates (dNTPs), and 1.7 U Taq polymerase. For each colony PCR, material from 2 to 3 well-isolated *P. multocida* colonies is collected using a sterile tip on a 20 µl micropipette (volume set at 20 µl) inserted into the middle of each colony. The collected material is then added to a 50 µl PCR mixture and mixed thoroughly by pipetting. For PCR using genomic DNA, approximately 50 ng of column-purified DNA is added to each PCR mixture. All reaction mixtures are mixed briefly then centrifuged for 10 seconds at 13,000 ***g***. For colony PCR, the cycling conditions are 96°C for 10 minutes, followed by 30 cycles of 96°C for 30 seconds, 52°C for 30 seconds, and 72°C for 2.5 minutes, with a final extension at 72°C for 5 minutes. For PCR using genomic DNA as the template, the cycling conditions are identical to those in the colony PCR except that the initial denaturation step at 96°C is reduced to 5 minutes. Amplified products are separated by 2% agarose gel electrophoresis.

1.3.6 Real-time PCR and loop-mediated isothermal amplification (LAMP) assays

HS-*est* real-time PCR based on *est* target gene has been developed (Petersen *et al.,* 2014). It specifically detects ST122, ST63, ST147 and ST162 associated with HS. However, it did not detect strains of ST151 with capsular type D isolated from pigs that were found positive with HS-causing type-B-specific PCR assay. The HS-*est* real-time PCR represents a fast and specific detection of the specific types of *P. multocida* involved in HS compared with other PCR assays.

Recently, the development of a Pm-LAMP assay and an HS-LAMP assay specific for serogroup B:2 strains associated with HS in cattle and buffalo were reported (Moustafa & Bennett, 2017). The preliminary findings suggest that the HS-LAMP assay has high sensitivity and specificity for detection of HS-associated *P. multocida*. It is presumed that the Pm-LAMP method should be a useful diagnostic tool for rapid and visible detection of *P. multocida* from diverse clinical materials of various host species. However, it needs further evaluation directly on clinical specimens, along

with its utility in the field as a simple alternate to PCR assays, which are sophisticated laboratory-based methodologies.

1.3.7. Genotypic differentiation of isolates

Once presumptive (or definitive) identification has been made, further differentiation of isolates can be achieved by genotypic fingerprinting methods. Restriction endonuclease analysis (REA) with the enzyme *Hha*I has proved useful for characterisation of type B HS-serotypes. Among 71 *P. multocida* capsule serogroup B isolates, 20 DNA fingerprint profiles were observed. With HS-causing serogroup strains, 13 unique profile among 54 isolates resembling the profile of the somatic serotype 2 reference strain have been reported (Wilson *et al.*, 1992). In contrast, while a single *Hha*I profile was observed among 13 serogroup E isolates, differentiation of these strains was possible following *Hpa*II digestion. *Hpa*II appear to generate finer subdivisions than those achieved with the use of *Hha*I (Wilson *et al.*, 1995). Ribotyping and large DNA separation by pulsed-field gel electrophoresis also provide useful discrimination of serogroup B and E *P. multocida* isolates (Townsend *et al.*, 1997a). Genetic diversity of HS-causing *Pasteurella multocida* strains of animal or bird origin could be obtained by sequence analysis of the 16S rRNA gene. A study in the United Kingdom using 79 field isolates recovered from various species revealed nineteen 16S rRNA types that clustered into two distinct phylogenetic lineages (Davies, 2004). On the other hand, sequence analyses of Indian isolates of *P. multocida* serogroup B from different animal species did not reveal considerable variation (Dey *et al.*, 2007). Multilocus sequence typing (MLST), a sequence-based typing system based on seven housekeeping genes has been used to identify strain diversity of bovine isolates of *P. multocida* as well as isolates from other host species (Davies *et al.*, 2004; Hotchkiss *et al.,* 2011; Sarangli *et al.,* 2016; Subaaharan *et al.,* 2010). MLST of 55 isolates of *P. multocida* associated with HS found that the majority include sequence type (ST) 122 (n = 50), and rarely ST63, ST147 and ST162 in comparison with other members of the species isolated from different lesion types and hosts (Petersen *et al.,* 2014).

PCR fingerprinting is feasible for any laboratory with PCR capability, with several methods previously used for *P. multocida* differentiation. Random amplified polymorphic DNA (RAPD) analysis and arbitrarily primed PCR (AP-PCR), respectively, have been shown to be useful for epidemiological studies of *P. multocida* isolated from rabbits (Chaslus-Dancla *et al.*, 1996). Repetitive sequence PCR analysis of *P. multocida* has proved useful for discrimination of avian and swine isolates, although all HS-causing strains analysed demonstrated similar profiles (Townsend *et al.*, 1997b; 1998b). However, molecular variability among HS-causing strains of *P. multocida* belonging to serogroup B has been found recently. Using repetitive extragenic palindromic (REP)-PCR, enterobacterial repetitive intragenic consensus (ERIC)-PCR and single primer PCR, genotypic differentiation among five *P. multocida* serogroup B isolates have been reported (Biswas *et al.*, 2004). RAPD and AP-PCR analysis of HS-causing *P. multocida* isolates have not been previously described.

2. Serological tests

Serological tests for detecting antibodies are not normally used for diagnosis. The IHA test can be used for this purpose, following a method broadly similar to that described for capsular typing above. High titres detected by the IHA test are indicative of recent exposure to HS. As HS is a disease that occurs mainly in animals reared under unsophisticated husbandry conditions, where disease-reporting systems are also poor, there is often considerable delay in notification of outbreaks. Deaths occur very suddenly and no carcasses are available for examination when notification is made. In such situations, high IHA titres from 1/160 up to 1/1280 or higher among in-contact animals surviving in affected herds, are indicative of recent exposure to HS for the purpose of diagnosis.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

The three types of vaccines used against HS are bacterins, alum-precipitated vaccine (APV) and oil-adjuvanted vaccine (OAV). To provide sufficient immunity with bacterins, repeated vaccination is

required. Administration of dense bacterins can give rise to shock reactions, which are less frequent with the APV and almost nonexistent with the OAV.

A live HS vaccine prepared using an avirulent *P. multocida* strain B:3,4 (Fallow deer strain) has been used for control of the disease in cattle and water buffaloes over 6 months of age in Myanmar since 1989. It is administered by intranasal aerosol application (Carter *et al.*, 1991; Myint *et al.*, 2005). The vaccine has been recommended by the Food and Agriculture Organization of the United Nations (FAO) as a safe and potent vaccine for use in Asian countries. However, there is no report of its use in other countries and killed vaccines are the only preparations in use by the countries affected with HS.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

A local isolate of *P. multocida* representing the prevalent serotype is used. A well-capsulated, stable culture that produces large colonies of approximately 2 mm in diameter on CSY blood agar must be maintained. Seed cultures should be stored as semisolid nutrient agar stab cultures at room temperature, or as lyophilised cultures.

A calf is infected with the culture, and, within 2–3 hours of its death, blood is collected aseptically from the heart and stored at –20°C in 1 ml aliquots. A fresh aliquot is used for each new batch of vaccine. It is permissible to subculture this aliquot once or twice, provided the colony size does not diminish. A blood aliquot is thawed, plated on to CSY blood agar, and the growth is tested for agglutinability by the appropriate antiserum on a slide. A good culture will give a coarse floccular agglutination in under 30 seconds. A poor culture will yield only a fine granular agglutination.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Seed lots must be shown to be:

i) *Pure:* Free from adventitious agents.

ii) *Safe:* Produce no adverse reaction in the target species when given as recommended.

iii) *Efficacious:* Stimulate effective immunity as indicated by potency tests.

The necessary tests are described in Section C.2.2.4 below.

2.2. Method of manufacture

2.2.1. Procedure

For vaccine production, dense suspensions of bacteria are necessary. They should have a minimum bacterial content of 1.5 g dry weight per litre of suspension. There are two methods of producing dense suspensions. The first is to culture on solid medium in Roux flasks and harvest in formalinised physiological saline, by which means suspensions of any density can be achieved. This is laborious as each flask must be harvested separately and tested for purity. The second and recommended method is the use of a large vessel with aerated cultures in a medium that specifically supports *P. multocida*.

There are two types of aeration process – by vortexing and sparging. Sterile air is provided by a compressor. In vortex aeration, the culture is stirred by an impeller shaft operating in the air stream, whereas in sparging aeration, the air is dispersed through a sparger. Intermittent aeration seems to produce denser growth (Thomas, 1968). The more finely dispersed the air, the better is the bacterial growth. Vessels of 20–40 litres are usually employed, and incubation is at 37°C. In continuous culture systems, once a maximum density has been reached, usually within 15 hours,

about 25% of the working volume is harvested and replaced hourly. The harvests of continuous cultures are collected in relatively small volumes into separate vessels, but, after several days, the density diminishes, presumably through loss of capsular antigen. For this reason, batch cultures are preferred. If batch culture vessels are inoculated at a rate of 50 ml/litre of medium, maximum turbidity is obtained within 15–18 hours, when the growth can be terminated by the addition of formalin to a final concentration of 0.5%. This procedure, where a large inoculum is employed and the growth is terminated within a short period, helps to minimise the chances of contamination. The turbidity is standardised against a reference containing the equivalent dry weight/volume of 1.5 g/litre.

Dense cultures are also obtained using fermenters, where heat sterilisation of the tanks and culture can be carried out *in situ*, with automatic temperature, pH and aeration control devices. Liquid sterilisation systems by filtration, for heat-labile components, can also be built into the fermenter. A 100 litre batch fermenter will yield a minimum of 66,000 doses (each of 3 ml) of OAV, and even more doses if the density is high enough for dilution to a reference standard equivalent to 1.5 g/litre, dry weight/volume.

OAV is made by the emulsification of equal volumes of a light mineral oil and the bacterial suspension, with 5% pure anhydrous lanolin as emulsifying agent. The mineral oil and lanolin are first sterilised and, on cooling to 40°C, 0.5% formalin is added to the mixture. The bacterial suspension is added slowly and emulsification is continued for a further 10 minutes. Following overnight storage, the mixture is re-emulsified, bottled and stored at 4°C for 2 weeks prior to use.

APV is prepared by first adjusting the turbidity of the suspension to the reference standard as above, and diluting it with an equal volume of 0.5% formalinised physiological saline. The pH is adjusted to 6.5, and a hot 20% solution of potash alum is added to give a final concentration of 1% alum. After overnight storage with continuous agitation, the vaccine is bottled for use.

2.2.2. Requirements for substrates and media

A suitable sterilised medium for the aerated culture method is casein hydrolysate (2 g), sucrose (6 g), yeast extract (6 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (8.6 g), anhydrous potassium dihydrogen orthophosphate (1.36 g), and distilled water to 1 litre. A denser growth is obtained if the casein, sucrose and yeast are prepared as a concentrate, filter-sterilised or autoclaved for 10 minutes at 107°C, and transferred aseptically into the tank that has previously been heat-sterilised with the rest of the ingredients.

2.2.3. In-process controls

Proper concentration of bacterial growth, the capsulation of the bacteria, purity of culture and efficient inactivation all need to be checked.

2.2.4. Final product batch tests

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

Two seronegative cattle are vaccinated with twice the recommended dose and observed for 10–14 days for adverse effects.

Five mice are inoculated intramuscularly with 0.2 ml each of the vaccine, and observed for 5 days. The blood of any mouse that dies is cultured for *P. multocida*.

iii) Batch potency

Potency tests can be carried out by any of the following methods:

a) Vaccination of cattle followed by direct challenge or passive mouse protection tests using the bovine sera. This procedure is not very feasible as cattle take a long time to develop adequate immunity after OAV;

b) Vaccination of rabbits followed by direct challenge or passive mouse protection test using the rabbit sera; or

c) Potency tests in mice, the most feasible method of the three.

Each of 50 mice is vaccinated intramuscularly with 0.2 ml of vaccine, and again 14 days later. On day 21, the mice are divided into ten groups of five, each group being challenged with respective dilutions of a 6–8-hour broth culture of a field strain in the range 10^{-1}–10^{-10}; 50 unvaccinated controls are similarly challenged, and all mice are observed for 5 days. The median lethal dose (LD_{50}) can then be calculated in order to obtain an indication of the dose that is sufficient to protect cattle: vaccines prepared in the manner described give at least 10^4 units protection in the vaccinated mice.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

See chapter 1.1.8.

ii) Reversion-to-virulence for attenuated/live vaccines

See chapter 1.1.8.

iii) Environmental consideration

See chapter 1.1.8.

2.3.2. Efficacy requirements

i) For animal production

A single dose of vaccine administered to young calves 4–6 months of age will protect susceptible animals for 3–4 months when APV is used, and for 6–9 months when OAV is used.

The vaccine should be administered by deep intramuscular injection. The use of nylon 5 ml volume syringes for a 3 ml dose and a gauge 14–15 needle is advised, and the recommended age for primary vaccination is 4–6 months. For routine, prophylactic vaccination, a single dose of OAV at 4–6 months, a booster 3–6 months later, and annual revaccination thereafter, is recommended. Where husbandry practices are such that reaching individual animals at appropriate times is impracticable, annual vaccination of all animals over 4 months of age, preferably before the breeding season, and vaccination of all calves under 1 year of age, 6 months later, is recommended. In the face of an outbreak in vaccinated animals, one dose of APV, followed by one dose of OAV, is recommended.

Leakage of OAV into subcutaneous tissue can occasionally give rise to fibrous lumps at sites of injection. Rarely, abscesses may develop if sterility conditions are not observed, though most animals are resistant to such infections. APV may occasionally cause shock reactions.

ii) For control and eradication

Not applicable.

2.3.3. Stability

The OAV emulsion should be pure white, and should stick to glass like paint. If the emulsion shows signs of cracking, it should be discarded. Separation of a thin layer of oil on the surface is permissible. It can be stored at 4–8°C for 6 months without any significant loss of potency. It must not be frozen. Increase in the content of lanolin improves stability, but also increases the viscosity

– a distinct disadvantage. Use of other emulsifying agents such as 'Arlacel' helps to produce thinner, stable emulsions.

3. Vaccines based on biotechnology

Not applicable at present. However, several attempts are being made to develop defined live mutant vaccines, bacterial ghosts, as well as recombinant subunit vaccine with suitable adjuvants (Shivachandra *et al*., 2011).

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NB: At the time of publication (2021) there were no WOAH Reference Laboratories for haemorrhagic septicaemia (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989; MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.4.11.

INFECTIOUS BOVINE RHINOTRACHEITIS/ INFECTIOUS PUSTULAR VULVOVAGINITIS

SUMMARY

Description of disease: *Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine herpesvirus 1 (BoHV-1), is a disease of domestic and wild cattle. The virus is distributed world-wide, but has been eradicated from a number of European countries and others have active eradication programmes.*

The disease is characterised by clinical signs of the upper respiratory tract, such as a (muco)purulent nasal discharge, hyperaemia of the muzzle (red nose disease) and by conjunctivitis. Signs of general illness are fever, depression, inappetence, abortions and reduced milk yield. The virus can also infect the genital tract and cause pustular vulvovaginitis and balanoposthitis. Post-mortem examinations reveal rhinitis, laryngitis and tracheitis. Mortality is low, and most infections run a subclinical course. Secondary bacterial infections can lead to more severe respiratory disease, and BoHV-1 could play a role in multifactor diseases such as 'shipping fever'.

Identification of the agent: *The virus can be isolated from nasal or genital swabs from animals with respiratory signs, vulvovaginitis or balanoposthitis, taken during the acute phase of the infection, and, in severe cases, from various organs collected at post-mortem. Following infection, BoHV-1 may persist in infected animals in a latent state in sensory neurons, e.g. in the trigeminal or sacral ganglia. The virus can be reactivated and this results in virus shedding (re-excretion) without exhibition of clinical disease. Because of this latency phenomenon, antibody-positive animals have to be classified as infected with BoHV-1 (with two exceptions: serological responses induced by vaccination with an inactivated vaccine or by colostral antibodies).*

For virus isolation, various cell cultures of bovine origin are used, for example, secondary lung or kidney cells or the Madin–Darby bovine kidney cell line. The virus produces a cytopathic effect in 2–4 days. It is identified by neutralisation or antigen detection methods using monospecific antisera or monoclonal antibodies. BoHV-1 isolates can be further subtyped by DNA restriction enzyme analysis into subtypes 1.1 and 1.2. BoHV-1.2 isolates can be further differentiated into 2a and 2b. Development of rhinotracheitis or vulvovaginitis/balanoposthitis depends more on the route of infection than on the subtype of the virus. The virus previously referred to as BoHV-1.3, a neuropathogenic agent, is now classified as BoHV-5.

For virus DNA detection, the polymerase chain reaction (PCR) technique is increasingly used in routine diagnosis especially the real-time PCR.

Serological tests: *The virus neutralisation test and various enzyme-linked immunosorbent assays (ELISA; indirect or blocking) are most widely used for antibody detection. With the ELISAs, antibodies can be detected in serum or plasma, and with lower sensitivity in milk or bulk milk samples. The use of a gE-antibody-ELISA makes it possible to distinguish field virus infected cattle from cattle vaccinated with a gE-deleted marker vaccine (DIVA strategy).*

Requirements for vaccines: *Inactivated and attenuated live vaccines are available. The vaccines protect cattle clinically in case of infection and markedly reduce the subsequent shedding of field virus. Although vaccination may not prevent field virus infection of individual animals, spreading of wild-type virus in infected herds is efficiently reduced. The vaccines must not induce disease, abortion, or any local or systemic reaction, and must be genetically stable. BoHV-1 glycoprotein E deleted mutant marker vaccines are now generally available (live or inactivated) and can be used as part of a DIVA strategy.*

A. INTRODUCTION

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine herpesvirus 1 (BoHV-1), is a disease of domestic and wild cattle. BoHV-1 is a member of the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae*, which belongs to the *Herpesviridae* family, order *Herpesvirales*. The viral genome consists of double-stranded DNA that encodes for about 70 proteins, of which 33 structural and more than 15 nonstructural proteins have been identified. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity. BoHV-1 can be differentiated into subtypes 1.1, 1.2a and 1.2b (Metzler *et al*., 1985). The BoHV-1.2 subtypes may be less virulent than subtype 1.1 (Edwards *et al*., 1990). The former BoHV-1.3, which may act as a neuropathogenic agent in calves, has been re-classified as BoHV-5 (Magyar *et al*., 1993). BoHV-1 shares antigenic and genetic close relationships with other ruminant alphaherpesviruses: BoHV-5, caprine herpesvirus 1, cervid herpesvirus 1 (red deer), cervid herpesvirus 2 (reindeer), bubaline herpesvirus 1 and elk herpesvirus 1 (Thiry *et al*., 2006).

After an incubation period of 2–4 days, serous nasal discharge, salivation, fever, inappetence, and depression become evident. Within a few days the nasal and ocular discharges change to mucopurulent. Where natural mating is practised, genital infection can lead to pustular vulvovaginitis or balanoposthitis. However, most infections run a very mild or subclinical course (Van Oirschot *et al*., 1993). Uncomplicated cases of respiratory or genital disease caused by BoHV-1 last about 5–10 days. Secondary bacterial or viral agents may contribute to a multifactor disease complex resulting in severe respiratory disease of young animals ('shipping' or 'crowding fever').

After infection via the airborne route, BoHV-1 replicates to high titres in mucous membranes of the upper respiratory tract and in the tonsils. Subsequently, the virus disseminates to conjunctivae and reaches the trigeminal ganglia by neuronal axonal transport. After genital infection, BoHV-1 replicates in the mucous membranes of the vagina or prepuce, and becomes latent in the sacral ganglia. The viral DNA remains in the neurons of the ganglia, probably for the entire life of the host (status of latency). Stress, such as transport and parturition, but also the application of corticosteroids can induce reactivation of the latent infection. Consequently, the virus may switch between latent and lytic infection and may be shed intermittently into the environment and spread to contact animals.

BoHV-1 infection elicits an antibody response and a cell-mediated immune response within 7–14 days. The immune response is presumed to persist life-long, although it may fall below the detection limit of some tests after a number of years. Maternal antibodies are transferred via colostrum to the young calf, which is consequently protected against BoHV-1-induced clinical disease (Mechor *et al*., 1987). Maternal antibodies have a biological half-life of about 3 weeks, but may be detected occasionally in animals for several months.

The virus is distributed worldwide, with the exception of the BoHV-1-free countries, paralleling the distribution of domestic cattle. Other *Artiodactyla* (e.g. goats, sheep, water buffaloes, camelids) may be infected with BoHV-1. After infection, nasal viral shedding is detected for 5–14 days, with peak titres of 10^8–10^{10} $TCID_{50}$ (50% tissue culture infective doses) per ml of nasal secretion. The semen of an infected bull may contain BoHV-1, and the virus can thus be transmitted by natural mating and artificial insemination (Parsonson & Snowdon, 1975).

Vaccines usually prevent the development of clinical signs and markedly reduce the shedding of virus after infection, but do not completely prevent infection. Several eradication campaigns have been carried out or are currently running in different countries including test-and-removal programmes or vaccination campaigns (see Section C).

BoHV-1 infection may be suspected on the basis of clinical, pathological and epidemiological findings. However, to make a definite diagnosis, laboratory examinations (serology or virus detection) are required. A complete diagnostic procedure in the laboratory is aimed at detecting the causative virus (or viral components) and the specific antibodies they induce. Nevertheless, because of latent infection induced by BoHV-1, detection of antibodies could be sufficient for the determination of the BoHV-1 status of individual animals.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of BoHV-1 and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[a]						
Virus isolation	–	+[b]	+	++	–	–
Real-time PCR	–	+[b]	+	+++	–	–
Detection of immune response						
ELISA	+++	+++	+++	++	+++	+++
VN	++	++	++	+	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.
(a)A combination of agent identification methods applied on the same clinical sample is recommended.
[b]Method particularly applicable to testing of semen.

1. Identification of the agent

1.1. Collection and processing of specimens

Nasal swabs, preferably foam or flocked swabs, are collected from several (from five to ten) affected cattle in the early phase of the infection. These cattle still have serous rather than mucopurulent nasal discharge. In cases of vulvovaginitis or balanoposthitis, swabs are taken from the genitals. The swabs should be vigorously rubbed against the mucosal surfaces. The prepuce can also be washed with saline; the washing fluid is then collected. The specimens are suspended in transport medium (cell culture medium containing antibiotics and 2–10% BoHV-1-free fetal bovine serum to protect the virus from inactivation), cooled at 4°C, and rapidly submitted to the laboratory.

During necropsy, mucous membranes of the respiratory tract, and samples of the tonsil, lung and bronchial lymph nodes are collected for virus detection. In cases of abortion, the fetal liver, lung, spleen, kidney and placental cotyledons are examined. Samples should be kept on ice and sent to the laboratory as quickly as possible.

After arrival at the laboratory, swabs are agitated at room temperature for 30 minutes in the transport medium to elute virus. Following removal of the swabs, the transport medium is clarified by centrifugation at 1500 ***g*** for 10 minutes. Tissues are homogenised to a 10–20% (w/v) suspension in cell culture medium before centrifugation at 1500 ***g*** for 10 minutes. The supernatants of these specimens are filtered through 0.45 μm filters and used for virus isolation.

The isolation of virus from semen needs some special adaptations, because the seminal fluid contains enzymes and other factors that are toxic to the cells and inhibit viral replication (see below).

1.2. Virus isolation

For virus isolation, bovine cells of various origins can be used. Primary or secondary bovine kidney, lung or testis cells, cell strains derived from bovine fetal lung, turbinate or trachea, and established cell lines, such as the Madin–Darby bovine kidney cell line (MDBK), are suitable for BoHV-1 propagation.

Cell cultures can be grown in glass or plastic tubes, plates or dishes. When 24-well plastic plates are used, a 100–200 µl volume of the supernatants described above is inoculated into these cell cultures. After a 1-hour adsorption period, the cultures are rinsed and maintenance medium is added. The serum used as a medium supplement in the maintenance medium should be free of antibodies against BoHV-1. The cell cultures are observed daily for cytopathic effect (CPE), which usually appears within 3 days after inoculation. It is characterised by grape-like clusters of rounded cells gathered around a hole in the monolayer; sometimes giant cells with several nuclei may be observed. Experience is needed to recognise this characteristic appearance. When, after 7 days, no CPE has appeared, a blind passage must be made. The cell culture is freeze–thawed and clarified by centrifugation, and the supernatant is used for inoculation of fresh monolayers (Brunner *et al*., 1988; Edwards *et al*., 1983).

To identify the recovered virus as BoHV-1, the supernatant of the culture should be neutralised with a monospecific BoHV-1 antiserum or neutralising monoclonal antibody (MAb). For this purpose, serial tenfold dilutions of the test supernatant are made, and to each dilution monospecific BoHV-1 antiserum or negative control serum is added. Following incubation at 37°C for 1 hour, the mixtures are inoculated into cell cultures; 3–5 days later, the neutralisation index is calculated. The neutralisation index is the virus titre (in $\log_{10}$) in the presence of negative control serum minus the virus titre in the presence of specific antiserum. If the neutralisation index is greater than 1.5, the isolate may be considered to be BoHV-1. To shorten the virus isolation procedure, two specimens may be inoculated into cell culture: one that has been pre-incubated with monospecific antiserum and another that has been preincubated with negative control serum. If the CPE is inhibited by the monospecific antiserum, the isolate can be considered to be BoHV-1, although definitive confirmation would require molecular characterisation to distinguish it from related ruminant alphaherpesviruses.

An alternative method of virus identification is the direct verification of BoHV-1 antigen in cells around the CPE by an immunofluorescence or immunoperoxidase test (Kaashoek *et al*., 1994) with conjugated monospecific antiserum or MAb. Furthermore, the supernatant can be used as template for restriction endonuclease fragment length polymorphism (RFLP) (see Section B.1.4) and polymerase chain reaction (PCR) analyses (see Section B.1.3).

1.2.1. Virus isolation from semen

0.05 to 0.1 ml of raw semen should be tested with two passages in cell culture. Raw semen is generally cytotoxic and should be prediluted (e.g. 1/10) before being added to cell cultures. A similar problem may sometimes arise with extended semen. For extended semen, an approximation should be made to ensure that the equivalent of a minimum of 0.1 ml raw semen is examined (e.g. a minimum of 0.5 ml extended semen). Multiple diluted samples may need to be tested with this procedure to reach a volume equivalent to 0.1 ml raw semen (e.g. 5 × 1 ml of a 1/10 diluted sample of extended semen). A suitable test procedure is given below. See also Brunner *et al.* (1988).

1.2.1.1. Test procedure

i) Dilute 200 µl fresh semen in 2 ml fetal bovine serum (free from antibodies against BoHV-1) with antibiotics.

ii) Mix vigorously and leave for 30 minutes at room temperature.

iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus isolation above) in a six-well tissue culture plate.

iv) Incubate the plates for 1 hour at 37°C.

v) Remove the mixture, wash the monolayer twice with 5 ml maintenance medium, and add 5 ml maintenance medium to each well.

vi) Include BoHV-1 negative and positive controls in the test. Special caution must be taken to avoid accidental contamination of test wells by the positive control, for example always handling the control last, and using separate plates.

vii) Observe plates under a microscope daily for the appearance of a CPE. If a CPE appears, confirmatory tests for BoHV-1 are made by specific neutralisation or immunolabelling methods (see above).

viii) If there is no CPE after 7 days, the cultures are frozen and thawed, clarified by centrifugation, and the supernatant is used to inoculate fresh monolayers.

ix) The sample is considered to be negative, if there is no evidence of a CPE after 7 days' incubation of the passaged cultures.

1.3. Nucleic acid detection

During the past decade, various methods for the detection of BoHV-1 DNA in clinical samples have been described, including DNA–DNA hybridisation and PCR. PCR is increasingly being used in routine diagnostic submissions (Moore *et al*., 2000). Compared with virus isolation, PCR has the primary advantages of being more sensitive and more rapid: it can be performed in 1–2 days. It is also possible to detect episomal DNA of non-replicating virus in sensory ganglia (Van Engelenburg *et al*., 1993), such as the trigeminal ganglion, in the latent phase of infection. The disadvantage is that PCR analyses are prone to contamination and therefore precautions have to be taken to prevent false-positive results. Risk of contamination is markedly reduced by new PCR techniques, such as real-time quantitative PCR (see below) (Abril *et al*., 2004; Lovato *et al*., 2003; Wernike *et al*., 2011) and the PCR method is recommended in all cases for direct BoHV-1 detection.

So far, PCR has been used mainly to detect BoHV-1 DNA in artificially (Kramps *et al*., 1993) or naturally (Van Engelenburg *et al*., 1993) infected semen samples. It is important to thoroughly optimise the PCR conditions, including the preparation of the samples, the concentration of test components e.g. primers and polymerase, and the cycle programmes. The target region for amplification must be present in all BoHV-1 strains, and its nucleotide sequence must be conserved. The TK, gB, gC, gD and gE genes have been used as targets for PCR amplification. In addition, PCRs based on detection of gE sequences can be used to differentiate between wild-type virus and gE-deleted vaccine strains (Fuchs *et al*., 1999; Schynts *et al*., 1999; Wernike *et al.,* 2011). Discrimination between infection with virulent IBR strains and infection with live attenuated (non-marker) strains may not be easily achievable with the PCR technique, and RFLP or high throughput sequencing (HTS) can be used for this purpose. Specific PCRs have been developed that are able to discriminate between BoHV-1, BoHV-5 and other related alphaherpesviruses (Ashbaugh *et al*., 1997; Ros *et al*., 1999).

Experimentally, PCR was found to be more sensitive than virus isolation: in egg yolk-extended semen samples obtained from experimentally infected bulls, PCR detected five times as many positives as did virus isolation (Van Engelenburg *et al*., 1995). The detection limit of validated PCR assays amounts to only a few genome copies per PCR reaction. Nevertheless, false-negative results cannot be excluded. To identify possible false-negative results, it is recommended to spike an internal control template into the reaction tube of the semen sample to be amplified by the same primers. Such a control template may be constructed by inserting, for example, a 100 base-pair fragment into the target region. This control template also makes it possible to semi-quantify the amount of DNA that is detected (Ros *et al*., 1999; Van Engelenburg *et al*., 1993). When using an internal control, extensive testing is necessary to ensure that PCR amplification of the added internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity (see also Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*). DNA extraction and quality of the DNA preparations can also be controlled by amplification of cellular sequences (housekeeping genes, Wernike *et al.,* 2011) or by addition of 'artificial' DNA sequences prior to extraction procedures (e.g. green fluorescent protein gene, non-BoHV-related viruses) as internal controls. To enhance the sensitivity and specificity of the BoHV-1 PCR, real-time PCR systems are the methods of choice.

1.3.1. Real-time polymerase chain reaction

The following real-time PCR test method has been developed to detect BoHV-1 in extended bovine semen destined for trade. The method has been validated according to chapter 1.1.6, and includes a comprehensive international inter-laboratory comparison involving six collaborating laboratories with specialist status in IBR testing (Wang *et al.,* 2008).

A number of studies have shown that PCR assays are more sensitive than virus isolation (Smits *et al*., 2000; Van Engelenburg *et al*., 1995; Vilcek *et al*., 1994; Wang *et al*., 2008; Wiedmann *et al*., 1993). Real-time PCR has been used for the detection of BoHV-1 and BoHV-5 in experimentally infected cattle and mice (Abril *et al*., 2004; Lovato *et al*., 2003) and a series of conventional PCR assays have been used for the detection of BoHV-1 DNA in artificially or naturally infected

bovine semen samples (Deka *et al*., 2005; Grom *et al*., 2006; Masri *et al*., 1996; Van Engelenburg *et al*., 1993; Weiblen *et al*., 1992; Wiedmann *et al*., 1993; Xia *et al*., 1995). Conventional detection of amplified PCR products relies on gel electrophoresis analysis (Rola *et al*., 2003). Sequence-specific primers have been selected to amplify different parts of conserved glycoprotein genes of the BoHV-1 genome, including glycoprotein B (gB) gene (Grom *et al*., 2006; Santurde *et al*., 1996), gC gene (Smits *et al*., 2000; Van Engelenburg *et al*., 1995), gD gene (Smits *et al*., 2000; Wiedmann *et al*., 1993), gE gene (Grom *et al*., 2006), and the thymidine kinase (tk) gene (Moore *et al*., 2000; Yason *et al*., 1995). Furthermore, a triplex real-time PCR for the detection of BoHV-1 gD and gE genes together with a house-keeping gene was validated and reported in 2011 (Wernike *et al.,* 2011). This PCR also allows the easy and fast differentiation of BoHV-1-field strains from gE-deleted vaccine strains.

The real-time PCR described here uses a pair of sequence-specific primers for amplification of target DNA and a 5'-nuclease oligoprobe for detection of amplified products. The oligoprobe is a single, sequence-specific oligonucleotide, labelled with two different fluorophores, the reporter/donor 5-carboxyfluorescein (FAM) at the 5' end, and the acceptor/quencher 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. This real-time PCR assay is designed to detect viral DNA of all BoHV-1 strains, including subtype 1 and 2, from extended bovine semen. The assay selectively amplifies a 97 base-pair sequence of the glycoprotein B (gB) gene. Details of the primers and probes are given in the protocol outlined below.

1.3.1.1. Sample preparation, equipment and reagents

i) The samples used for the test are, typically, extended bovine semen stored in liquid nitrogen. The semen samples can be transported to the laboratory in liquid nitrogen, or shipped at 4°C, and stored in liquid nitrogen or at –70°C (for long-term storage) or 4°C (for short-term storage). Storing semen at 4°C for a short period (up to 7 days) does not affect PCR test result.

ii) Three straws from each batch of semen should be processed. Duplicate PCR amplifications should be carried out for each DNA preparation (six amplifications in total) to ensure the detection of DNA in samples containing low levels of virus.

iii) The real-time PCR assay described here involves two separate procedures. Firstly, BoHV-1 DNA is extracted from semen using Chelex-100 chelating resin, along with proteinase K and DL-dithiothreitol (DTT). The second procedure is the PCR analysis of the extracted DNA template in a real-time PCR reaction mixture. A number of commercial real-time PCR amplification kits are available and any particular kit selected needs to be compatible with the chosen real-time PCR platform. The required primers and probes can be synthesised by various commercial companies.

1.3.1.2. Extraction of DNA

i) In a screw top 1.5 ml tube, add:

Chelex 100 sodium (10% w/v in distilled deionised water)	100 µl
Proteinase K (10 mg/ml)	11.5 µl
DL-dithiothreitol (1 M)	7.5 µl
Nuclease-free water	90 µl
Semen sample	10 µl

Mix gently by pipetting[1].

ii) The samples are incubated at 56°C for 30 minutes and then vortexed at high speed for 10 seconds.

iii) Subsequently, the tubes are incubated in a boiling water bath for 8 minutes and then vortexed at high speed for 10 seconds.

iv) The tubes are centrifuged at 10,000 ***g*** for 3 minutes.

1 It is important that the Chelex 100 solution is homogeneous while pipetting, as Chelex 100 sodium is not soluble. This can be achieved by putting the vessel containing Chelex-100 solution on a magnetic stirrer while pipetting.

v) The supernatant[2] is transferred into a new microtube and can be used directly for PCR, or stored at –20°C.

1.3.1.3. Preparation of reagents

The manufacturer's instructions should be followed for application and storage of the real-time PCR reaction mixture.

Working stock solutions for primers and probe are made with nuclease-free water at the concentration of 4.5 µM and 3 µM, respectively. The stock solutions are stored at –20°C and the probe solution should be kept in the dark. Single-use aliquots can be prepared to limit freeze–thawing of primers and probes and extend their shelf life.

1.3.1.4. Real-time PCR test procedure

i) *Primers and probe sequences*

Selection of the primers and probe are outlined in Abril *et al*. (2004) and described below.

Primer gB-F: 5'-TGT-GGA-CCT-AAA-CCT-CAC-GGT-3' (position 57499–57519 GenBank®, accession AJ004801)

Primer gB-R: 5'-GTA-GTC-GAG-CAG-ACC-CGT-GTC-3' (position 57595–57575 GenBank®, accession AJ004801)

Labelled Probe: 5'-FAM-AGG-ACC-GCG-AGT-TCT-TGC-CGC-TAMRA-3' (position 57525–57545 GenBank®, accession AJ004801)

ii) *Preparation of reaction mixtures*

The PCR reaction mixtures are prepared in a separate laboratory room. For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC, reagents only), appropriate negative controls, i.e. 1 per 10 test samples, and two positive controls (moderate and weak positive) should be included. Each test sample and control is tested in duplicate. The PCR amplifications are carried out in a volume of 25 µl.

a) PCR reagent mixtures are added in a clean room (no viral cultures, DNA extracts or post-amplification products should be handled here)

2 × real-time PCR reaction mixture	12.5 µl
ROX reference dye (optional)	0.5 µl
Forward primer (gB-F, 4.5 µM)	1 µl
Reverse primer (gB-R, 4.5 µM)	1 µl
Probe (3 µM)	1 µl
Nuclease free water	4 µl

b) 5 µl of the DNA template are added to the PCR reagent mixture to a final volume of 25 µl. DNA samples are prepared and added to the PCR mix in a separate room.

iii) Real-time polymerase chain reaction

The PCR tubes are placed in the real-time PCR detection system in a separate, designated PCR room. The PCR detection system is programmed for the test as follows:

PCR Reaction Parameters (may vary with different PCR platforms)

One cycle:	Hold 50°C	2 minutes
One cycle:	Hold 95°C	2 minutes
45 cycles:	Hold 95°C	15 seconds
	Hold 60°C	45 seconds

2 Some DNA samples can become cloudy and a thin white membrane may form occasionally after freezing and thawing. This appears to have no influence on the PCR performance. No heating or re-centrifuging of the samples is necessary.

iv) Analysis of real-time PCR data

The threshold level is usually set according to the manufacturers' instructions for the selected analysis software used. Alternatively, virus isolation negative semen samples, from seronegative animals, can be run alongside to determine the background signal associated with the detection system used.

1.3.1.5. Interpretation of results

i) Test controls

Positive and negative controls, as well as reagent controls, should be included in each PCR test. Negative semen, from seronegative bulls, can be used as a negative control. Positive semen from naturally infected bulls is preferable as a positive control. However, this might be difficult to obtain. Alternatively, positive controls can be derived from negative semen spiked with defined quantities of BoHV-1 virus.

ii) Test results

Positive result: Any sample that has a cycle threshold (Ct) value equal or less than 45 associated with an amplification curve is regarded as positive. The positive control should have a Ct value within an acceptable range (± 3 Ct values) as previously determined by repeatability testing. To minimise the risk of contamination by the positive control, a dilution resulting in a Ct value of about 30 to 33 should be used.

Negative result: Any sample that shows no Ct value is regarded as negative. Negative control and no template control should have no Ct values.

1.4. Viral antigen detection

Nasal, ocular or genital swabs can be directly smeared onto glass cover-slips, or, following centrifugation, the cell deposit (see Section B.1.1) may be spotted onto cover-slips. These cover-slips are subjected to a standard direct or indirect fluorescent antibody test. In a direct immunofluorescence test, the monospecific antiserum is conjugated to a fluorescent dye e.g. fluorescein isothiocyanate (FITC), whereas in the indirect procedure, the anti-species immunoglobulin secondary antibody is conjugated to a fluorochrome. To obtain reliable results, it is necessary to sample several animals in a herd that have fever and a slight, serous nasal discharge. Smears should be air-dried and fixed in acetone. Smears from nasal swabs from cattle with a purulent or haemorrhagic nasal discharge are often negative (Terpstra, 1979). The advantage of this antigen-detection technique is that it can lead to a same-day diagnosis. However, the sensitivity of this procedure is lower than that of virus isolation (Edwards *et al*., 1983) or PCR. Positive and negative controls must be included in each test.

Tissues collected at post-mortem can be examined for the presence of BoHV-1 antigen by immunofluoresence analyses of frozen sections. Immunohistochemistry may also be applied for BoHV-1 detection and determination of the antigen location in the tissues. MAbs are increasingly being used for detecting BoHV-1 antigen, leading to enhanced specificity of the test. However, such MAbs must be carefully selected, because they must be directed against conserved epitopes that are present on all isolates of BoHV-1.

Another possibility for direct rapid detection of viral antigen is the use of an enzyme-linked immunosorbent assay (ELISA). Antigen can be captured by MAbs or polyclonal antibodies coated on a solid phase, usually on microplates. Amounts of antigen equivalent to 10^4–10^5 $TCID_{50}$ of BoHV-1 are required in order to obtain reliable positive results. This may not be unrealistically high, because titres of 10^8–10^9 $TCID_{50}$/ml of nasal fluid can be excreted by cattle 3–5 days after infection with BoHV-1. Sensitivity can be increased by amplification systems (see Edwards & Gitao, 1987).

In contrast to virus isolation, no cell culture facilities are required for direct antigen detection techniques and a laboratory diagnosis can be made within 1 day. The disadvantages are the lower sensitivity of direct antigen detection and the extra requirement to perform additional virus isolation, if the isolate is required for further studies.

Therefore, real-time PCR is the state-of-the-art standard, and antigen detection should only be used if other methods are not available.

1.5. Differentiation of bovine herpesvirus 1 subtypes and of ruminant alphaherpesviruses related to bovine herpesvirus 1

By using appropriate MAbs for immunofluorescence, radioimmunoprecipitation, immunoperoxidase or immunoblot assays, BoHV-1 subtype 1 and subtype 2b can be differentiated (Rijsewijk *et al*., 1999; Wyler *et al*., 1989). Restriction endonuclease digestion of viral DNA enables differentiation between BoHV-1 subtypes. RFLP analysis includes extraction of the DNA from virions or from infected cells, digestion of the isolated DNA by restriction endonucleases, and separation of the resulting fragments by agarose gel electrophoresis. Differentiation of the BoHV-1 subtypes 1, 2a and 2b by Hind*III* endonuclease digestion is based on the molecular weight of three relevant DNA fragments (*I*, *K* and *L*) (Metzler *et al*., 1985). RFLP techniques are of limited diagnostic value, but may be useful in epidemiological studies. Furthermore, RFLP pattern of virus isolates can be compared with that of live vaccine strains.

When differentiation is required between antigenically and genetically related alphaherpesviruses (BoHV-1, BoHV-5, caprine herpesvirus 1, cervid herpesvirus 1 and 2, elk herpesvirus 1, bubaline herpesvirus 1), improved methods are available using monoclonal antibodies (Keuser *et al*., 2004) or PCR amplification and sequencing (Ros *et al*., 1999).

HTS techniques can also be used to reveal the whole genome of BoHV-1 and compare different outbreak strains. The HTS is likely to be used more routinely due to improved technology and protocols. For optimised results, the virus titre should be as high as possible, and purified virus preparations allow the best sequencing results. See also Chapter 1.1.7 *Standards for high throughput sequencing, bioinformatics and computational genomics.*

1.6. Interpretation of results

The isolation of BoHV-1 from a diseased animal does not unequivocally mean that this virus is the cause of the illness. It may, for instance, be a latent virus that has been reactivated due to stressful conditions. A confirmatory laboratory diagnosis must be made from a group of animals and must be accompanied by seroconversion from negative to positive, or a four-fold or higher increase in BoHV-1-specific antibody titres. Paired serum samples collected 3–4 weeks apart are examined in a serological test for the presence of specific antibodies (see Section B.2).

2. Serological tests

Serological tests can be used for several purposes:

i) To diagnose an acute infection: paired serum samples from the acute and convalescent stages of infection of the same animals are examined in one test. A seroconversion from negative to positive or a four-fold or higher increase in antibody titres is considered to prove an acute infection.

ii) To demonstrate absence of infection, for instance, for international trade purposes.

iii) To determine the prevalence of infection in seroepidemiological studies.

iv) To support eradication programmes and subsequent surveillance.

v) For research purposes, for instance, the evaluation of the antibody response after vaccination and challenge infection.

Virus neutralisation (VN) tests (Bitsch, 1978) and various ELISAs (Kramps *et al*., 1993) are usually used for detecting antibodies against BoHV-1 in serum. Because virus latency is a normal sequel to BoHV-1 infection, the identification of serologically positive animals provides a useful and reliable indicator of infection status. Any animal with antibodies to the virus is considered to be a carrier and potential intermittent excretor of the virus. The only exceptions are calves that have acquired passive colostral antibodies from their dam, and noninfected cattle vaccinated with inactivated vaccines. It has been reported that, under experimental conditions, calves infected under cover of maternal immunity may become serologically negative while carrying a reactivatible latent infection (Lemaire *et al*., 2000).

In general, BoHV-1 serological tests can be divided into conventional and marker tests. Up to now, the only serological marker tests available are the BoHV-1 gE-antibody blocking ELISAs (Van Oirschot *et al*., 1997). Animals vaccinated with gE-deleted marker vaccines can be discriminated from field-virus infected animals by a negative serological reaction for gE. For conventional serology, VNT, BoHV1-antibody blocking ELISAs or indirect ELISAs may be used.

For the detection of antibodies in (bulk) milk samples ELISAs, including the gE-ELISA, are mainly used (Wellenberg *et al*., 1998a), but have some limitations. By testing bulk milk, a positive gB-specific test indicates the presence of several infected animals in the herd (Frankena *et al*., 1997). With the gE-blocking ELISA, bulk milk gives a positive reaction only when more than 10–15% of the herd is infected (Wellenberg *et al*., 1998b) although the sensitivity can be increased by milk concentration protocols (Schroeder *et al.,* 2012). Consequently, it is not possible to declare a herd to be free from BoHV-1 infection with these tests on the basis of bulk or pooled milk samples, and a negative gE- or gB-ELISA bulk milk test should be followed up with individual blood samples from all cattle in the herd. However, indirect ELISAs optimised for use with bulk milk samples of up to 50 individual cows (or up to 100 animals in BoHV-1-free regions) can indicate reliably the BoHV-1 status of these animals. These test systems are able to detect one weak positive sample in a pool of 50 milk samples or one strong positive milk in a pool composed of 100 samples. For general surveillance purposes, bulk milk tank tests can give an estimate of BoHV-1 prevalence in a herd, an area or country (Nylin *et al*., 2000). These should be supplemented by serum testing (individual or pooled) from non-milking herds. For monitoring a BoHV-1 status in dairy herds, bulk milk samples of up to 50 (or up to 100 in BoHV-1-free regions) animals should be tested 3–4 times per year with a suitable indirect ELISA. In herds of more than 50 (or 100 in BoHV-1-free regions) cows, several bulks of milk from the animals should be tested. Positive bulk milk results have to be confirmed by testing individual blood or milk samples from all animals that contributed to the positive bulk milk sample.

In an extensive study, tests for the detection of antibodies routinely used by national reference laboratories in Europe were evaluated (Kramps *et al*., 2004). Twelve reference laboratories from 12 European countries participated in this study. Fifty-three serum samples and 13 milk samples, originating from several countries, were sent in duplicate under code to the participating laboratories. The serum samples included the three European reference sera EU1 (antibody positive), EU2 (antibody weak positive and defined as borderline sample) and EU3 (antibody negative) (Perrin *et al*., 1994). At that time, it was concluded that VNT and gB-specific ELISAs are the most sensitive tests for the detection of antibodies in serum. However, modern ELISA systems show a clearly improved sensitivity. Owing to the very high sensitivity of the gB-blocking ELISAs, gB-antibody weak positive results can often not be confirmed by alternative test systems (indirect ELISA, VNT). Indirect BoHV-1 ELISAs have now been developed that are also highly sensitive and specific. The results of these ELISAs are comparable with those obtained using gB-blocking ELISAs (Beer *et al*., 2003).

gE-ELISAs are less sensitive and specific than the conventional test systems. In addition, seroconversion against gE can be delayed, especially in vaccinated animals, and is often not detectable before day 21 to 35 post-infection. A minimum of three different commercial gE-ELISA systems is currently available, allowing the analysis of samples with different gE-ELISAs each with unique characteristics.

2.1. Virus neutralisation

VN tests are performed with various modifications. Tests vary with regard to the virus strain used in the test protocol, the starting dilution of the serum, the virus/serum incubation period (1–24 hours), the type of cells used, the day of final reading and the reading of the end-point (50% versus 100%) (Perrin *et al*., 1993). Among these variables, the virus/serum incubation period has the most profound effect on the sensitivity of the VNT. A 24-hour incubation period may score up to 16-fold higher antibody titres than a 1-hour incubation period (Bitsch, 1978), and is recommended where maximum sensitivity is required (e.g. for international trade purposes). Various bovine cells or cell lines are suitable for use in the VN test, including secondary bovine kidney or testis cells, cell strains of bovine lung or tracheal cells, or the established MDBK cell line.

2.1.1. Suitable protocol for a VN test

i) Inactivate sera, including control standard sera, for 30 minutes in a water bath at 56°C.

ii) Make doubling dilutions of test sera in cell culture medium. Start with undiluted serum and continue to 1/1024 horizontally in a 96-well flat-bottomed cell-culture grade microtitre plate, at least three wells per dilution and 50 µl volumes per well. Dilutions of a positive

control serum, and of weak positive and negative internal control sera, are also included in the test. An extra well with undiluted test serum is used for toxicity control of sera.

iii) Add 50 µl per well of BoHV-1 stock at a dilution in culture medium calculated to provide 100–200 $TCID_{50}$ per well. In the toxicity control wells, add 50 µl of culture medium in place of virus. Add 100 µl of culture medium to ten empty wells for cell controls.

iv) Make at least four tenfold dilutions of the residual virus stock (back titration) in culture medium, using 50 µl per well and at least four wells per dilution.

v) Incubate the plates for 24 hours at 37°C.

vi) Add 100 µl per well of the cell suspension at 3×10^4 cells per well.

vii) Incubate the plates for 3–5 days at 37°C.

viii) Read the plates microscopically for CPEs. Validate the test by checking the back titration of virus (which should give a value of 100 $TCID_{50}$ with a permissible range of 30–300 $TCID_{50}$), the control sera and the cell control wells. The positive control serum should give a titre of ± 1 twofold dilution (±0.3 $\log_{10}$ units) from its target value. The weak positive serum should be positive. The negative serum should give no neutralisation (equivalent to a final dilution of 1/2 at the neutralisation stage). In the cell control wells, the monolayers should be intact.

ix) The test serum results are expressed as the reciprocal of the dilution of serum that neutralised the virus in 50% of the wells. If 50% of the wells with undiluted serum neutralised the virus, the (initial dilution) titre is read as 1 (1/2 using the final dilution convention). If all the undiluted and 50% of the wells with 1/2 diluted serum neutralised the virus, the (initial dilution) titre is 2 (final dilution 1/4). For qualitative results, any neutralisation at a titre of 1 or above (initial dilution convention) is considered to be positive. If cytotoxicity is observed in the control wells, the sample is reported to be toxic (no result) unless neutralisation of the virus without cytotoxicity is observed at higher dilutions and a titre can be read without ambiguity. Where cytotoxicity of a serum interferes with the interpretation of the neutralising activity of the sample, changing the medium in the wells of the lowest two or three dilutions 16–24 hours after the addition of cells may remove the cytotoxic effects.

2.2. Enzyme-linked immunosorbent assay

ELISAs for the detection of antibody against BoHV-1 are widely used, however a standard procedure for ELISA has not been established. Several types of ELISA are commercially available, including indirect and blocking ELISAs, some of which are also suitable for detecting antibodies in milk (Kramps *et al*., 2004). For reasons of standardisation in a country or state, it may be desirable to compare the quality of the kits and to perform batch release tests by previously defined criteria in a national reference laboratory, before it is used by other laboratories in the country.

There are a number of variations in the ELISA procedures. The most common are: antigen preparation and coating, the dilution of the test sample, the incubation period of antigen and test sample, and the substrate/chromogen solution. Before being used routinely, an ELISA should be validated with respect to sensitivity, specificity and reproducibility (see chapter 1.1.6). For this purpose, a comprehensive panel of well defined (e.g. by VN test) strong positive, weak positive and negative sera has to be tested. However, it is recommended to use commercially available ELISAs that have been shown to perform better than home-made assays (Kramps *et al*., 2004).

2.2.1. Indirect enzyme-linked immunosorbent assay

The principle of an indirect ELISA is based on the binding of BoHV-1-specific antibodies present in the test sample to immobilised BoHV-1 antigen. The bound antibodies are detected using enzyme-labelled anti-bovine immunoglobulin antiserum. The presence of antibodies in the test sample will result in colour development after addition of the substrate/chromogen solution. This test principle is the most suitable for individual and pooled milk samples.

2.2.2. Blocking enzyme-linked immunosorbent assay

The principle of a blocking or competitive ELISA is based on blocking the binding of an enzyme-labelled BoHV-1 antiserum or anti-BoHV-1 MAb to immobilised antigen by antibodies in the test sample. The presence of antibodies in the test sample results in reduced colour development after addition of the substrate/chromogen solution. An example of a gB blocking ELISA procedure is given below:

i) Prepare the antigen by growing BoHV-1 in cell cultures. When extensive CPE is observed, cells and medium are frozen at –20°C. After thawing, the resulting cellular lysate is centrifuged for 4 hours at 8500 ***g***. The virus-containing pellet is suspended in a small volume of phosphate buffered saline (PBS), cooled on ice and disrupted using an ultrasonic disintegrator. The antigen preparation is then centrifuged for 10 minutes at 800 ***g***, and inactivated by adding detergent (final concentration of 0.5% Nonidet P 40). The antigen preparation is used at an appropriate dilution to coat the microtitre plates. Many alternative methods of antigen production are described in the published literature.

ii) Coat the microtitre plates with antigen by adding 100 µl of diluted antigen (in 0.05 M carbonate buffer, pH 9.6) to each well. Seal the plates with tape, incubate at 37°C overnight, and store at –20°C.

iii) Before the test is performed, wash the plates with 0.05% Tween 80. Add 100 µl negative serum (fetal calf serum, FCS), 100 µl of each of the serum test samples and 100 µl of positive, weak positive and negative control sera. Usually, serum samples are tested undiluted. Shake, seal the plates and incubate overnight at 37°C. If false-positive reactions are suspected (e.g. when epidemiologically implausible results are generated), it is recommended to retest such sera after heating for 30 minutes at 56°C following one freeze–thaw cycle.

iv) Wash the plates thoroughly and add 100 µl of an anti-BoHV-1-gB-monoclonal antibody/horseradish peroxidase conjugate at a predetermined dilution, and incubate again for 1 hour at 37°C. The monoclonal antibody must be selected carefully for its specificity to gB of BoHV-1.

v) Wash the plates and add freshly prepared substrate/chromogen solution (e.g. 0.05 M citric acid buffer, pH 4.5, containing 2,2'-azino-bis-[3-ethylbenzothiazoline]-6-sulphonic acid [ABTS; 0.55 mg/ml] and a 3% solution of freshly added H_2O_2 [5 µl/ml]), and incubate for the appropriate time (1–2 hours at room temperature).

vi) Measure the absorbance of the plates on a microplate photometer at 405 nm.

vii) Calculate for each test sample the blocking percentage [$(OD_{FCS} - OD_{test\ sample})/OD_{FCS} \times 100\%$]

vii) A test sample is considered to be positive if it has a blocking percentage of e.g. 50% of higher. The test is valid if the positive and weak positive control sera are positive and the negative control serum reacts negatively. The acceptable limits for control and cut-off values must be determined for the individual assay.

2.3. Standardisation

In each serological test, appropriate controls of strong positive, weak positive and negative serum should be included. A scientific group in Europe, initiated by the group of artificial insemination veterinarians of the European Union (EU), has agreed on the use of a strong positive (EU1), a weak positive (EU2) and negative serum (EU3) for standardisation of BoHV-1 tests in laboratories that routinely examine samples from artificial insemination centres (Perrin *et al*., 1994). These sera have been adopted as WOAH international standards for BoHV-1 tests and are available in limited quantities at the WOAH Reference Laboratories for IBR/IPV[3]. Tests that are suitable for certifying individual animals prior to movement (VN or ELISA) must be capable of scoring both the strong and weak positive standards (or secondary national standards of equivalent potency) as positive. Because of the limited

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availability of the international standard sera, there is a need to prepare a new extended panel of reference lyophilised serum (and milk) samples taken from infected as well as from vaccinated animals. This panel should be used to validate newly developed tests and to harmonise tests between laboratories. Additional reference sera are available in limited quantities from the WOAH Reference Laboratories (e.g. R1, R2 and R3 as positive, weak positive and very weak positive standard sera from the WOAH Reference Laboratory in Germany).

2.4. Nonspecific reactivity in BoHV-1-serology and 'pseudo-vaccines'

Nonspecific reactivity of sera in the BoHV-1-ELISAs should be taken into consideration, and is more often seen for the marker test than for the conventional serology. There are several reasons for nonspecific reactions:

i) Batch variation of the ELISA used;

ii) Samples were tested very early after collection (freshness phenomenon);

iii) Samples tested by gE ELISA were collected within 4 weeks after vaccination with a marker vaccine (vaccination phenomenon);

iv) Bad specimen quality (e.g. haemolysed samples).

Therefore the following measures should be considered:

i) Validation of each test batch, and batch release tests have to be implemented;

ii) Specimens should be stored at 4°C and should not be tested before 24–48 hours after sample collection;

iii) Samples should be subjected to a freeze–thaw cycle (–20°C); in some cases a subsequent heat inactivation (30 minutes/56°C) may eliminate nonspecific reactions of serum samples;

iv) Cattle should not be serologically tested for BoHV-1 prior to 4 weeks after any vaccination;

v) gE-ELISAs should not be used for classification of unvaccinated animals.

In IBR/IPV-free regions, implausible singleton reactors are occasionally detected in gB blocking and indirect ELISAs, which cause problems concerning the status of both the animal and the affected herd. It has been suggested that BoHV-2 infection could be the cause of this cross reactivity (Böttcher *et al.,* 2012). Interestingly, the gE-ELISAs are the most BoHV-1-specific serological tests available and cross reactivity with non-BoHV-1 herpesviruses is very low in these ELISAs. This is especially critical in BoHV-1-free regions where single animals with cross reactivity are detected and might be misinterpreted as BoHV-1 incursion into the herd. Therefore, in free regions, gE-ELISAs and VN tests should be used to further verify such singleton reactors. One recommendation is to repeat testing of those animals after 28 days in a gE-ELISA using a lower cut-off (e.g. positive/negative of 0.95) to increase the sensitivity. In the case of two negative gE-ELISA results, the animal is not classified as BoHV-1-positive, but it is recommended to slaughter the animal as diagnostic problems with other test systems may still occur (e.g. positive bulk milk results of the affected herd).

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Several attenuated and inactivated BoHV-1 vaccines are currently available. The vaccine strains have usually undergone multiple passages in cell culture. Some of the vaccine virus strains have a temperature-sensitive phenotype, i.e. they do not replicate at temperatures of 39°C or higher. Attenuated vaccines are administered intranasally or intramuscularly. Inactivated vaccines contain high levels of inactivated virus or portions of the virus particle (glycoproteins) supplemented with an adjuvant to stimulate an adequate immune response. Inactivated vaccines are given intramuscularly or

subcutaneously. Vaccination against BoHV-1 is used to protect animals from the clinical outcome of infection, and as an aid in control and eradication programmes.

Marker or DIVA (differentiation of infected from vaccinated animals) vaccines are now available in various countries. These attenuated or inactivated marker vaccines are based on deletion mutants (deletion of gE) or on a subunit of the virion, for instance glycoprotein D. The use of such marker vaccines in conjunction with companion diagnostic tests allows the distinction between infected and vaccinated cattle (DIVA principle), and provides the basis for BoHV-1 eradication programmes in countries or regions with a high prevalence of field-virus infected animals. Intensive vaccination programmes can reduce the prevalence of infected animals (Bosch *et al*., 1998; Mars *et al*., 2001), which could be monitored by using an appropriate diagnostic test. In situations where it is economically justifiable, the residual infected animals could be slaughtered, resulting in a region free from BoHV-1. Control and eradication of BoHV-1 was started in some countries in the early 1980s. Different policies have been used due to differences in herd prevalence, breeding practices and disease eradication strategies. To date, in the EU, only gE-deleted DIVA vaccines (live as well as killed) have been marketed and used for control or eradication programmes.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are of general nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The vaccine is prepared using a seed-lot-like system. Origin, passage history and storage conditions of the master seed virus (MSV) must be recorded. A virus identity test must be performed on the MSV. The seed lot contains BoHV-1 strains have to be attenuated to yield a live vaccine strain. The strains can be attenuated by multiple passages in cell cultures, by adapting virus to grow at low temperatures (temperature-sensitive mutants), or by genetic engineering, for example, by deleting one or more viral genes (e.g. the BoHV-1 glycoprotein E) that are nonessential for replication. There should be some means of distinguishing the live vaccine virus from field viruses (for example temperature-specific growth patterns or restriction fragment length polymorphisms). Strains used for the preparation of inactivated vaccines need not be attenuated. The seed lot must be free from contaminants.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The seed lot is tested for absence of extraneous viruses and absence from contamination with bacteria, fungi or mycoplasma. The following extraneous viruses should be specifically excluded in BoHV-1 vaccines: adenovirus, Akabane virus, Schmallenberg virus, bovine coronavirus, bovine herpesviruses 2, 4 and 5, bovine parvovirus, bovine respiratory syncytial virus, bovine viral diarrhoea virus and atypical pestiviruses, bovine rotavirus, vaccinia virus, and the viruses of Aujeszky's disease, bluetongue, bovine ephemeral fever, bovine leukaemia, bovine papilloma, bovine papular stomatitis, cowpox, foot and mouth disease, lumpy skin disease, malignant catarrhal fever, parainfluenza 3, rabies, rinderpest, and vesicular stomatitis. As bovine viral diarrhoea virus (either CPE or non-CPE) has regularly been found to be a contaminant of vaccines, special attention should be paid to the absence of BVDV. In addition, new atypical pestiviruses (e.g. HoBi or HoBi-like) have to be taken into consideration as possible contaminants.

2.2. Method of manufacture

2.2.1. Procedure

The cells used for vaccine production are prepared using a master-cell-lot system. The virus should be cultured on established cell lines that have been shown to be suitable for vaccine production, for example the Madin–Darby bovine kidney (MDBK) cell line. The history of the cell

line must be known. The cell line must be free from extraneous agents and may be tested for tumorigenicity.

2.2.2. Requirements for substrates and media

All substances used for the manufacture of vaccines must be free from contaminants. Cells should be used that are not further than 20 passages from the master cell stock. The seed virus should not be more than five passages from the MSV. Genetically engineered vaccine virus strains are treated in the same way as conventionally attenuated vaccine virus strains. When sufficient cells are grown, infection of the cell line with the vaccine virus takes place. The addition of antibiotics is normally restricted to cell culture fluids. The supernatant fluid is harvested at times when the virus (antigen) production peaks. For live vaccines, the supernatant is clarified, mixed with a stabiliser, freeze-dried and bottled. For the production of classical inactivated vaccines, the supernatant is homogenised before the inactivating agent is added in order to ensure proper inactivation. After the inactivation procedure, a test for ensuring complete inactivation of the virus is carried out. The test should include at least two passages in cells. The inactivated virus suspension is then mixed with an adjuvant and bottled. The manufacture of vaccines must comply with guidelines for Good Manufacturing Practice (GMP) and local regulations such as European Pharmacopoeia or United States Code of Federal Regulations (9CFR).

2.2.3. In-process controls

Working cell seed and working virus seed must have been shown to be free from contaminants. The cells must show inconspicuous morphology before being inoculated with virus. The CPE is checked during cultivation. Uninoculated control cells must have retained their morphology until the time of harvesting. A virus titration is performed on the harvested supernatant. During the production of inactivated vaccines, tests are performed to ensure inactivation. The final bulk must be tested for freedom from contaminants.

2.2.4. Final product batch tests

The following tests must normally be performed on each batch. Example guidelines for performing batch control can be found in EU directives, the European Pharmacopoeia and 9CFR.

i) Sterility/purity

Bacteria, fungi, mycoplasma and extraneous viruses must not be present. Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

For inactivated vaccines, a twofold dose of vaccine, and for live vaccines, a tenfold dose of vaccine, must not produce adverse effects in young BoHV-1 seronegative calves.

iii) Batch potency

It is sufficient to test one representative batch for efficacy, as described in Section C.2.3.2. In the case of live vaccines, the virus titre of each batch must be determined and must be not higher than 1/10 of the dose at which the vaccine has been shown to be safe, and no lower than the minimum release titre. In the case of inactivated vaccines, the potency is tested using another validated method, for instance, efficacy assessment in calves.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

A quantity of virus equivalent to ten doses of vaccine should (a) not induce significant local or systemic reactions in young calves; (b) not cause fetal infection or abortion, and (c) not revert to virulence during five serial passages in calves. For inactivated vaccine, a double

dose is usually administered. The reversion to virulence test is not applicable to inactivated vaccines.

ii) Reversion-to-virulence for attenuated/live vaccines

The selected final vaccine strain should not revert to virulence during a minimum of five serial passages in calves.

iii) Environmental consideration

Attenuated vaccine strains should not be able to perpetuate autonomously in a cattle population (R0 <1).

2.3.2. Efficacy requirements

i) For animal production

This must be shown in vaccination challenge experiments under laboratory conditions. Example guidelines are given in a monograph of the European Pharmacopoeia (Third Edition (1997). Briefly, the vaccine is administered to ten 2–3-month-old BoHV-1 seronegative calves. Two calves are kept as controls. All the calves are challenged intranasally 3 weeks later with a virulent strain of BoHV-1 that gives rise to typical clinical signs of a BoHV-1 infection. The vaccinated calves should show no or only very mild signs. The maximum (peak) virus titre in the nasal mucus of vaccinated calves should be at least 100 times lower than that in control calves. The virus excretion period should be at least 3 days shorter in vaccinated than in control calves.

An efficacious BoHV-1 vaccine should induce protective immunity for at least 1 year, although many existing vaccines have not been tested to this standard.

ii) For control and eradication

In addition to the above-mentioned criteria, BoHV-1-vaccines for control and eradication should be marker vaccines (e.g. gE-deleted vaccines) allowing the differentiation of infected from vaccinated animals (DIVA-strategy). Several gE-deleted vaccines (inactivated preparations as well as modified live vaccines) are commercially available.

2.3.3. Stability

For live vaccines, virus titrations should be carried out 3 months beyond the indicated shelf life. In addition, tests for determining moisture content, concentrations of preservatives, and pH are performed. For inactivated vaccines, the viscosity and stability of the emulsion are also tested.

The efficacy of preservatives should be demonstrated. The concentration of the preservative and its persistence throughout shelf life should be checked. The concentration must be in conformity with the limits set for the preservative.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Glycoprotein E (gE)-deleted inactivated and modified live vaccines are available that are based on recombinant strains. The vaccines are comparable to other gE-deleted vaccines and are licensed by European Medicines Agency (EMA) for use in the EU.

Additional recombinant vaccines like gD-subunits or genetically engineered deletion mutants of BoHV-1 (e.g. with deletions of gE and/or gG) are described and available as prototypes.

Advantages of BoHV-1 vaccines based on biotechnology could be the possibility of additional marker features for the differentiation of infected from vaccinated animals (DIVA; e.g. gB-antibody-ELISAs for gD-subunit vaccines or gG-antibody-ELISAs for the respective deletion mutants).

3.2. Special requirements for biotechnological vaccines, if any

Recombinant vaccines, which are destined for use in the EU have to be licensed by EMA.

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NB: There are WOAH Reference Laboratories for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis

NB: FIRST ADOPTED IN 1990 AS INFECTIOUS BOVINE RHINOTRACHEITIS. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 3.4.12.

LUMPY SKIN DISEASE

SUMMARY

Description of the disease: *Lumpy skin disease (LSD) is a poxvirus disease of cattle characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death. The disease is of economic importance as it can cause a temporary reduction in milk production, temporary or permanent sterility in bulls, damage to hides and, occasionally, death. Various strains of capripoxvirus are responsible for the disease. These are antigenically indistinguishable from strains causing sheep pox and goat pox yet distinct at the genetic level. LSD has a partially different geographical distribution from sheep and goat pox, suggesting that cattle strains of capripoxvirus do not infect and transmit between sheep and goats. Transmission of LSD virus (LSDV) is thought to be predominantly by arthropods, natural contact transmission in the absence of vectors being inefficient. Lumpy skin disease is endemic in many African and Middle Eastern countries. Between 2012 and 2022, LSD spread into south-east Europe, the Balkans, Russia and Asia as part of the Eurasian LSD epidemic.*

Pathology: *the nodules are firm, and may extend to the underlying subcutis and muscle. Acute histological key lesions consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis. Chronic key histological lesions consist of fibrosis and necrotic sequestra.*

Detection of the agent: *Laboratory confirmation of LSD is most rapid using a real-time or conventional polymerase chain reaction (PCR) method specific for capripoxviruses in combination with a clinical history of a generalised nodular skin disease and enlarged superficial lymph nodes in cattle. Ultrastructurally, capripoxvirus virions are distinct from those of parapoxvirus, which causes bovine papular stomatitis and pseudocowpox, but cannot be distinguished morphologically from orthopoxvirus virions, including cowpox and vaccinia viruses, both of which can cause disease in cattle, although neither causes generalised infection and both are uncommon in cattle. LSDV will grow in tissue culture of bovine, ovine or caprine origin. In cell culture, LSDV causes a characteristic cytopathic effect and intracytoplasmic inclusion bodies that is distinct from infection with Bovine herpesvirus 2, which causes pseudo-lumpy skin disease and produces syncytia and intranuclear inclusion bodies in cell culture. Capripoxvirus antigens can be demonstrated in tissue culture using immunoperoxidase or immunofluorescent staining and the virus can be neutralised using specific antisera.*

A variety of conventional and real-time PCR tests as well as isothermal amplification tests using capripoxvirus-specific primers have been published for use on a variety of samples.

Serological tests: *The virus neutralisation test (VNT) and enzyme-linked immunosorbent assays (ELISAs) are widely used and have been validated. The agar gel immunodiffusion test and indirect immunofluorescent antibody test are less specific than the VNT due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of LSDV with test sera is both sensitive and specific, but is difficult and expensive to carry out.*

Requirements for vaccines: *All strains of capripoxvirus examined so far, whether derived from cattle, sheep or goats, are antigenically similar. Attenuated cattle strains, and strains derived from sheep and goats, have been used as live vaccines against LSDV.*

A. INTRODUCTION

Lumpy skin disease (LSD) was first seen in Zambia in 1929, spreading into Botswana by 1943 (Haig, 1957), and then into South Africa the same year, where it affected over eight million cattle causing major economic loss. In 1957 it entered Kenya, at the same time as an outbreak of sheep pox (Weiss, 1968). In 1970 LSD spread north into the Sudan, by 1974 it had spread west as far as Nigeria, and in 1977 was reported from Mauritania, Mali, Ghana and Liberia. Another epizootic of LSD between 1981 and 1986 affected Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, with reported mortality rates in affected cattle of 20%. The occurrence of LSD north of the Sahara desert and outside the African continent was confirmed for the first time in Egypt and Israel between 1988 and 1989, and was reported again in 2006 (Brenner *et al.,* 2006). In the past decade, LSD occurrences have been reported in the Middle Eastern, European and Asian regions (for up-to-date information, consult WOAH WAHIS interface[1]). Lumpy skin disease outbreaks tend to be sporadic, depending upon animal movements, immune status, and wind and rainfall patterns affecting vector populations. The principal method of transmission is thought to be mechanical by various arthropod vectors (Tuppurainen *et al*., 2015).

Lumpy skin disease virus (LSDV) belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae*, and genus *Capripoxvirus*. In common with other poxviruses LSDV replicates in the cytoplasm of an infected cell, forming distinct perinuclear viral factories. The LSD virion is large and brick-shaped measuring 293–299nm (length) and 262–273nm (width). The LSDV genome structure is also similar to other poxviruses, consisting of double-stranded linear DNA that is 25% GC-rich, approximately 150,000 bp in length, and encodes around 156 open reading frames (ORFs). An inverted terminal repeat sequence of 2200–2300 bp is found at each end of the linear genome. The linear ends of the genome are joined with a hairpin loop. The central region of the LSDV genome contains ORFs predicted to encode proteins required for virus replication and morphogenesis and exhibit a high degree of similarity with genomes of other mammalian poxviruses. The ORFs in the outer regions of the LSDV genome have lower similarity and likely encode proteins involved in viral virulence and host range determinants.

Phylogenetic analysis shows the majority of LSDV strains group into two monophyletic clusters (cluster 1.1 and 1.2) (Biswas *et al.,* 2020; Van Schalkwyk *et al.,* 2021). Cluster 1.1 consists of LSDV Neethling vaccine strains that are based on the LSDV/Neethling/LW-1959 vaccine strain (Kara *et al.,* 2003; Van Rooyen *et al.,* 1959; van Schalkwyk *et al.,* 2020) and historic wild-type strains from South Africa. Cluster 1.2 consists of wild-type strains from southern Africa, Kenya, the northern hemisphere, and the Kenyan KSGP O-240 commercial vaccine. In addition to these two clusters, there have recently been recombinant LSDV strains isolated from clinical cases of LSD in the field in Russia and central Asia (Flannery *et al.,* 2021; Sprygin *et al.,* 2018; 2020; Wang *et al.,* 2021). These recombinant viruses show unique patterns of accessory gene alleles, consisting of sections of both wild-type and "vaccine" LSDV strains.

The severity of the clinical signs of LSD is highly variable and depends on a number of factors, including the strain of virus, the age of the host, immunological status and breed. *Bos taurus* is generally more susceptible to clinical disease than *Bos indicus;* the Asian buffalo (*Bubalus spp.*) has also been reported to be susceptible. Within *Bos taurus,* the fine-skinned Channel Island breeds develop more severe disease, with lactating cows appearing to be the most at risk. However, even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the clinical signs presented, ranging from subclinical infection to death (Carn & Kitching, 1995). There may be failure of the virus to infect the whole group, probably depending on the virulence of the virus isolate, immunological status of the host, host genotype, and vector prevalence.

The incubation period under field conditions has not been reported, but following experimental inoculation is 6–9 days until the onset of fever. In the acutely infected animal, there is an initial pyrexia, which may exceed 41°C and persist for 1 week. All the superficial lymph nodes become enlarged. In lactating cattle there is a marked reduction in milk yield. Lesions develop over the body, particularly on the head, neck, udder, scrotum, vulva and perineum between 7 and 19 days after virus inoculation (Coetzer, 2004). The characteristic integumentary lesions are multiple, well circumscribed to coalescing, 0.5–5 cm in diameter, firm, flat-topped papules and nodules. The nodules involve the dermis and epidermis, and may extend to the underlying subcutis and occasionally to the adjacent striated muscle. These nodules have a creamy grey to white colour on cut section, which may initially exude serum, but over the ensuing 2 weeks a cone-shaped central core or sequestrum of necrotic material/necrotic plug ("sit-fast") may appear within the nodule. The acute histological lesions consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis. The inclusion bodies are numerous, intracytoplasmic, eosinophilic, homogenous to occasionally granular and they may occur in endothelial cells, fibroblasts, macrophages, pericytes, and keratinocytes. The dermal lesions include vasculitis with fibrinoid necrosis, oedema, thrombosis, lymphangitis, dermal-epidermal separation, and mixed inflammatory infiltrate. The chronic lesions are

1 https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/

characterised by an infarcted tissue with a sequestered necrotic core, often rimmed by granulation tissue gradually replaced by mature fibrosis. At the appearance of the nodules, the discharge from the eyes and nose becomes mucopurulent, and keratitis may develop. Nodules may also develop in the mucous membranes of the mouth and alimentary tract, particularly the abomasum and in the trachea and the lungs, resulting in primary and secondary pneumonia. The nodules on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly ulcerate, and by then all secretions, ocular and nasal discharge and saliva contain LSD virus (LSDV). The limbs may be oedematous and the animal is reluctant to move. Pregnant cattle may abort, and there is a report of intrauterine transmission (Rouby & Aboulsoudb, 2016). Bulls may become permanently or temporarily infertile and the virus can be excreted in the semen for prolonged periods (Irons *et al*., 2005). Recovery from severe infection is slow; the animal is emaciated, may have pneumonia and mastitis, and the necrotic plugs of skin, which may have been subject to fly strike, are shed leaving deep holes in the hide (Prozesky & Barnard, 1982).

The main differential diagnosis is pseudo-LSD caused by bovine herpesvirus 2 (BoHV-2). This is usually a milder clinical condition, characterised by superficial nodules, resembling only the early stage of LSD. Intra-nuclear inclusion bodies and viral syncytia are histopathological characteristics of BoHV-2 infection not seen in LSD. Other differential diagnoses (for integumentary lesions) include: dermatophilosis, dermatophytosis, bovine farcy, photosensitisation, actinomycosis, actinobacillosis, urticaria, insect bites, besnoitiosis, nocardiosis, demodicosis, onchocerciasis, pseudo-cowpox, and cowpox. Differential diagnoses for mucosal lesions include: foot and mouth disease, bluetongue, mucosal disease, malignant catarrhal fever, infectious bovine rhinotracheitis, and bovine papular stomatitis.

LSDV is not transmissible to humans. However, all laboratory manipulations must be performed at an appropriate containment level determined using biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of LSD and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Virus isolation	+	++	+	+++	+	–
PCR	++	+++	++	+++	+	–
Transmission electron microscopy	–	–	–	+	–	–
Detection of immune response						
VNT	++	++	++	++	++	++
IFAT	+	+	+	+	+	+
ELISA	++	++	++	++	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; VNT = virus neutralisation test;
IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

1. Detection of the agent

1.1. Specimen collection, submission and preparation

Material for virus isolation and antigen detection should be collected as biopsies or from skin nodules at post-mortem examination. Samples for virus isolation should preferably be collected within the first week of the occurrence of clinical signs, before the development of neutralising antibodies (Davies, 1991; Davies *et al*., 1971); however virus can be isolated from skin nodules for at least 3–4 weeks thereafter. Samples for genome detection using conventional or real-time polymerase chain reaction (PCR) may be collected when neutralising antibody is present. Following the first appearance of the skin lesions, the virus can be isolated for up to 35 days and viral nucleic acid can be demonstrated via PCR for up to 3 months (Tuppurainen *et al*., 2005; Weiss, 1968). Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of LSD (before generalisation of lesions or within 4 days of generalisation), can also be used for virus isolation.

Blood samples with anticoagulant for virus isolation from the buffy coat should be placed immediately on ice after gentle mixing and processed as soon as possible. In practice, the samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues for virus isolation and antigen detection should be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size (e.g. 1 g in 10 ml) that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation.

Samples for histology should include the lesion and tissue from the surrounding (non-lesion) area, be a maximum size of 2 cm^3, and be placed immediately following collection into ten times the sample volume of 10% neutral buffered formaldehyde. Tissues in formalin have no special transportation requirements in regard to biorisks. Material for histology should be prepared using standard techniques and stained with haematoxylin and eosin (H&E) (Burdin, 1959).

Lesion material for virus isolation and antigen detection is minced using a sterile scalpel blade and forceps and then macerated in a sterile steel ball-bearing mixer mill, or ground with a pestle in a sterile mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) or serum-free modified Eagle's medium containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (amphotericin, 2.5 μg/ml) and neomycin (200 IU/ml). The suspension is freeze–thawed three times and then partially clarified using a bench centrifuge at 600 ***g*** for 10 minutes. In cases where bacterial contamination of the sample is expected (such as when virus is isolated from skin samples), the supernatant can be filtered through a 0.45 μm pore size filter after the centrifugation step. Buffy coats may be prepared from unclotted blood using centrifugation at 600 ***g*** for 15 minutes, and the buffy coat carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 ***g*** for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml growth medium, such as Glasgow's modified Eagle's medium (GMEM). After centrifugation at 600 ***g*** for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample by using a Ficoll gradient.

1.2. Virus isolation on cell culture

LSDV will grow in tissue culture of bovine, ovine or caprine origin. MDBK (Madin–Darby bovine kidney) cells are often used, as they support good growth of the virus and are well characterised (Fay *et al.,* 2020). Primary cells, such as lamb testis (LT) cells, also support viral growth, but care needs to be taken to ensure they are not contaminated with viruses such as bovine viral diarrhoea virus. One ml of clarified supernatant or buffy coat is inoculated onto a confluent monolayer in a 25 cm^2 culture flask at 37°C and allowed to adsorb for 1 hour. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing appropriate cells and a flying cover-slip, or tissue culture microscope slides, are also infected.

The flasks/tissue culture tubes are examined daily for 7–14 days for evidence of cytopathic effects (CPE). Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from

surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 2 days after infection; over the following 4–6 days these expand to involve the whole cell monolayer. If no CPE is apparent by day 14, the culture should be freeze–thawed three times, and clarified supernatant inoculated on to a fresh cell monolayer. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are diagnostic for poxvirus infection. PCR may be used as an alternative to H&E for confirmation of the diagnosis. The CPE can be prevented or delayed by adding specific anti-LSDV serum to the medium. In contrast, the herpesvirus that causes pseudo-LSD produces a Cowdry type A intranuclear inclusion body. It also forms syncytia.

An ovine testis cell line (OA3.Ts) has been evaluated for the propagation of capripoxvirus isolates (Babiuk *et al.*, 2007), however this cell line has been found to be contaminated with noncytopathic pestivirus and should be used with caution.

1.3. Polymerase chain reaction

The conventional gel-based PCR method described below is a simple, fast and sensitive method for the detection of capripoxvirus genome in EDTA blood, semen or tissue culture samples (Tuppurainen *et al*., 2005).

1.3.1. Test procedure

The extraction method described below can be replaced using commercially available DNA extraction kits.

i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM Tris/HCl (pH 8); and 0.5 ml Tween 20.

ii) Cut skin and other tissue samples into fine pieces using a sterile scalpel blade and forceps. Grind with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned lysis buffer.

iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K (20 mg/ml) to tissue samples. Incubate at 56°C for 2 hours or overnight, followed by heating at 100°C for 10 minutes. Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to the samples in a 1:1 ratio. Vortex and incubate at room temperature for 10 minutes. Centrifuge the samples at 16,060 ***g*** for 15 minutes at 4°C. Carefully collect the upper, aqueous phase (up to 200 µl) and transfer into a clean 2.0 ml tube. Add two volumes of ice cold ethanol (100%) and 1/10 volume of 3 M sodium acetate (pH 5.3). Place the samples at –20°C for 1 hour. Centrifuge again at 16,060 ***g*** for 15 minutes at 4°C and discard the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and centrifuge at 16,060 ***g*** for 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in 30 µl of nuclease-free water and store immediately at –20°C (Tuppurainen *et al*., 2005). Alternatively a column-based extraction kit may be used.

iv) The primers for this PCR assay were developed from the gene encoding the viral attachment protein. The size of the expected amplicon is 192 bp (Ireland & Binepal, 1998). The primers have the following gene sequences:

Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'

Reverse primer 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.

v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl of $MgCl_2$ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl of nuclease-free water. The volume of DNA template required may vary and the volume of nuclease-free water must be adjusted to the final volume of 50 µl.

vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at 95°C, 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold at 4°C until analysis.

vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder. Electrophoretically separate the products using approximately 8–10 V/cm for 40–60 minutes and visualise with a suitable DNA stain and transilluminator.

Quantitative real-time PCR methods have been described that are reported to be faster and have higher sensitivity than conventional PCRs (Balinsky *et al*., 2008; Bowden *et al*., 2008). A real-time PCR method that differentiates between LSDV, sheep pox virus and goat pox virus has been published (Lamien *et al.,* 2011).

Quantitative real-time PCR assays have been designed to differentiate between Neethling-based LSDV strains, which are often used for vaccination, and wild-type LSDV strains from cluster 1.2 (Agianniotaki *et al.,* 2017; Pestova *et al.,* 2018; Vidanovic *et al.,* 2016). These "DIVA" assays (DIVA: differentiation of infected from vaccinated animals) enable, for example, differentiation of "Neethling response" caused by vaccination with a LSDV Neethling vaccine strain from disease caused by infection with a cluster 1.2 wild-type virus. However, these DIVA PCR assays cannot distinguish between a LSDV Neethling vaccine strain and the novel recombinant LSDV strains recently isolated from disease outbreaks in Asia (Byadovskaya *et al.,* 2021; Flannery *et al.,* 2021). These DIVA assays are also not capable of discriminating between LSDV Neethling vaccine strains and recently characterised (historic) wild-type viruses from South Africa belonging within cluster 1.1 (Van Schalkwyk *et al.,* 2020; 2021). Consequently, in regions where recombinant strains (currently Asia and possibly elsewhere) or wild-type cluster 1.1 strains (currently South Africa and possibly elsewhere) are circulating, these DIVA assays are not suitable for distinguishing vaccine and wild-type virus. Thus, in order to overcome these constraints, whole genome sequencing is recommended.

1.4. Transmission electron microscopy

The characteristic poxvirus virion can be visualised using a negative staining preparation technique followed by examination with an electron microscope. There are many different negative staining protocols, an example of which is given below.

1.4.1. Test procedure

Before centrifugation, material from the original biopsy suspension is prepared for examination under the transmission electron microscope by floating a 400-mesh hexagonal electron microscope grid, with pioloform-carbon substrate activated by glow discharge in pentylamine vapour, onto a drop of the suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The capripox virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

The capripox virions are indistinguishable from those of orthopoxvirus, but, apart from vaccinia virus and cowpox virus, which are both uncommon in cattle and do not cause generalised infection, no other orthopoxvirus is known to cause lesions in cattle. However, vaccinia virus may cause generalised infection in young immunocompromised calves. In contrast, orthopoxviruses are a common cause of skin disease in domestic buffalo causing buffalo pox, a disease that usually manifests as pock lesions on the teats, but may cause skin lesions at other sites, such as the perineum, the medial aspects of the thighs and the head. Orthopoxviruses that cause buffalo pox cannot be readily distinguished from capripoxvirus by electron microscopy. The parapoxvirus virions that cause bovine papular stomatitis and pseudocowpox are smaller, oval in shape and each is covered in a single continuous tubular element that appears as striations over the virion. Capripoxvirions are also distinct from the herpesvirus that causes pseudo-LSD (also known as "Allerton" or bovine herpes mammillitis).

1.5. Fluorescent antibody tests

Capripoxvirus antigen can be identified on infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test using immune cattle sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from capripox) or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control as cross-reactions can cause problems due to antibodies to cellular components (pre-absorption of these from the immune serum helps solve this issue).

1.6. Immunohistochemistry

Immunohistochemistry using F80G5 monoclonal antibody specific for capripoxvirus ORF 057 has been described for detection of LSDV antigen in the skin of experimentally infected cattle (Babiuk *et al.,* 2008).

1.7. Isothermal genome amplification

Molecular tests using loop-mediated isothermal amplification to detect capripoxvirus genomes are reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and lower cost (Das *et al.,* 2012; Murray *et al.,* 2013). Field validation of the Das *et al.* method was reported by Omoga *et al.* (2016).

2. Serological tests

All the viruses in the genus *Capripoxvirus* share a common major antigen for neutralising antibodies and it is thus not possible to distinguish strains of capripoxvirus from cattle, sheep or goats using serological techniques.

2.1. Virus neutralisation

A test serum can either be titrated against a constant titre of capripoxvirus (100 $TCID_{50}$ [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the accurate and repeatable seeding of 100 $TCID_{50}$/well, the neutralisation index is the preferred method in most laboratories, although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue-culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes.

2.1.1. Test procedure

i) Test sera, including a negative and a positive control, are diluted 1/5 in Eagle's/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) buffer and inactivated at 56°C for 30 minutes.

ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES buffer (without serum) is placed in columns 11 and 12, and to all wells in row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over $\log_{10}$ 6 $TCID_{50}$ per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log dilution series of $\log_{10}$ 5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5 $TCID_{50}$ per ml (equivalent to $\log_{10}$ 3.7, 2.7, 2.2, 1.7, 1.2, 0.7, 0.2 $TCID_{50}$ per 50 µl).

iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

v) The plates are covered and incubated for 1 hour at 37°C.

vi) An appropriate cell suspension (such as MDBK cells) is prepared from pregrown monolayers as a suspension of 10^5 cells/ml in Eagle's medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum controls.

vii) The microtitre plates are covered and incubated at 37°C for 9 days.

viii) Using an inverted microscope, the monolayers are examined daily from day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, by way of example, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated using the Kärber method. If left longer, there is invariably a 'breakthrough' of virus in which virus that was at first neutralised appears to disassociate from the antibody.

ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because the immunity to capripoxviruses is predominantly cell mediated, a negative result, particularly following vaccination, after which the antibody response may be low, does not imply that the animal from which the serum was taken is not protected.

Antibodies to capripoxvirus can be detected from 1 to 2 days after the onset of clinical signs. These remain detectable for about 7 months.

2.2. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) for the detection of capripoxviral antibodies are widely used and are available in commercial kit form (Milovanovic *et al.,* 2019; Samojlovic *et al.,* 2019).

2.3. Indirect fluorescent antibody test

Capripoxvirus-infected tissue culture grown on cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/20 or 1/40, and positive samples are identified using an anti-bovine gamma-globulin conjugated with fluorescein isothiocyanate. Antibody titres may exceed 1/1000 after infection. Sera may be screened at 1/50 and 1/500. Cross-reactions can occur with orf virus (contagious pustular dermatitis virus of sheep), bovine papular stomatitis virus and perhaps other poxviruses.

2.4. Western blot analysis

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out.

Capripoxvirus-infected LT cells should be harvested when 90% CPE is observed, freeze–thawed three times, and the cellular debris pelleted using centrifugation. The supernatant should be decanted, and the proteins should be separated using SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous gel system, using a stacking gel made up of acrylamide (5%) in Tris (125 mM), pH 6.8, and SDS (0.1%), and a resolving gel made up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use with a glycine running buffer containing Tris (250 mM), glycine (2 M), and SDS (0.1%). Samples of supernatant should be prepared by boiling for 5 minutes with an appropriate lysis buffer prior to loading. Alternatively, purified virus or recombinant antigens may replace tissue-culture-derived antigen.

Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should be transferred electrophoretically to a nitrocellulose membrane (NCM). After transfer, the NCM is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin (BSA) in PBS, or 5% skimmed milk powder in PBS, on a rotating shaker at 4°C overnight. The NCM can then be separated into strips by employing a commercial apparatus to allow the concurrent testing of multiple

serum samples, or may be cut into strips and each strip incubated separately thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating shaker, and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate serum at a dilution of 1/50 in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk powder and 0.05% Tween 20 in PBS). The membrane is again thoroughly washed and incubated (in blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated immunoglobulins at a dilution determined using titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidine tetrahydrochloride (10 mg in 50 ml of 50 mM Tris/HCl, pH 7.5, and 20 µl of 30% [v/v] hydrogen peroxide) is added. Incubation is then undertaken for approximately 3–7 minutes at room temperature on a shaker with constant observation, and the reaction is stopped by washing the NCM in PBS before excessive background colour is seen. A positive and negative control serum should be used on each occasion.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights 67, 32, 26, 19 and 17 kDa – the major structural proteins of capripoxvirus – whereas negative serum samples will not react with all these proteins. Hyperimmune serum prepared against parapoxvirus (bovine papular stomatitis or pseudocowpox virus) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

C. REQUIREMENTS FOR VACCINES [THIS SECTION IS UNDER REVIEW IN THE 2023/2024 REVIEW CYCLE]

1. Background: rationale and intended use of the product

Live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (Brenner *et al*., 2006; Capstick & Coakley, 1961; Carn, 1993). Capripoxviruses are cross-reactive within the genus. Consequently, it is possible to protect cattle against LSD using strains of capripoxvirus derived from sheep or goats (Coakley & Capstick, 1961). However, it is recommended to carry out controlled trials, using the most susceptible breeds, prior to introducing a vaccine strain not usually used in cattle. The duration of protection provided by LSD vaccination is unknown.

Capripoxvirus vaccine strains can produce a large local reaction at the site of inoculation in *Bos taurus* breeds (Davies, 1991), which some stock owners find unacceptable. This has discouraged the use of vaccine, even though the consequences of an outbreak of LSD are invariably more severe. Risk–benefit of vaccination should be assessed following stakeholder discussion.

2. Outline of production and minimum requirements for conventional vaccines

General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*. The documentation should include standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batch and the final product.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Each seed strain of capripoxvirus used for vaccine production must be accompanied by records clearly and accurately describing its origin, isolation and tissue culture or animal passage history. Preferably, the species and strain of capripoxvirus are characterised using PCR or DNA sequencing techniques.

A quantity of master seed vaccine virus should be prepared, frozen or desiccated and stored at low temperatures such as –80°C and used to produce a consistent working seed for regular vaccine production.

Each master seed strain must be non-transmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. It must produce a minimal clinical reaction in cattle when given via the recommended route.

The necessary safety and potency tests are described in Section C.2.2.4 *Final product batch tests*.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi or mycoplasmas.

The general procedures for sterility or purity tests are described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

2.2. Method of manufacture

The method of manufacture should be documented as the Outline of Production.

2.2.1. Procedure

Vaccine batches are produced on an appropriate cell line such as MDBK. The required number of vials of seed virus is reconstituted with GMEM or other appropriate medium and inoculated onto a monolayer. Cells should be harvested after 4–8 days when they exhibit 50–70% CPE for maximum viral infectivity, or earlier if CPE is extensive and cells appear ready to detach. Techniques such as sonication or repeated freeze–thawing are used to release the intracellular virus from the cytoplasm. The lysate may then be clarified to remove cellular debris (for example by use of centrifugation at 600 ***g*** for 20 minutes, with retention of the supernatant). A second passage of the virus may be required to produce sufficient virus for a production batch.

An aliquot of the virus suspension is titrated to check the virus titre. The virus-containing suspension is then mixed with a suitable protectant such as an equal volume of sterile, chilled 5% lactalbumin hydrolysate and 10% sucrose (dissolved in double-distilled water or appropriate balanced salt solution), and transferred to individually numbered bottles for storage at low temperatures such as –80°C, or for freeze–drying. A written record of all the procedures followed must be kept for all vaccine batches.

2.2.2. Requirements for substrates and media

The specification and source of all ingredients used in the manufacturing procedure should be documented and the freedom of extraneous agents (bacteria, fungi, mycoplasma and viruses) should be tested. The detailed testing procedure is described in Chapter 1.1.9. The use of antibiotics must meet the requirements of the licensing authority.

2.2.3. In-process control

i) Cells

Records of the source of the master cell stocks should be maintained. The highest and lowest passage numbers of the cells that can be used for vaccine production must be indicated in the Outline of the Production. The use of a continuous cell line (such as MDBK, etc.) is strongly recommended, unless the virus strain only grows on primary cells. The key advantage of continuous over primary cell lines is that there is less risk of introduction of extraneous agents.

ii) Serum

Serum used in the growth or maintenance medium must be free from antibodies to capripoxvirus and free from contamination with pestivirus or other viruses, extraneous bacteria, mycoplasma or fungi.

iii) Medium

Media must be sterile before use.

iv) Virus

Seed virus and final vaccine must be titrated and pass the minimum release titre set by the manufacturer. For example, the minimum recommended field dose of the South African Neethling strain vaccines (Mathijs *et al.,* 2016) is $\log_{10}$ 3.5 $TCID_{50}$, although the minimum protective dose is $\log_{10}$ 2.0 $TCID_{50}$. Capripoxvirus is highly susceptible to inactivation by sunlight and allowance should be made for loss of activity in the field.

The recommended field dose of the Romanian sheep pox vaccine for cattle is $\log_{10}$ 2.5 sheep infective doses (SID_{50}), and the recommended dose for cattle of the RM65-adapted strain of Romanian sheep pox vaccine is $\log_{10}$ 3 $TCID_{50}$ (Coakley & Capstick, 1961).

2.2.4. Final product batch tests

i) Sterility/purity

Vaccine samples must be tested for sterility/purity. *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* may be found in Chapter 1.1.9.

ii) Safety and efficacy

The efficacy and safety studies should be demonstrated using statistically valid vaccination–challenge studies using seronegative young LSDV susceptible dairy cattle breeds. The group numbers recommended here can be varied if statistically justified. Fifteen cattle are placed in a high containment level large animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Two cattle are inoculated with 10 times the recommended field dose of the vaccine, and eight cattle are inoculated with the recommended field dose. The remaining five cattle are unvaccinated control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the animals are again serum sampled and challenged with a known virulent capripoxvirus strain. The challenge virus solution should also be tested free from extraneous viruses. The clinical response is recorded during the following 14 days. Animals in the unvaccinated control group should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinates other than a raised area in the skin at the site of vaccination, which should disappear after 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to selected viral diseases that could have contaminated the vaccine, and the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus. Because of the variable response in cattle to LSD challenge, generalised disease may not be seen in all of the unvaccinated control animals, although there should be a large local reaction.

Once the efficacy of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.

iii) Batch potency

Potency tests in cattle must be undertaken for vaccine strains of capripoxvirus if the minimum immunising dose is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of hair. Log_{10} dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals may develop an

initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference in titre >$\log_{10}$ 2.5 is taken as evidence of protection.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

The vaccine must be safe to use in all breeds of cattle for which it is intended, including young and pregnant animals. It must also be non-transmissible and remain attenuated after further tissue culture passage.

Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.

ii) Reversion-to-virulence for attenuated/live vaccines

The selected final vaccine should not revert to virulence during further passages in target animals.

iii) Environmental consideration

Attenuated vaccine should not be able to perpetuate autonomously in a cattle population. Strains of LSDV are not a hazard to human health.

2.3.2. Efficacy requirements

i) For animal production

The efficacy of the vaccine must be demonstrated in statistically valid vaccination challenge experiments under laboratory conditions. The group numbers recommended here can be varied if statistically justified. Fifteen cattle are placed in a high containment level large animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Two cattle are inoculated with 10 times the field dose of the vaccine, eight cattle are inoculated with the recommended field dose. The remaining five cattle are unvaccinated control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the animals are again serum sampled and challenged with a known virulent capripoxvirus strain using intravenous and intradermal inoculation (the challenge virus solution should also be tested and shown to be free from extraneous viruses). The clinical response is recorded during the following 14 days. Animals in the unvaccinated control group should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinates other than a raised area in the skin at the site of vaccination which should disappear after 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to selected viral diseases that could have contaminated the vaccine, and the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus. Because of the variable response in cattle to challenge with LSDV, generalised disease may not be seen in all of the unvaccinated control animals, although there should be a large local reaction.

Once the potency of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.

ii) For control and eradication

Vaccination is the only effective way to control LSD outbreaks in endemic countries and recent experiences of the disease in Eastern Europe and the Balkans suggests this is also true for outbreaks in non-endemic countries. Unfortunately, currently no marker vaccines

allowing a DIVA strategy are available, although to a limited extent PCR can be used for certain vaccines.

The duration of immunity produced by LSDV vaccine strains is currently unknown.

2.3.3. Stability

All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life period to determine the vaccine stability.

Properly freeze-dried preparations of LSDV vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported. No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

3. Vaccines based on biotechnology

A new generation of capripox vaccines is being developed that uses the LSDV as a vector for the expression and delivery of immuno-protective proteins of other ruminant pathogens with the potential for providing dual protection (Boshra *et al*., 2013; Wallace & Viljoen, 2005), as well as targeting putative immunomodulatory genes for inducing improved immune responses (Kara *et al.,* 2018).

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NB: There are WOAH Reference Laboratories for lumpy skin disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for lumpy skin disease

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.4.13.

MALIGNANT CATARRHAL FEVER

SUMMARY

Description and importance of the disease: *Malignant catarrhal fever* (*MCF*) *is an acute, generalised and usually fatal disease affecting many species of* Artiodactyla*. The disease has been most often described as affecting species of the subfamily* Bovinae *and family* Cervidae*, but is also recognised in domestic pigs as well as giraffe and species of antelope belonging to the subfamily* Tragelaphinae*. MCF is characterised by subepithelial lymphoid cell accumulations and infiltrations, vasculitis and generalised lymphoid proliferation and necrosis. At least ten MCF viruses have been reported, including two well characterised viruses:* Alcelaphine gammaherpesvirus-1 (*AlHV-1*) *and* Ovine gammaherpesvirus-2 (*OvHV-2*)*. AlHV-1, which is maintained by inapparently infected wildebeest, causes the disease in cattle in regions of Africa and in a variety of ruminant species in zoological collections world-wide. OvHV-2, which is prevalent in domestic sheep as a subclinical infection, is the cause of MCF in most regions of the world. In both forms of the disease, animals with clinical disease are not a source of infection as virus is only excreted by the natural hosts, wildebeest and sheep, respectively.*

MCF usually appears sporadically and affects few animals, though both AlHV-1 and OvHV-2 can give rise to epizootics. There is a marked gradation in susceptibility to the OvHV-2 form of MCF ranging from the relatively resistant Bos taurus *and* B. indicus*, through water buffalo, North American bison and many species of deer, to the extremely susceptible Père David's deer, and Bali cattle. The disease may present a wide spectrum of clinical manifestations ranging from the acute form, when minimal changes are observed prior to death, to the more florid cases characterised by high fever, bilateral corneal opacity, profuse catarrhal discharges from the eye and nares, necrosis of the muzzle and erosion of the buccal epithelium. Diagnosis is normally achieved by observing the characteristic histopathological changes, though detection of viral DNA in either form of the disease has become the preferred option.*

Identification of the agent: *AlHV-1 may be recovered from clinically affected animals using peripheral blood leukocytes or lymphoid cell suspensions, but cell viability must be preserved during processing, as infectivity cannot be recovered from dead cells. Virus can also be recovered from wildebeest, either from peripheral blood leukocytes or from cell suspensions of other organs. Most monolayer cultures of ruminant origin are probably susceptible to AlHV-1 and develop cytopathic effect* (*CPE*)*. Primary isolates typically produce multinucleated CPE in which viral antigen can be identified by immunofluorescence or immunocytochemistry using suitable antisera or monoclonal antibodies. The OvHV-2 agent has never been isolated in culture, although lymphoblastoid cell lines propagated from affected animals contain OvHV-2-specific DNA. Both agents have been transmitted experimentally to rabbits and hamsters, which develop lesions characteristic of MCF.*

Viral DNA has been detected in clinical material from cases of MCF caused by both AlHV-1 and OvHV-2 using PCR, and this is becoming the method of choice for diagnosing both forms of the disease.

Serological tests: *Infected wildebeest, the natural host, consistently develop antibody to AlHV-1, which can be detected in a variety of assays including virus neutralisation, immunoblotting, enzyme-linked immunosorbent assay* (*ELISA*) *and immunofluorescence. Antibody to OvHV-2 can be detected by using AlHV-1 as the source of antigen. Domestic sheep consistently have antibody that can be detected by immunofluorescence, ELISA or immunoblotting. For both viruses the antibody response in MCF-affected animals is limited, with no neutralising antibody developing.*

Requirements for vaccines: *No vaccine is currently available for this disease.*

A. INTRODUCTION

1. Description and impact of the disease

Malignant catarrhal fever (MCF) is a generally fatal disease of cattle and many species of *Artiodactyla* that occurs following infection with certain herpesviruses of the genus *Macavirus*. At least six herpesviruses can cause MCF, the best characterised being *Alcelaphine gammaherpesvirus-1* (AlHV-1) and *Ovine gammaherpesvirus-2* (OvHV-2). MCF is characterised by systemic lymphoproliferation and is usually fatal, with infected cells being detectable in blood and most tissues at necropsy by PCR. Natural hosts of these viruses, including wildebeest (*Connochaetes* spp. of the subfamily *Alcelaphinae*) for AlHV-1 and domestic sheep for OvHV-2, experience no clinical disease following infection.

The clinical signs of MCF are highly variable and range from peracute to chronic with, in general, the most obvious manifestations developing in the more protracted cases. In the peracute form, either no clinical signs are detected, or depression followed by diarrhoea and dysentery may develop for 12–24 hours prior to death. In general, the onset of signs is associated with the development of a high fever, increased serous lachrymation and nasal exudate, which progresses to profuse mucopurulent discharges. Animals may be inappetent and milk yields may drop. Characteristically, progressive bilateral corneal opacity develops, starting at the periphery. In some cases skin lesions appear (characterised by ulceration and exudation), which may form hardened scabs associated with necrosis of the epidermis, and are often restricted to the perineum, udder and teats. Nervous signs may be present. Salivation associated with hyperaemia may be an early sign, progressing to erosions of the tongue, hard palate, gums and, characteristically, the tips of the buccal papillae. Superficial lymph nodes may be enlarged and limb joints may be swollen.

In addition, a number of cases of MCF with dermatological presentation have been described in sika deer infected with caprine herpesvirus 2 (CpHV-2; Foyle *et al*., 2009 and references cited therein). These cases exhibited cutaneous lesions combined with lymphocytic vasculitis characteristic of MCF, with CpHV-2 being detected by PCR and DNA sequencing.

Wildebeest-associated (WA-) MCF occurs in the cattle-raising regions of eastern and southern Africa where wildebeest and cattle are grazed together. The disease, however, can also affect a variety of other ruminant species in zoological collections world-wide and so, apart from antelope of the subfamilies *Alcelaphinae* and *Hippotraginae*, it is advisable to regard all ruminants as susceptible.

Sheep-associated (SA-) MCF occurs world-wide in cattle and other species, normally appearing sporadically and affecting only one or a few animals. However, on occasion, incidents occur in which multiple animals become affected. The disease can also infect and cause substantial losses in North American bison (*Bison bison*), red deer (*Cervus elaphus*), other deer species and water buffalo (*Bubalus bubalis*) and even more readily in Père David's deer (*Elaphurus davidianus*) and Bali cattle (*Bos javanicus*). OvHV-2 is also responsible for causing MCF in zoological collections, where disease has been reported in a variety of species including giraffe.

Reports from several countries, and in particular from Norway, that MCF affects domestic pigs have been confirmed by the detection of virus DNA in affected animals (Loken *et al*., 1998). Experimental infection of pigs with OvHV-2 has also been documented (Li *et al.*, 2012). Signs are very similar to those seen in acutely affected cattle.

The more resistant species tend to experience a more protracted infection and florid lesions, while in the more susceptible species the disease course tends to be shorter and the clinical signs less dramatic. Some studies also suggest that substantial numbers of animals may become infected without developing clinical disease (for an example see Lankester *et al.,* 2016).

Gross pathological changes reflect the severity of clinical signs, but are generally widespread and may involve most organ systems. Erosions and haemorrhages may be present throughout the gastrointestinal tract, and lymph nodes are enlarged, although the extent of lymph node involvement varies within an animal. Catarrhal accumulations, erosions and the formation of a diphtheritic membrane are often observed in the respiratory tract. Within the urinary tract characteristic echymotic haemorrhages of the epithelial lining of the bladder are often present, especially in bison.

Histological changes have been the basis for confirming cases of MCF and are characterised by epithelial degeneration, vasculitis, hyperplasia and necrosis of lymphoid organs, and widespread interstitial accumulations of lymphoid cells in nonlymphoid organs. Vasculitis is generally present and may be pronounced in the brain, affecting veins, arteries, arterioles and venules. It is characterised by lymphoid cell infiltration of the tunica adventitia and media, often associated with fibrinoid degeneration. The brain may also show a nonsuppurative meningoencephalitis with lymphocytic perivascular cuffing and a marked increase in the cellularity of the cerebrospinal fluid. Lymph-node hyperplasia is characterised by an expansion of lymphoblastoid cells in the paracortex, while degenerative lesions are generally associated with the follicles. The interstitial accumulation of lymphoid cells in nonlymphoid organs, in particular the renal cortex and periportal areas of the liver, is typical, and in the case of the kidney may be very extensive with development of multiple raised white foci, each 1–5 mm in diameter.

The pathological features of MCF, irrespective of the agent involved, are essentially similar. However, apart from histological examination, the methods available for diagnosing AlHV-1- and OvHV-2-induced disease tend to be virus-specific and are indicated below for each virus.

2. Nature and classification of the pathogen

MCF is caused by viruses of the genus *Macavirus*, subfamily *Gammaherpesvirinae*, family *Herpesviridae*, which share features of sequence and antigenicity and infect three subfamilies of *Bovidae* (*Alcelaphinae*, *Hippotraginae* and *Caprinae*). Disease caused by AlHV-1 is restricted to those areas of Africa where wildebeest are present and to zoological collections elsewhere, and has been referred to as WA-MCF. The OvHV-2 form of the disease occurs world-wide wherever sheep husbandry is practised and has been described as SA-MCF. Both forms of the disease may present a wide spectrum of clinical entities, though the characteristic histopathological changes are very similar in all cases. On rare occasions macaviruses other than AlHV-1 and OvHV-2 have been identified as a cause of MCF.

3. Differential diagnosis

The clinical signs of the 'head and eye' form of MCF resemble those of other diseases that cause oral lesions (Holliman, 2005). Thus BVD/mucosal disease, rinderpest, foot and mouth disease, bluetongue and vesicular stomatitis may be considered as potential differential diagnoses where MCF is suspected. A clear diagnosis of MCF may be supported by additional evidence such as detection of MCF virus DNA, virus-specific antibody response and/or histopathology consistent with MCF.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of malignant catarrhal fever (AlHV-1 and OHV-2) and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
PCR	+	+	+	+++	++	–
Virus isolation	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)	–
Detection of immune response						
C-ELISA	+++	+++	+++	++	+++	++

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Virus neutralisation	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)	–	–	+(AlHV-1)
IFAT	+	+	–	+	–	–
Immuno-peroxidase test	+	+	–	+	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; C-ELISA = competitive inhibition enzyme-linked immunosorbent assay;
IFAT = indirect fluorescent antibody test.
Note that virus isolation and virus neutralisation have only been documented for AlHV-1.

It must be emphasised that the viral cause of SA-MCF cannot be isolated reliably and evidence for OvHV-2 as a cause of MCF relies on: (a) the presence of antibody in sera of all domestic sheep that cross-reacts with AlHV-1 antigens (Hart *et al*., 2007, Li *et al.*, 2001); (b) the development of antibody that cross-reacts with AlHV-1 antigens in most cattle with SA-MCF and in experimentally infected animal models; (c) the detection of OvHV-2 sequences by PCR of DNA from peripheral blood or affected tissues of animals with SA-MCF; (d) the sequencing of OvHV-2 from sheep nasal secretions and their use to induce MCF with characteristic clinical signs and histopathology in rabbits, cattle and bison (Li *et al*., 2011; Taus *et al*., 2006; 2007).

Diagnosis of MCF based on clinical signs and gross pathological examination cannot be relied on as these can be extremely variable. Histological examination of a variety of tissues including, by preference, kidney, liver, urinary bladder, buccal epithelium, cornea/conjunctiva and brain, are necessary for reaching a more certain diagnosis. However, detection of antibody to the virus and/or viral DNA can now also be attempted and these are rapidly becoming the methods of choice.

Most laboratory-based tests to detect virus-specific antibody have relied on one attenuated isolate of AlHV-1 (WC11) that has been subjected to many laboratory passages as a source of viral antigen and DNA (Plowright *et al*., 1960). The full nucleotide sequence of the virulent low passage virus (C500) is now available and will form the basis of further studies of this virus (Ensser *et al*., 1997). Laboratory passage of the AlHV-1 C500 strain leads to attenuation of virulence and the ability to propagate in a cell-free manner, accompanied by genomic changes (Wright *et al*., 2003). This high passage derivative of AlHV-1 C500 has been used as a candidate vaccine for wildebeest-associated MCF and as a source of antigen for serological analysis (Haig *et al*., 2008; Russell *et al*., 2012). Individual MCF virus antigens, expressed either in bacteria or in mammalian cell culture, have recently been shown to be recognised by sera from MCF-affected animals and, in the case of OvHV-2 glycoproteins, induce virus-specific antibodies in hyperimmune rabbits (Bartley *et al.,* 2014; Cunha *et al.,* 2015; Dry *et al.,* 2016).

1. Identification of the agent

1.1. Clinically affected animals

1.1.1. Cell culture or Isolation

A striking feature of MCF is the lack of detectable viral antigen or herpesvirus-specific cytology within lesions. Confirmation of infection by virus recovery can only be performed for AlHV-1 to date, while attempts to recover the disease-causing virus from clinical cases of SA-MCF have failed consistently. However, lymphoblastoid cell lines have been generated from affected cattle and deer, some of which transmit MCF following inoculation into experimental animals (Reid *et al*., 1989).

Generally, AlHV-1 infectivity is strictly cell associated and thus isolation can be achieved only from cell suspensions either of peripheral blood leukocytes, lymph nodes or other affected tissues. Cell suspensions are prepared in tissue culture fluid, approximately $5 \times 10_6$ cells/ml, and

inoculated into preformed monolayer cell cultures. Bovine thyroid cells have been used extensively, but most primary and low passage monolayer cell cultures of ruminant origin will probably provide a suitable cell substrate for isolating AlHV-1. Following 36–48 hours' incubation, culture medium should be changed and monolayers should be examined microscopically (×40) for evidence of cytopathic effects (CPE). These appear characteristically as multinucleate foci within the monolayers, which then progressively retract forming dense bodies with cytoplasmic processes that may detach. This is followed by regrowth of normal monolayers. A CPE may take up to 21 days to become visible and is seldom present before day 7. Infectivity at this stage tends to be largely cell associated and thus any further passage or storage must employ methods that ensure that cell viability is retained. Identification of the isolate should be determined by PCR analysis or MCF-specific antibodies in fluorescence or immunocytochemical tests.

1.1.2. Molecular methods – detection of viral nucleic acids

Characteristically, little viral DNA can be detected within affected tissues, hence it is necessary to amplify the viral genome either by conventional culture (in the case of AlHV-1) or by polymerase chain reaction (PCR).

The full sequence of the C500 isolate of AlHV-1 and of two isolates of OvHV-2 have been published, permitting the design of primers for PCR reactions from conserved regions of the genome (Ensser *et al*. 1997; Hart *et al.,* 2007; Taus *et al*., 2007). Nested and real-time PCR assays have been developed for AlHV-1 and OvHV-2 (Baxter *et al*., 1993; Flach *et al*., 2002; Hussy *et al*., 2001; Traul *et al*., 2005) while a pan-herpesvirus PCR (VanDevanter *et al*., 1996) has been used to identify CpHV-2 in Sika deer with MCF (Foyle *et al*., 2009) and a virus associated with MCF in white-tailed deer (Li *et al*., 2000). This assay targets the viral DNA polymerase gene sequence and has been employed for phylogenetic comparison of AlHV-1 and related viruses (Li *et al*., 2005).

1.1.2.1. PCR protocols

These protocols are based on published nested PCR assays designed to detect OvHV-2 (Baxter *et al*., 1993) or to distinguish AlHV-1 and OvHV-2 (Flach *et al*., 2002) in DNA samples from natural hosts or MCF-affected species. Silica-based genomic DNA extraction methods have been extensively used and appear reliable. Methods for extraction of DNA from fixed tissue samples should be validated before use in these assays. An example protocol is given below but optimal reaction conditions should be validated for each system of enzymes and buffers. Protocols for real-time PCR to detect MCF virus DNA (Hussy *et al*., 2001; Traul *et al*., 2005) are not given as these should be optimised for each reagent set and analysis system used.

1.1.2.2. Protocol 1: Hemi-nested PCR to detect OvHV-2 DNA (Baxter *et al.*, 1993)

i) Primers

Name	Length	Sequence
556	30 mer	5'-AGT CTG GGT ATA TGA ATC CAG ATG GCT CTC-3'
555	28 mer	5'-TTC TGG GGT AGT GGC GAG CGA AGG CTTC-3'
755	30 mer	5'-AAG ATA AGC ACC AGT TAT GCA TCT GAT AAA-3'

ii) Primary amplification (product size 422 bp)

Prior to PCR, a master mix is made up, comprising all components except template DNA. This is then dispensed into PCR tubes containing test or control DNA. This approach minimises pipetting errors when assaying large numbers of samples. The master mix comprises (per reaction): 10× PCR buffer, 5 µl; $MgCl_2$ (25 mM), 1 µl; dNTP mix (1 mM), 5 µl; primer 556 (10 µM), 1 µl; primer 755 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; and nuclease-free water, 31.875 µl; making a total of 45 µl per reaction. Samples of 5 µl, containing up to 1 µg of test or control DNA, are placed in PCR tubes and 45 µl of master mix are added to each tube. The tubes are then used for PCR according to the following protocol, using a thermal cycler with heated lid. To use a thermal cycler without a heated lid, mineral oil should be overlaid on each PCR reaction to prevent evaporation.

Suggested cycling conditions are: Hot-start activation or denaturation at 95°C for up to 15 minutes; followed by 15 cycles of 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds; with a final extension at 72°C for 10 minutes. The conditions should be adjusted according to the Taq polymerase and the thermal cycler used.

iii) Secondary amplification (product size 238 bp)

The master mix for the secondary amplification comprises (per reaction): 10× PCR buffer, 5 µl; $MgCl_2$ (25 mM), 1 µl; dNTP mix (1 mM), 5 µl; primer 556 (10 µM), 1 µl; primer 555 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; and nuclease-free water, 33.875 µl; making a total of 48 µl. Samples of 2 µl of each primary amplification product are placed in PCR tubes and 48 µl of master mix are added to each tube. Cycling conditions for the secondary PCR are the same as for the primary PCR, except that 30 cycles of amplification are used. After amplification approximately 10 µl of each secondary PCR reaction should be run on a 1.8 % agarose gel to visualise the PCR products.

1.1.2.3. Protocol 2: Hemi-nested PCR to distinguish AlHV-1 and OvHV-2 DNA (Flach *et al.,* 2002)

i) Primers

Name	Length	Sequence*
Primer POL1	24-mer	5'-GGC (CT)CA (CT)AA (CT)CT ATG CTA CTC CAC-3'
Primer POL2	21-mer	5'-ATT (AG)TC CAC AAA CTG TTT TGT-3'
Primer OHVPol	20-mer	5'-AAA AAC TCA GGG CCA TTC TG-3'
Primer AHVPol	20-mer	5'-CCA AAA TGA AGA CCA TCT TA-3'

*base positions in parentheses are degenerate – the oligonucleotide will contain either of the two indicated bases at these positions.

The primers POL1 and POL2 target a segment of the DNA polymerase gene which is conserved in both OvHV-2 and AlHV-1, amplifying a fragment of 386bp. OHVPol and AHVPol are specific primers for OvHV-2 and AlHV-1 respectively, which amplify 172 bp products.

ii) Primary amplification

Master mix, per reaction: 10× buffer, 2.5 µl; $MgCl_2$ (25 mM), 0.5 µl; dNTP mix (1 mM), 2.5 µl; primer POL1 (10 µM), 1 µl; primer POL2 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; nuclease-free water, 12.375 µl (to 25 µl). Samples of 5 µl, containing up to 1 µg of test or control DNA, are placed in PCR tubes and 20 µl of master mix is added to each tube. The tubes are then used for PCR according to the following protocol: Hot-start activation or denaturation at 95°C for up to 15 minutes; followed by 25 cycles of 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds; with a final extension at 72°C for 10 minutes. The conditions should be adjusted according to the Taq polymerase and the thermal cycler used.

iii) Secondary amplification

Master mix, per reaction: 10× buffer, 2.5 µl; $MgCl_2$ (25 mM), 0.5 µl; dNTP mix (1 mM), 2.5 µl; primer AHVpol or OHVpol (10 µM), 1 µl; primer POL2 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; nuclease-free water, 12.375 µl (to 25 µl). Samples of 2 µl of each primary amplification product are placed in PCR tubes and 23 µl of master mix are added to each tube. Cycling conditions for the secondary PCR are the same as for the primary PCR, except that 30 cycles of amplification are used. After amplification approximately 10 µl of each secondary PCR reaction should be run on a 1.8% agarose gel.

1.2. Natural hosts

It is almost certain that all free-living wildebeest are infected with AlHV-1 by 6 months of age, virus having been spread intensively during the perinatal period (Lankester *et al.,* 2015). The species

Connochaetes taurinus taurinus, *C.t. albojubatus* and *C. gnu* are all assumed to be infected with the same virus. Infection also appears to persist in most groups of wildebeest held in zoological collections. However, it is possible that infection may be absent in animals that have been isolated during calf-hood or that live in small groups.

Following infection there is a brief period when virus is excreted in a cell-free form and can be isolated from nasal swabs. Virus can also be isolated from blood leukocytes at this time, but in older animals this is less likely to be successful unless the animal is immunosuppressed either through stress or pharmacological intervention.

The domestic sheep is the natural host of OvHV-2 and probably all sheep populations are infected with the virus in the absence of any clinical response. Studies of the dynamics of infection within sheep flocks have however, generated conflicting results with some suggesting productive infection occurs in the first weeks of a lamb's life while others suggest infection of most lambs does not occur until 3 months of age with excretion of infectious virus occurring between 5 and 6 months (Li *et al*., 2004). There is also evidence that some lambs may become infected *in utero* while other studies suggest that removal of lambs from their dams during the first week permits the establishment of virus-free animals. There may therefore be considerable variation in the dynamics of infection in different flocks. However, circumstantial evidence of the occurrence of MCF in susceptible species does suggest that the perinatal sheep flock is the principal source of infection, but that periodic recrudescence of infection may occur in sheep of all ages.

In latently infected adult sheep or wildebeest, the very low circulating viral load may reduce the reliability of PCR tests. However in these animals, a clear virus-specific antibody response should be detectable.

In addition to domestic sheep, domestic goats and other members of the subfamily *Caprinae* have antibody that reacts with AlHV-1 in a similar pattern to sheep serum. This implies that these species are infected with viruses similar to OvHV-2. Some goats have been found to be OvHV-2 positive by PCR, while a few cases of MCF caused by CpHV-2 have been reported (Foyle *et al*., 2009). Other large antelope of the subfamilies *Alcelaphinae* and *Hippotraginae* are also infected with antigenically closely related gammaherpesviruses (Li *et al*., 2005). These similarities have been supported by recent analysis of cross-neutralisation of AlHV-1 and OvHV-2 viruses by sera from ovine, caprine, hippotragine or alcelaphine reservoir host species, which suggests that AlHV-1 may be neutralised by hippotragine or alcelaphine sera while OvHV-2 may be neutralised by ovine or caprine sera (Taus *et al.,* 2015). This implies a hierarchy of relatedness among the MCF viruses that may influence the reliability of serological tests.

2. Serological tests

2.1. Clinically affected animals

The antibody response of clinically affected animals is limited, with no neutralising antibody developing. Antibody to OvHV-2 has historically been detected using AlHV-1 as the source of antigen, although detection of recombinant OvHV-2 proteins by sera from MCF-affected cattle has been reported (Bartley *et al.,* 2014). Antibody to AlHV-1 can be detected in 70–80% of clinically affected cattle by indirect fluorescent antibody (IFA) or immunoperoxidase test (IPT) procedures, but may not be present in affected deer or animals that develop acute or peracute disease. A competitive inhibition enzyme-linked immunosorbent assay (C-ELISA) using a monoclonal antibody (MAb) (15-A) that targets an epitope conserved among MCF viruses has been employed to detect antibody in serum of wild and domestic ruminants in North America (Section B.2.2.2.) (Li *et al*., 2001). A comparative study of MCF diagnosis by histopathology, C-ELISA and OvHV-2-specific PCR showed that most cattle classified as MCF-positive by histopathology also had detectable MCF virus-specific antibodies and OvHV-2 DNA in the blood (Muller-Doblies *et al*., 1998). Direct ELISAs based on antigens from AlHV-1 strains WC11 or C500 high-passage (HP) have also been reported (Fraser *et al*. 2006; Russell *et al.,* 2012).

2.2. Natural hosts

Antibody appears to develop consistently in wildebeest following infection and can be identified by neutralisation assays using the cell-free isolates WC11 or C500 HP, or by immunofluorescence, using

anti-bovine IgG, which has been shown to react with wildebeest IgG. The Minnesota MCF virus strain, which is indistinguishable from the WC11 strain of AlHV-1, is used for C-ELISA antigen production.

There has been no attempt so far to standardise the IFA test and the IPT, but the two methods below are given as examples. The C-ELISA may be available as a commercial kit.

2.2.1. Virus neutralisation

Tests have been developed for detecting antibodies to AlHV-1 in both naturally infected reservoir and indicator hosts. The first of these is a virus neutralisation (VN) test using cell-free virus of the WC11 strain, and another uses a hartebeest isolate (AlHV-2). The attenuated (HP) strain of AlHV-1 C500 may also be used. These viruses have cross-reactive antigens and therefore either strain can be used in the test. The test can be performed in microtitre plates using low passage cells or cell lines. The main applications have been in studying the range and extent of natural macavirus infection in wildlife, captive species in zoos and, to a lesser extent, sheep populations. It has also been useful in attempts to develop vaccines, including the recent AlHV-1 vaccine that induced neutralising antibodies in cattle blood plasma and nasal secretions (Haig *et al*., 2008). The VN test is of no value as a diagnostic test in clinically affected animals as no VN antibody develops in clinically susceptible species.

AlHV-1 stock virus is grown in primary or secondary cell cultures of bovine kidney, bovine thyroid, bovine turbinate, low passage bovine testis, or another permissive cell type. The virus is stored in aliquots at –70°C. The stock is titrated to determine the dilution that will give 100 $TCID_{50}$ (50% tissue culture infective dose) in 25 µl under the conditions of the test.

2.2.1.1. Test procedure

i) Inactivate the test sera for 30 minutes in a water bath at 56°C.

ii) Make doubling dilutions of test sera in cell culture medium from 1/2 to 1/16 using a 96-well flat-bottomed cell-culture grade microtitre plate, four wells per dilution and 25 µl volumes per well. Positive and negative control sera are also included in the test. No standard sera are available, but internal positive standards should be prepared and titrated in an appropriate range.

iii) Add 25 µl per well of AlHV-1 virus stock at a dilution in culture medium calculated to provide 100 $TCID_{50}$ per well.

iv) Incubate for 1 hour at 37°C. The residual virus stock is also incubated.

v) Back titrate the residual virus in four tenfold dilution steps, using 25 µl per well and at least four wells per dilution.

vi) Add 50 µl per well of permissive cell suspension at $3 \times 10_5$ cells/ml.

vii) Incubate the plates in a humidified CO_2 atmosphere at 37°C for 7–10 days.

viii) Read the plates microscopically for CPE. Validate the test by checking the back titration of virus (which should give a value of 100 $TCID_{50}$ with a permissible range 30–300) and the control sera. The standard positive serum should give a titre within 0.3 log_{10} units of its predetermined mean.

ix) The test serum results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of the wells.

x) A negative serum should give no neutralisation at the lowest dilution tested (1/2 equivalent to a dilution of 1/4 at the neutralisation stage).

2.2.2. Competitive inhibition enzyme-linked immunosorbent assay (C-ELISA)

The C-ELISA targets an epitope on the major surface glycoprotein that appears to be conserved among all MCF viruses. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and elsewhere. The test has detected antibody to the following MCF viruses: AlHV-1, AlHV-2, OvHV-2, CpHV-2 and the herpesvirus of unknown origin observed to cause classic MCF in white-tailed deer, as well as the MCF-group viruses not yet reported to be pathogenic, such as those carried by the oryx, muskox, and others (Li *et al*.

2005). The C-ELISA has the advantage of being faster and more efficient than the IFA or IPT. Comparison between C-ELISA, PCR and histopathological diagnosis of SA-MCF (Muller-Doblies *et al*., 1998) suggested that the three approaches had good concordance.

The complete reagent set for the C-ELISA, including pre-coated plates, labelled MAb and control sera, may be commercially available. For laboratories wishing to prepare their own antigen-coated plates, the following protocol is provided. ELISA plates are coated at 4°C (39°F) for 18–20 hours with 50 µl of a solution containing 0.2 µg per well of semi-purified MCF viral antigens (Minnesota or WC11 isolates of AlHV-1) in 50 mM carbonate/bicarbonate buffer (pH 9.0). The coated plates are blocked at room temperature (21–25°C, 70–77°F) for 2 hours with 0.05 M phosphate buffered saline (PBS) containing 2% sucrose, 0.1 M glycine, 0.5% bovine serum albumin and 0.44% NaCl (pH 7.2). After blocking, wells are emptied and the plates are then dried in a low humidity environment at 37°C for 18 hours, sealed in plastic bags with desiccant, and stored at 4°C (39°F) (Li *et al*., 2001). MAb 15-A is conjugated with horseradish peroxidase using a standard periodate method.

2.2.2.1. Test procedure

i) Dilute positive and negative controls and test samples (either serum or plasma) 1/5 with dilution buffer (PBS containing 0.1% Tween 20, pH 7.2).

ii) Add 50 µl of diluted test or control samples to the antigen-coated plate (four wells for negative control and two wells for positive control). Leave well A1 empty and for use as a blank for the plate reader.

iii) Cover the plate with parafilm and incubate for 60 minutes at room temperature, (21–25°C, 70–77°F).

iv) Using a wash bottle, wash the plate three times with wash buffer (same as dilution buffer: PBS containing 0.1% Tween 20, pH 7.2).

v) Prepare fresh 1 × antibody-peroxidase conjugate in dilution buffer according to previous titration/optimisation for each conjugate preparation, or to the manufacturer's instructions.

vi) Add 50 µl of diluted antibody-peroxidase conjugate to each sample well. Cover the plate with parafilm and incubate for 60 minutes at room temperature (21–25°C, 70–77°F).

vii) Wash the plate with wash buffer three times.

viii) Add 100 µl of tetramethylbenzidine substrate solution to each sample well. Incubate for 60 minutes at room temperature (21–25°C; 70–77°F). Do not remove the solution from the wells.

ix) Add 100 µl of stop solution (0.18 M sulphuric acid) to each well. Do not remove the solution from the wells.

x) Read the optical densities (OD) on an ELISA plate reader at 450 nm.

xi) Calculating % inhibition:

$$100 - \frac{\text{Sample OD (Average)} \times 100}{\text{Mean negative control OD}} = \%\text{inhibition}$$

xii) *Interpreting the results:* If a test sample yields equal to or greater than 25% inhibition, it is considered positive. If a test sample yields less than 25% inhibition, it is considered negative.

xiii) *Test validation:* The mean OD of the negative control must fall between 0.40 and 2.10. The mean of the positive control must yield greater than 25% inhibition.

2.2.3 Immunofluorescence

The IFA test is less specific than virus neutralisation (VN); it can be used to demonstrate several varieties of 'early' and 'late' antigens in AlHV-1-infected cell monolayers. Antibodies reacting in the IFA test or the IPT develop in cattle and experimentally infected rabbits during the incubation period, and later in the clinical course of the disease, though cross-reactions with

some other bovine herpesviruses, as well as OvHV-2, reduce the differential diagnostic value. Detection of such cross-reacting antibodies can sometimes be useful in supporting a diagnosis of SA-MCF.

2.2.3.1. Preparation of fixed slides

Inoculate nearly or newly confluent cell cultures with AlHV-1 (strain WC11). Uninoculated control cultures should be processed in parallel. At about 4 days – when the first signs of CPE are expected to appear but before overt CPE is visible – treat the cultures as follows: discard the medium, wash with PBS, remove the cells with trypsin–versene solution, spin down cells at approximate 800 ***g*** for 5 minutes, discard the supernatant fluid, and resuspend the cells in 10 ml of PBS for each 800 ml plastic bottle of cell culture.

Make test spots of the cell suspension on two wells of a polytetrafluoroethylene-coated multiwell slide; air-dry and fix in acetone. Stain the spots with positive standard serum and conjugated anti-IgG to the appropriate species. Examine the incidence of positive and negative cells under a fluorescence microscope. Adjust the cell suspension by adding noninfected cells and/or PBS to give a suitable concentration that will form a single layer of cells when spotted on to the slide, with clearly defined positive cells among a background of negative cells.

Spot the adjusted positive cell suspension and the control negative suspensions on to multiwell slides in the desired pattern, and air-dry. Fix in acetone for 10 minutes. Rinse, dry and store over silica gel in a sealed container at –70°C.

An alternative procedure, which is easier to evaluate, is to prepare monolayers of infected and noninfected cells in Leighton tubes or chamber slides. The cell monolayers are infected with from 150 to 200 $TCID_{50}$ of virus that has been diluted in cell culture medium. The infected and noninfected slides are fixed in acetone and stored, as above, at –70°C.

2.2.3.2. Test procedure

i) Rehydrate the slides for 5 minutes with PBS, rinse in distilled water and air-dry.

ii) Dilute sera 1/20 in PBS. Samples that give high background staining may be retested at higher dilutions. Apply diluted fluids to one MCF virus-positive cell spot and one negative control spot for each sample. Include positive and negative serum controls. Ideally, the test should be validated by titrating the control positive to determine its end-point.

iii) Incubate at 37°C for 30 minutes in a humid chamber.

iv) Drain the fluids from the spots. Wash the slides in two changes of PBS, for 5 minutes each.

v) Wash in PBS for 1 hour with stirring, and then air-dry the slides.

vi) Apply rabbit anti-bovine IgG fluorescein isothiocyanate (FITC) conjugate at a predetermined working dilution.

vii) Incubate at 37°C for 20 minutes, drain the slides, and wash twice in PBS for 10 minutes each.

viii) Counterstain in Evans blue 1/100,000 for 30 seconds, and wash with PBS for 2 minutes. Dip in distilled water, dry and mount in PBS/glycerol (50/50).

ix) Examine by fluorescence microscopy for specific binding of antibody to the infected cells.

2.2.4. Immunoperoxidase test

A dilution of bovine turbinate (BT) cell-cultured AlHV-1 containing approximately 10_3 $TCID_{50}$ is made in a freshly trypsinised suspension of BT cells and seeded into Leighton tubes containing glass cover-slips, 1.6 ml per tube, or four-chambered slides, 1.0 ml per chamber.

Observe the cell cultures at 4–6 days for CPE and fix the cultures with acetone when signs of CPE begin. Remove the plastic chambers, but not the gaskets, from the slide chambers before

fixation, and use acetone (e.g. UltimAR grade) that will not degrade the gasket. Store the fixed cells at –70°C.

2.2.4.1. Test procedure

i) Prepare IPT diluent (21.0 g NaCl and 0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2) and washing fluid (0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2).

ii) Dilute the serum to be tested 1/20 in IPT diluent and overlay 150–200 µl on to a fixed virus-infected cover-slip or slide chamber.

iii) Incubate the cover-slip in a humid chamber at 37°C for 30 minutes.

iv) Dip the cover-slip three times in washing fluid.

v) Overlay 150–200 µl of diluted (1/5000 in IPT diluent) peroxidase-labelled anti-bovine IgG on to the cover-slip or slide chamber.

vi) Incubate the cover-slip or slide chamber in a humid chamber at 37°C for 30 minutes.

vii) Dip the cover-slip three times in washing fluid.

viii) Dilute the AEC substrate (3-amino-9-ethylcarbazole, 20 mg/ml in dimethyl formamide) in distilled water (5 ml of distilled water, 2 drops 50 mM sodium acetate buffer pH 5.0, 2 drops hydrogen peroxide (30%), and 3 drops AEC) and apply to the cover-slip or slide chamber.

ix) Incubate in a humid chamber at 37°C for 8–10 minutes.

x) Dip the cover-slip in distilled water, air-dry, and mount on a glass slide. Slide chambers are read dry.

xi) The slide is read on a light microscope. The presence of a reddish-brown colour in the nuclei of the infected cells indicates a positive reaction.

C. REQUIREMENTS FOR VACCINES

At present no vaccine has been licensed for this disease.

Vaccination against MCF could be considered for use in those farmed species that have higher exposure or susceptibility to MCF, such as cattle in regions of East and South Africa where breeding wildebeest are prevalent, Bali cattle, bison in North America, farmed deer worldwide and susceptible species in zoological collections. Vaccination of reservoir hosts, such as wildebeest or sheep, is unlikely to be commercially viable and this is also the case for the majority of cattle herds that are at risk of sporadic SA-MCF. Numerous attempts to produce a protective vaccine against the AlHV-1 form of the disease have met with disappointing results. However, recent trials that focussed on stimulating high titres of neutralising antibody in nasal secretions of cattle have produced encouraging results (Haig *et al*., 2008). This live attenuated vaccine induced protection against intranasal experimental challenge with pathogenic AlHV-1. Protection was also found to persist for at least 6 months (Russell *et al*., 2012). This approach is likely to be the target for further research, including field trials.

As OvHV-2 cannot be successfully propagated in the laboratory no attempts have been made to develop a vaccine. However, recent work has developed a challenge system for OvHV-2 using virus from sheep nasal secretions (Taus *et al.,* 2006), which makes the testing of OvHV-2 vaccine candidates a possibility.

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NB: At the time of publication (2018) there were no WOAH Reference Laboratories for malignant catarrhal fever (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.4.14.

NAGANA: INFECTIONS WITH SALIVARIAN TRYPANOSOMOSES (EXCLUDING *TRYPANOSOMA EVANSI* AND *T. EQUIPERDUM*)

SUMMARY

Description and importance of the disease: *Nagana is a disease complex caused by several species of protozoan parasites of the genus* Trypanosoma*, section salivaria, transmitted mainly cyclically by flies of the genus* Glossina (*tsetse flies*) in sub-Saharan Africa (latitudes 10° North to 20–30° South) and some pockets of the Arabian peninsula, *but also mechanically transmitted by several biting flies* (*tabanids,* Stomoxys*, etc.*) in Africa and some other parts of the world. *The disease can affect various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis is particularly important in cattle though other hosts such as horses, donkeys, camels, goats, sheep, pigs and dogs may be affected. Nagana is mainly caused by* Trypanosoma congolense (*subgenus Nannomonas*)*,* T. vivax (*subgenus Duttonella*) *and, to a lesser extent,* T. brucei brucei (*subgenus Trypanozoon*) *however, other* Trypanosoma *species must be considered, such as* T. simiae (*mostly found in pigs*), *and the two zoonotic subspecies* T. b. gambiense *and* T. b. rhodesiense *notably found in humans, cattle and pigs. Other* Trypanozoon *species derived from the* T. brucei *lineage that are not transmissible by tsetse flies, e.g.* T. evansi (*responsible for "surra", mechanically transmitted by biting insects*) *and* T. equiperdum (*responsible for "dourine", venereally transmitted amongst equids*) *are presented in chapters 3.1.21 and 3.6.3, respectively.*

Animal infections with these salivarian trypanosomosis is a classically acute or chronic disease that causes intermittent fever and is accompanied by anaemia, oedema, lacrimation, enlarged lymph nodes, abortion, decreased fertility, loss of appetite and weight, leading to early death in acute forms or to digestive or nervous signs with emaciation and eventually death in chronic forms. Subclinical or healthy carriers of the parasites are frequently observed in enzootic areas, however, there are seasonal variations in transmission and clinical emergence.

Detection of the agents: *Several parasite detection techniques can be used, including the microscopic examination of the wet or dry-stained thick or thin blood films. Diagnostic sensitivity is increased significantly by concentrating the parasites prior to examination in combination with a phase-contrast or dark-ground microscope. The centrifugation parasite concentration techniques have the added advantage that the packed cell volume, and hence the level of anaemia, can be determined at the individual animal and/or herd level. A highly specific and more sensitive test, used in an increasing number of laboratories, is the polymerase chain reaction* (*PCR*), *which can identify parasites at the genus, species, subspecies or type level, depending on the cases. Highly specific primers or sequencing of PCR products allow the zoonotic* T. brucei *sub-spp. to be identified, which brings new information on the role of domestic and wild fauna in maintenance of some sleeping sickness foci. Additionally, in some geographical areas where nagana, surra and dourine may occur, there is a need to identify the non-tsetse transmitted* Trypanozoon *at the species level as control measures might be different from those of nagana.*

Serological tests: *The indirect fluorescent antibody test and the antibody-detection enzyme-linked immunosorbent assay* (*ELISA*) *are routinely used for the detection of antibodies against* Trypanosoma *in cattle. They have high sensitivity and genus specificity, but can only be used for the presumptive diagnosis of trypanosomosis. The antibody-detection ELISA in particular lends itself to automation and should allow a high degree of standardisation using recombinant antigens or, better, in-vitro-produced blood forms of the parasites, once their development and validation are completed. However, ELISA for antibodies to* T. congolense, T. vivax *and* T. brucei brucei *are at the*

present time carried out with native soluble antigens of trypanosomes grown in rodents and give reasonable sensitivity and specificity. In areas where several species of trypanosomes are present (*including* T. cruzi, T. evansi *and* T. equiperdum) *mixed infections may not be detected because cross-reactions amongst pathogenic trypanosomes may occur with any serological test employed, and an agent detection test may provide false negative results; in this situation the exact status of an animal with regard to the* Trypanosoma *species it is carrying cannot be established.*

Requirements for vaccines: *No vaccines are in use at the present time.*

A. INTRODUCTION

Trypanosomes are flagellate protozoans that inhabit the blood plasma, the lymph and various tissues of their hosts. The genus *Trypanosoma* belongs to the protozoan branch, order Kinetoplastida, family Trypanosomatidae. It is divided into two sections, Stercorarian (which implement their cyclical development in the posterior part of the digestive tract of their vector), to which belongs *Trypanosoma cruzi* a zoonotic parasites present in America, and Salivarian (which implement their cyclical development in the anterior part of the digestive tract of their vector), to which belong all animal trypanosomes originating from Africa (subgenera and species or subspecies): *Nannomonas* for *T. congolense* and *T. simiae*, Duttonella for *T. vivax* and *T. uniforme*, and *Trypanozoon* for *T. brucei brucei, T. brucei gambiense, T. brucei rhodesiense, T. evansi and T. equiperdum.*

Nagana is a disease complex caused by one or several of these trypanosomes, transmitted mainly cyclically by flies of the genus *Glossina* (tsetse flies), but also mechanically by biting flies such as tabanids and *Stomoxys* spp. (Baldacchino *et al.,* 2014). Tsetse flies infest 10 million square kilometres and affect 37 countries, in sub-Saharan Africa (between latitude 10° North and 20–30° South) and in some pockets of the Arabian Peninsula. The disease, known as 'nagana', affects various species of wild and domestic mammals but, from an economic point of view, African trypanosomosis is particularly important in cattle (also referred to as tsetse-fly disease in southern Africa). The most prevalent and pathogenic Trypanosome species in African cattle is *Trypanosoma congolense* (Savannah sub-type); however *T. vivax* and *T. brucei brucei* are also important causative agents (Bengaly *et al.,* 2002).

Other hosts such as horses, donkeys, camels, goats, sheep, pigs, dogs, cats, non-human primates and even humans may be affected, and other *Trypanosoma* species must be considered as well as other means of transmission. *Trypanosoma congolense* type forest and Kenya coast are mild pathogens for cattle, but their epidemiology is not fully elucidated. *Trypanosoma uniforme*, and *T. simiae*, a pig parasite, are other less common tsetse-transmitted *Trypanosoma* spp. *Trypanosoma vivax* is also mechanically transmitted by biting flies, among which tabanids and *Stomoxys* are presumed to be the most important, as exemplified by its presence in South and Central America, but also as observed in some areas of Africa free or cleared of tsetse flies (in Ethiopia, Chad, Senegal, Sudan, etc.). Tsetse-transmitted trypanosomosis can affect horses and camels and act as a natural barrier preventing the introduction of camelids into the southern Sahel region of West Africa. Very rare human cases have been observed caused by animal *Trypanosoma* spp. of African origin such as *T. congolense* and *T. brucei brucei*, but most of them are due to *T. evansi* (other human infections by animal trypanosomes are due to *T. lewisi*, a cosmopolitan rat parasite). Infection with *T. brucei gambiense* and *T. brucei rhodesiense* cause chronic or acute sleeping sickness in humans, respectively. A large range of wild and domestic animals, including cattle and pigs, can act as reservoirs of these human parasites, especially for the latter. Consequently, laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*) especially when handling cattle, pig or wild fauna samples. Under the expanding "One health" concept, identification of these zoonotic agents is essential for the control of human African trypanosomosis (Holmes, 2015). Similarly, in Latin America, and even in the USA, special care should be taken when handling animal samples due to the potential presence of *T. cruzi* (in cattle, sheep, horse dogs etc.), as recently shown in a horse exhibiting neurological clinical signs in Texas (Bryan *et al.,* 2016).

Two other *Trypanozoon* species, derived from the *T. brucei* lineage, are not transmitted by tsetse flies: (i) *T. evansi*, the causative agent of "surra", especially pathogenic to camels and horses in Africa, and to horses, cattle, buffaloes and others in Latin America and Asia*;* it is mechanically transmitted by tabanids and *Stomoxys* spp., but may be found in the same hosts and sometimes in the same areas as the agents of nagana; only highly specific molecular diagnosis allows distinction of the agent of surra from other *Trypanozoon* involved in nagana; and (ii) of *T. equiperdum*, the causative agent of dourine, a venereal disease with a relatively global distribution, transmitted to horses and mules. Diagnostic procedures for these parasites are presented in chapters 3.1.21 and 3.6.3, respectively.

In the present chapter, the following animal infections with salivarian trypanosomes are considered: *T. congolense, T. brucei*, *T. vivax* and *T. simiae* – the agents of Nagana.

Clinical signs of Nagana may include intermittent fever, anaemia, oedema, abortion, decreased fertility and emaciation. Anaemia usually develops in affected animals and is followed by loss of body condition, reduced productivity and often mortality. Post-mortem signs may include emaciation, enlarged lymph nodes, enlarged liver and spleen, excessive fluid in the body cavities, and petechial haemorrhages. In animals that died during the chronic phase of the disease, the lymphoid organs are usually no longer enlarged and severe myocarditis is a common finding. Neither clinical nor post-mortem signs of animal infections with these salivarian trypanosomes are pathognomonic. Therefore, diagnosis must rely on direct techniques that confirm the presence of trypanosomes, either by microscopic visualisation, or by indirect serological techniques, or by polymerase chain reaction (PCR), complemented in some cases by sequencing and sequence analysis. Clinically, infections of animals with salivarian trypanosomes can be confused with babesiosis, anaplasmosis, theileriosis, haemonchosis and even ehrlichiosis, rabies, plant intoxications or *T. cruzi* infection in Latin America (Bryan *et al.,* 2016; Desquesnes 2004). Final diagnosis is aided by clinical observations, and the epidemiological context, but it is essentially based on laboratory diagnosis.

B. DIAGNOSTIC TECHNIQUES

A variety of diagnostic tests are available and researchers are still working to improve existing tests and develop new ones. Current diagnostic tests vary in their sensitivity and specificity, the ease with which they can be applied and their cost. The choice of a particular test will be guided by economic principles and the availability of expertise, but especially by the diagnostic requirement. For example, different degrees of sensitivity and specificity are applied to the confirmation of the infection in an individual animal as compared with the detection of infection at the herd level. Similarly, the diagnostic test(s) to establish the parasitological prevalence of trypanosomosis are different from those required to establish the presence or absence of the disease in an area. Reliable diagnosis may be achieved by combining appropriate diagnostic tests. Reliable interpretation of results from diagnostic tests will depend on test validity as well as on proper sample selection/collection, the sample size, and the way the diagnostic tests are conducted (see Table 1). Detailed diagnosis techniques (including figures) of all tests described in section B can be found in the "Compendium of standard diagnostic protocols for Animal Trypanosomoses of African Origin", available online[1].

Table 1. Test methods available for the diagnosis of animal trypanosomosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
GSBS	–	+	–	+++	+	–
DNA detection/PCR	+++	+++	+++	+++	+++	–
Wet blood film	–	–	–	++	–	–
TGSBF	–	–	–	+	+	–
HCT (Woo)	+++	+++	+++	+++	+++	–
BCT (Murray)	–	–	++	++	++	–
AECT	–	+[(b)]	++	++[(a)]	–	–

1 http://www.oie.int/nttat/Attached%20files/A16-REC-COMPENDIUM_PROTOCOLES_TRYPANO-En.pdf

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
IFAT[c],[d]	++	++	++	–	++	–
ELISA[d]	+++	+++	+++	–	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
GSBS = thin Giemsa stained blood smear; PCR = polymerase chain reaction; TGSBS = thick Giemsa stained blood film;
HTC = haematocrit centrifuge technique; BCT = buffy coat technique; AECT = anion exchange chromatography technique;
IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.
[a]A combination of agent identification tests applied on the same clinical sample is recommended.
[b]Although expensive and time consuming, the mini-AECT can be useful for the detection of low parasitaemic animals.
[c]CATT (card agglutination test for *T. evansi*) and IFAT or ELISA *T. evansi* should be used if a *T. evansi* infection is suspected (as such or in a mixed infection).
[d]Choice of the antigen(s), *T. vivax, T. congolense* type savannah and/or *T. brucei brucei* (± *T. evansi*) must be adapted to the epizootiological situation; 1 to 4 IFATs or ELISAs may be justified.

1. Detection of the agent

Identification of the agents of nagana can be made by visualisation of the parasite with a microscope or by demonstration of its DNA. Molecular techniques are highly sensitive and their specificity can be very high; they are increasingly used and they have considerably improved the diagnosis of African trypanosomosis. Parasitological methods however, remain the most employed; they are easy and inexpensive to carry out, and parasite visualisation remains the best method for diagnostic certainty.

Parasite detection techniques are subgenus specific, but their sensitivity is relatively low (i.e. the proportion of false-negative results recorded is high), which give them a low negative predictive value (NPV). Sensitivity is especially low when results are considered at the individual animal level rather than the herd level. Sensitivity is highly variable during the course of the infection: (i) in the early phase, the sensitivity is high as parasites are actively multiplying in the blood in the absence of immunological control; (ii) during the chronic phase the sensitivity is low as, due to the immune response of the host, parasites are scanty, thus rarely seen in the blood; (iii) finally the sensitivity is almost nil in healthy carriers, where parasites are never seen. At the population level these variations mean that parasite detection techniques are highly sensitive during epizootic outbreaks (when most of the animals are in the early stages of infection), and are of low or very low sensitivity in stable enzootic areas (most of the animals are in the chronic stages of infection), especially during subclinical phases of the infection, which makes the animals appear like healthy carriers. Due to this low sensitivity, the apparent parasitological prevalence of trypanosomosis can be slightly or much lower than the true parasitological prevalence. The low diagnostic sensitivity also makes it difficult to detect trypanosomosis when present at low prevalence and it is impossible to establish the absence of the disease with a high degree of confidence. Moreover, in areas where trypanocidal drugs are used extensively, parasites may not be detected during the post-treatment period.

Rodent inoculation is expensive and of ethical concern. It should no longer be used as a diagnostic method, and should be restricted to antigen production for use in serological diagnostic tests

Several parasite detection techniques are available, exhibiting variable sensitivity. The choice will depend on the laboratory facilities available and the aim of the diagnosis accordingly to the recommendations indicated in Table 1.

1.1. Direct examination techniques

The simplest techniques are examination of wet, thick or thin films of fresh blood, usually obtained from the ear vein, jugular vein or the tail. Amongst the direct examination techniques, stained thin blood films are generally regarded as more specific but less sensitive than the other two. The actual specificity and sensitivity of these techniques is directly dependent on the volume of blood actually examined and the

skill and experience of the microscopist. Examination of Giemsa-stained blood smear (GSBS) remains the classic and most certain reference diagnostic test for trypanosome infection.

1.1.1. Wet blood films

These are made by placing a droplet of blood (about 2 µl) on a clean microscope slide and covering with a cover-slip (22 × 22 mm). The blood is examined microscopically at ×400 total magnification with condenser aperture, phase-contrast or interference contrast. Approximately 50–100 fields are examined. Trypanosomes can be recognised by their movement among the red blood cells (RBCs).

The method is simple, inexpensive and gives immediate results. Depending on the trypanosome size and movements, a presumptive diagnosis can be made of the trypanosome species. Final confirmation of the species must be made by the examination of the stained preparation (GSBS).

The diagnostic sensitivity of the method is generally low but depends on the examiner's experience and the level of parasitaemia (the test may be positive when the parasitaemia is above 10^4 parasites/ml). Sensitivity can be improved significantly by lysing the RBCs before examination using a haemolytic agent.

Due to its very low sensitivity, this technique is generally used to follow-up experimental infections (in which high parasitaemias are expected), rather than to detect infections in field samples.

1.1.2. Thick blood films

These are made by placing a drop of blood (5–10 µl) on a clean microscope slide and spreading it over an area of approximately 2 cm in diameter, using the corner of another slide. The thickness of the resultant film should be such that, when dry, the figures on a wristwatch dial can just be read through it. The film is dried thoroughly by rapidly waving in the air and, without fixation, is de-haemoglobinised by immersion in distilled water for a few seconds and dried before staining. A dry smear should be kept dry and protected from dust, heat, flies and other insects. It is stained for 30 minutes with 4% diluted Giemsa stain in phosphate buffered saline, pH 7.2. Staining time and stain dilution may vary with stain and individual technique. Therefore, it is important to start with the manufacturer's directions and to vary staining time and stain concentration to obtain the optimal result. The stained smear is then washed with buffered water and examined at ×500 to ×1000 total magnification.

The method is simple and relatively inexpensive, but results are delayed because of the staining process; however commercial kits are available for quick staining. Trypanosomes are recognised by their general morphology, but may be damaged during the staining process. This may make it difficult to identify the subgenus. The test is positive when the parasitaemia is above 10^4–10^5/ml. Thin blood smears are generally preferred to thick ones, because of the lower specificity of the latter.

1.1.3. Thin blood smear films: Giemsa-stained blood smear (GSBS)

Thin blood smears are made by placing a small drop of blood (about 3 µl), for example from a microhaematocrit capillary tube, on a clean microscope slide approximately 20 mm from one end (allowing for space to apply the thick smear when both techniques are applied) and spreading with the edge of another slide (spreader). This slide is placed at an angle of approximately 30° to the first slide and drawn back to make contact with the blood droplet. The blood is allowed to run along the edge of the spreader, which is then pushed to the other end of the slide in a fairly rapid but smooth motion. The blood is thus pulled (by capillary action) by the spreader slide. If the correct amount of blood is used, the slide should be covered with a film of blood with no surplus when the end of the slide is reached, and the smear should take the shape of a bullet. Ideally, thin films should be prepared so that the RBCs are fairly close to each other but not overlapping. The slide is dried quickly by waving in the air and protected from dust, flies and other insects. The slide is fixed for 3 minutes in methanol, and stained as for thick blood smears. After staining, the slide is washed gently under tap water and allowed to dry. A variation of this method is to fix in pure methanol for 2 minutes, apply May–Grünwald stain for 2 minutes, then add an equal volume of

buffered water, pH 7.2, incubate for 8 minutes and drain off. Finally some rapid methods use staining by 4–5 dips of 1 second each, serially into methanol, eosinophilic and basophilic solutions. Approximately 50–100 fields of the stained thin smear are examined, with a ×50 or ×100 oil-immersion objective lens (total magnification ×500–×1000), before the specimen is considered to be negative. Even after a trypanosome has been detected, approximately 20 extra fields are investigated to determine if more than one species is present. The sharp extremity of the smear must be extensively explored as, because of their capillary properties, trypanosomes may be concentrated in this area (especially true for large species like *T. brucei* and *T. vivax*).

The technique described above can also be used for biopsy samples of lymph obtained from punctured lymph nodes or for oedema fluids. It can also help with differential diagnosis from other haemoparasites such as *Anaplasma, Babesia* and *Theileria*.

Although it is more time-consuming, both a thin and a thick smear may be made from the same sample. Thick smears contain more blood than thin smears and, hence, have a higher diagnostic sensitivity. Thin smears, on the other hand, allow *Trypanosoma* subgenus identification; the test is positive when the parasitaemia is around or above 10^5/ml. Trypanosome subgenera or species can be identified by the following morphological characteristics:

i) Duttonella: *Trypanosoma vivax*, 20–27 μm long, undulating membrane is medium or not obvious, free flagellum present at the anterior end, posterior end rounded, kinetoplast large and terminal. *Trypanosoma uniforme* presents the same characteristics although it is smaller (12–20 μm long);

ii) Trypanozoon: *Trypanosoma brucei* (e.g. *T. brucei brucei*, *T. b. gambiense* & *T. b. rhodesiense*) is a polymorphic trypanosome species. Two distinctly different forms can be distinguished, i.e. a long slender form and a short stumpy form. Often, intermediate forms, possessing characteristics of both the slender and stumpy forms, are observed. The cytoplasm often contains basophilic granules in stained specimens.

a) *Trypanosoma brucei* long slender form: 17–30 μm long and about 2.8 μm wide, undulating membrane is conspicuous, free flagellum present at the anterior end, posterior end pointed, kinetoplast small and subterminal. *Trypanosoma evansi* and *T. equiperdum* can be confused with the slender form of *T. brucei*.

b) *Trypanosoma brucei* short stumpy form: 17–22 μm long and about 3.5 μm wide, undulating membrane is conspicuous, free flagellum absent, posterior end pointed, kinetoplast small and subterminal.

iii) Nannomonas: *Trypanosoma congolense* is 8–25 μm (small species), undulating membrane not obvious, free flagellum absent, posterior end rounded, kinetoplast is medium sized and terminal, often laterally positioned. Although *T. congolense* is considered to be monomorphous, a degree of morphological variation is sometimes observed. In *Nannomonas*, a number of morphotypes have been described so far; from the slender to the stumpiest: hyperleptomorph (rodhaini-form, very long and slender, with a free flagellum), leptomorph (this is *T. simiae*-form, slender, with a free flagellum), isomorph (*congolense*-form, short, without a free flagellum), pachymorph (montgomeryi-form, short and stout; 0.25<WLr<0.34, without a free flagellum), and hyperpachymorph ('hyper-montgomeryi-form', short and very stout; 0.35<WLr<0.7, without a free flagellum) (Desquesnes *et al.,* 2012). Additionally, sphaeromorph and rosettes have also been described. Within *T. congolense*, different types or subgroups exist (savannah, forest, Kilifi or Kenya coast) that have a different pathogenicity (Bengaly *et al.,* 2002); also there is a large variation in pathogenicity within the savannah subgroup. These types can only be distinguished using PCR. Finally, the pig and monkey parasite, *T. simiae,* is pleomorphic, appearing from hyperleptomorph to pachymorph, most often like a long parasite (leptomorph), with well developed undulating membrane, occasionally exhibiting a free flagellum, it may also appear like a classical *T. congolense*.

iv) Megatrypanum*:* Megatrypanum are not tsetse transmitted; they are Stercorarian parasites, cyclically transmitted by tabanids; they are however, regularly found in bovine blood samples and must be distinguished from pathogenic *Trypanosoma* spp. *Trypanosoma theileri* is typically 60–70 μm (large species), but individual organisms can range from 19 to 120 μm, undulating membrane is conspicuous, long free flagellum present, posterior end is

long, sharp-pointed and rigid, kinetoplast is large and positioned near the nucleus and in a marginal position. *Trypanosoma theileri* is normally non-pathogenic, but its presence can confuse the parasitological diagnosis. In western Europe and Japan, *T. theileri* is the only trypanosome species occurring in cattle. As a consequence of its cyclical transmission by tabanids – highly cosmopolite and abundant vectors – this parasite is very common worldwide and has a very high prevalence in bovines. Detection is rare however, due to very low parasitaemia. Other related species, such a *T. ingens* (found in cattle [not pathogenic] and wild ruminants [reservoir hosts])[2], can be distinguished by a typical unstained transversal band inside the nucleus on GSBS.

v) Other species: in the area of distribution of salivarian trypanosomes, other *Trypanosoma* spp. may be found in blood samples and should be identified. Although they may be highly polymorphic, the most characteristic identification criteria of two common parasites are described hereafter. *Trypanosoma lewisi* (rat parasite) can be found in rodents and sometimes in primate samples including humans; it is characterised by a large size (30 μm), a posterior nucleus, a free flagellum, a large sub-terminal kinetoplast and a very sharp posterior extremity in the C shape adult form. *Trypanosoma cruzi* is a medium-sized parasite (16–25 μm) with central nucleus, free flagellum, a very large and protruding sub-terminal kinetoplast, and a C-or S-shape adult form; it should be noted that amastigote parasites may be found, notably in spinal fluid and muscles; *T. cruzi* can be found in all mammal species in Latin America and the southern part of the USA.

As stated above, these criteria allow identification of the parasites on GSBS at the subgenus level by microscopic visualisation. In a number of epizootiological contexts, they allow the species to be identified; for example a Duttonella is almost always linked to *T. vivax*, a Megatrypanum to *T. theileri*, a Nannomonas to *T. congolense* savannah in cattle, etc. However, only molecular techniques allow identification of the species with certainty (as opposed to microscopic visualisation, which brings diagnostic certainty at the genus level).

1.2. Parasite concentration techniques

The probability of detecting trypanosomes in a sample from an infected animal depends largely on the amount of blood examined and the level of parasitaemia. The amount of blood examined with direct examination techniques is low (2–10 μl) and parasites are often very scanty in the blood of an infected animal. Both of these factors contribute to the low sensitivity of direct examination techniques. Sensitivity can be improved by increasing the volume of blood to be examined and by concentrating the trypanosomes. Amongst these methods, the haematocrit centrifuge technique (HCT) is the classic and most certain reference diagnostic test for detection of living trypanosomes.

1.2.1. Haematocrit centrifuge technique (HCT, Woo method)

The haematocrit centrifuge technique (HCT), or Woo method, is widely used for the diagnosis of animal trypanosomosis. It is based on the separation of the different components of the blood depending on their specific density and shape. The method is as follows:

i) Usually about 70 μl of fresh blood, preferably from the ear vein, is collected into heparinised capillary tubes (75 × 1.5 mm); when the blood is collected from a larger vein in an anti-coagulant tube, a dry capillary tube can be filled.

ii) One end of the capillary tube is sealed with cristaseal (plasticine).

iii) The sealed capillary tubes are placed in a microhaematocrit centrifuge with the sealed ends pointing towards the outside. To ensure good balance, the tubes are loaded symmetrically. The capillary tubes are centrifuged at 9000 ***g*** for 5 minutes.

iv) A tube carrier is made from a slide on which two pieces of glass 25 × 10 × 1.2 mm have been fixed, 1.5 mm apart, to form a groove. The capillary tube is placed in the groove, a cover-slip is placed on top and the interface is flooded with water. Alternatively, examination can be

2 FAO Guide, Table 2: http://www.fao.org/3/X0413E/X0413E02.htm

done without flooding the interface with water, but in such case, the light condenser must be lowered in such a way that cells become refringents.

v) The interface of the plasma and buffy coat (platelets and white blood cells [WBCs]) is examined by slowly rotating the tube 6–7 times for about 60 degrees of angle. Trypanosome movement can first be detected using the ×10 objective lens with reduced condenser aperture; the trypanosomes can be seen more clearly using the ×40 objective lens preferably with a long working distance to allow adequate depth of focus through the capillary tube.

The HCT is more sensitive than the direct examination techniques. In the case of *T. vivax* infections, the sensitivity of the Woo methods approaches 100% when the parasitaemia is >700 trypanosomes/ml blood. Sensitivity decreases to 50% when parasitaemia varies between 60 and 300 trypanosomes/ml blood. Trypanosomes become very difficult to detect when the parasitaemia is lower than 60 trypanosomes/ml blood (Desquesnes, 2004). Sensitivity is higher with large trypanosomes such as Trypanozoon, and lower with small ones such as Nannomonas. Overall, HCT is considered to be positive when the parasitaemia is around or above 10^2–10^3. Identification of trypanosome species is difficult. As the specific decantation parameters of *T. congolense* are very close to those of WBCs and platelets, parasites are often found inside the buffy coat. To improve the separation of blood cells and parasites, and increase the sensitivity for *T. congolense*, the specific density of blood cells can be increased by the addition of glycerol.

1.2.2. Dark-ground or phase-contrast buffy coat technique (Murray method)

The buffy coat technique (BCT) or Murray method derives from the Woo method. It is carried out following steps (i) to (iii) above (Section B.1.2.1), after which the capillary tube is cut with a diamond-tipped pencil, 0.5 mm below the buffy coat, to include the top layer of RBCs. The buffy coat and the uppermost layer of RBCs are extruded on to a clean microscope slide (it is important to check that the buffy coat is not sticking to the capillary tube; it should be visible on the slide before covering it with a cover-slip [22 × 22 mm]). Approximately 200 fields of the preparation are examined for the presence of motile trypanosomes with a dark-ground or a phase-contrast microscope with a ×40 objective lens (×400 total magnification). Trypanosome species can be identified by reference to the following criteria:

i) *Trypanosoma vivax:* Large, extremely active, traverses the whole field very quickly, pausing occasionally.

ii) *Trypanosoma brucei:* Various sizes, rapid movement in confined areas; undulating membrane traps the light into 'pockets' moving along the body.

iii) *Trypanosoma congolense:* Small, sluggish, adheres to RBCs by anterior end.

iv) *Trypanosoma theileri:* More than twice the size of pathogenic trypanosomes, tends to rotate; the posterior end is clearly visible, very long, sharp and obviously rigid.

As with the HCT, the BCT is more sensitive than direct examination techniques. The sensitivity of HCT and BCT can be improved by using the buffy coat double-centrifugation technique. A total amount of 1500–2000 µl of blood is centrifuged, after which the buffy coat is aspired into a microhaematocrit capillary tube and centrifuged again. The upper buffy coat is directly examined (HCT) or collected and examined (BCT). However, collection of the buffy coat after the initial centrifugation is a delicate step and results may vary from one technician to another.

Compared with the HCT, the BCT has the added advantage that preparations can be fixed and stained for more accurate species identification and for retention as a permanent record. However, repeatability of the method is lower than HCT as the procedure for dropping the buffy coat from the capillary tube to the slide is uncertain and consequently its success varies from time to time, and from one technician to another. Most often the buffy coat sticks to the wall of the capillary tube and thus may be missed and the examination is negative. Additionally, BCT is more time consuming than HCT. For these reasons, preference is given to HCT as a reference method in routine use.

Both the HCT and BCT give direct results but HCT can better be used for screening large numbers of animals. They require specialised equipment and an electricity supply making the test more

expensive compared with the examination of the wet blood film. However, this is compensated for by increased sensitivity. Both parasite concentration techniques rely on the detection of live, motile trypanosomes. As trypanosomes can lose their vigour and die rather quickly once the blood sample is drawn, samples collected in capillary tubes should be cooled immediately and not be allowed to overheat in the microhaematocrit centrifuge or on the microscope stage. Capillary tubes should be kept vertically just after centrifugation, to avoid spreading of the buffy coat. Blood samples should, be tested as soon as possible after collection, preferably within a couple of hours.

The HCT and BCT are particularly useful in that the packed cell volume (PCV) can be assessed at the same time. To determine the PCV after centrifugation, the microhaematocrit capillary tube (containing ear vein or jugular vein blood) is placed in a haematocrit reader. The length of the packed RBC + buffy coat column is expressed as a percentage of the total volume of blood. Measuring the PCV is useful for determining the degree of anaemia. Anaemia can be caused by factors other than trypanosomosis, however, it remains one of the most important indicators of the disease in cattle. As trypanosomosis is a herd problem, the PCV-profile of a herd is influenced by the number of trypanosome-infected animals and can be used to indicate differences in disease challenge. The average PCV is also influenced by the age and level of genetic susceptibility of cattle.

1.2.3. Anion exchange

The miniature anion-exchange chromatography technique (m-AECT) is widely used for the diagnosis of human sleeping sickness caused by *T. b. gambiense* (Lumsden *et al*., 1979). Blood is passed through a diethyl amino-ethyl (DEAE)-cellulose column equilibrated with a phosphate buffered saline (PBS) solution of an ionic strength suited to the blood of the animal species under examination (Lanham & Godfrey, 1970). As the RBCs are more negatively charged than the trypanosomes, they are held in the column and the trypanosomes pass through with the eluate, which is collected, centrifuged to concentrate the trypanosomes, and the pellet is collected, spread on a slide and examined under the microscope. The positivity threshold is around 10^2–10^3 parasites/ml of blood.

Large volumes of blood can be examined from each animal and, therefore, the method may exhibit higher sensitivity. However, the technique is cumbersome and is not suitable for the examination of a large number of animals because it is very expensive and time consuming. It can be occasionally used when diagnostic certainty is required.

1.4. DNA amplification tests

A PCR method has been developed as a tool for the diagnosis of infections with African trypanosomes in humans and animals, as well as tsetse flies. Specific highly repetitive nuclear DNA sequences (also called satellite-DNA, presenting 10,000–20,000 serial repeats in the genome) can be amplified for *T. vivax* and three types of *T. congolense*; a common primer set is available for detection of all *Trypanozoon* taxa, including the three *T. brucei* spp., *T. evansi* and *T. equiperdum* (Desquesnes & Davila, 2002). DNA preparation is a determining step; several methods including commercial kits are available, however, a resin preparation method is generally recommended (Penchenier *et al.,* 1996). Similarly to parasitological examinations, a concentration technique by centrifugation allows enrichment of blood samples; it is therefore recommended to carry out the DNA preparation step on buffy coats to increase the sensitivity of parasite DNA detection. Using the correct DNA preparation method on buffy coat samples, and satellite DNA primers, PCRs are generally positive when the parasitaemia is around or above 1–10 trypanosomes/ml of blood.

Well validated primer sets available for the different trypanosome subgenera, species and types are referred to as follows (Table 2): *Trypanozoon* subgenus – TBR1 and TBR2; *T. congolense* savannah type – TCS1 and TCS2; *T. congolense* forest type – TCF1 and TCF2; *T. congolense* Kenya Coast type – TCK1 and TCK2; *T. simiae*– TSM1 and TSM2, and *T. vivax*– TVW1 and TVW2 (Masiga *et al*., 1992). Other sets of primers are available to distinguish *T. evansi* type A (RoTat1.2 primers) and B (EVAB primers) from *T. brucei* sspp. and *T. equiperdum*, but some strains are cross-reacting (Claes *et al.,* 2004; Njiru *et al.,* 2006). More specific methods are also available to identify *T. b. gambiense* and *T. b. rhodesiense* (Radwanska *et* 2002a; 2002b), which allow investigations of the animal reservoir of sleeping sickness (Hamill *et al.,* 2013; Karshima *et al.,* 2016). Due to the multiplicity of these taxon-specific primers in tsetse

flies or cattle, a complete *Trypanosoma* species identification may require three to six or more PCR tests be carried out per sample, which considerably increases the time and cost of diagnosis. In the USA and Latin America, primers for detection of *T. cruzi* – TCZ1 & TCZ2 (Moser *et al*., 1989) might also be required for trypanosome identification, notably in horses (Bryan *et al.,* 2016).

Amplifications of ITS1 of ribosomal DNA have also been developed that allow the identification of all *Trypanosoma* species as single or mixed infections using one single test (Desquesnes *et al*., 2001, Desquesnes & Davila, 2002; Njiru *et al.,* 2005). These tests are useful for screening, however sizing of the PCR product(s) on gels is sometimes not reliable, thus, sequencing is most often required to confirm species identification, which is not suitable for routine diagnosis. The ITS1 sequence being repeated 500–800 times only in a genome, the sensitivity of this PCR is lower than that of the satellite DNA primers; the test is generally positive when the parasitaemia is above 50–100 trypanosomes/ml of blood. Loop-mediated isothermal amplification has also been developed for trypanosome diagnosis (Kuboki *et al*., 2003), however, so far its limited use did not allow full validation for veterinary purposes.

Table 2. Well validated primer sequences for animal Trypanosomes identification

Specificity	Primer sequences (5' → 3')	References
Trypanozoon (*T. brucei brucei, T. b. gambiense, T. b. rhodesiense, T. evansi & T. equiperdum*)	TBR1: CGA-ATG-AAT-ATT-AAA-CAA-TGC-GCA-G	Masiga *et al*, 1992; Moser *et al.,* 1989
	TBR2: AGA-ACC-ATT-TAT-TAG-CTT-TGT-TGC	
T. congolense type savannah	TCS1: CGA-GCG-AGA-ACG-GGC-AC	Masiga *et al.*, 1992
	TCS2: GGG-ACA-AAC-AAA-TCC-CGC	
T. congolense type forest	TCF1: GGA-CAC-GCC-AGA-AGG-TAC-TT	Masiga *et al.*, 1992
	TCF2: GTT-CTC-GCA-CCA-AAT-CCA-AC	
T. congolense type "Kilifi" (or Kenya coast)	TCK1: GTG-CCC-AAA-TTT-GAA-GTG-AT	Masiga *et al.*, 1992
	TCK2: ACT-CAA-AAT-CGT-GCA-CCT-CG	
T. simiae	TSM1: CCG-GTC-AAA-AAC-GCA-TT	Masiga *et al.*, 1992
	TSM2: AGT-CGC-CCG-GAG-TCG-AT	
T. vivax	TVW 1: CTG-AGT-GCT-CCA-TGT-GCC-AC	Masiga *et al.*, 1992
	TVW 2: CCA-CCA-GAA-CAC-CAA-CCT-GA	
T. cruzi	TCZ1: CGA-GCT-CTT-GCC-CAC-ACG-GGT-GCT	Moser *et al.*, 1989
	TCZ2: CCT-CCA-AGC-AGC-GGA-TAG-TTC-AGG	
Specificity	**Other commonly used Primer sequences (5' → 3')**	**References**
*T. evansi**	TEPAN1: AGT-CAC-ATG-CAT-TGG-TGG-CA	Panyim *et al.*, 1993.
	TEPAN2: GAG-AAG-GCG-TTA-CCC-AAC-A	
*T. evansi**	ESAG6/7F: ACA-TTC-CAG-CAG-GAG-TTG-GAG	Holland *et al.*, 2001.
	ESAG6/7R: CAC-GTG-AAT-CCT-CAA-TTT-TGT	
T. evansi (type A)**	RoTat1.2F: GCG-GGG-TGT-TTA-AAG-CAA-TA	Claes *et al*., 2004
	RoTat1.2R: ATT-AGT-GCT-GCG-TGT-GTT-CG	
*T. evansi (*type B)	EVAB1: CAC-AGT-CCG-AGA-GAT-AGA-G	Njiru *et al.*, 2006
	EVAB2: CTG-TAC-TCT-ACA-TCT-ACC-TC	

Specificity	Other commonly used Primer sequences (5' → 3')	References
T. brucei gambiense	Tgs-GP F: GCT-GCT-GTG-TTC-GGA-GAG-C	Radwanska *et al.*, 2002a
	TgsGP R: GCC-ATC-GTG-CTT-GCC-GCT-C	
T. brucei rhodesiense	Tbr F: ATA-GTG-ACA-AGA-TGC-GTA-CTC-AAC-GC	Radwanska *et al.*, 2002b
	Tbr R: AAT-GTG-TTC-GAG-TAC-TTC-GGT-CAC-GCT	
Pan-tryp.: *T. vivax, Trypanozoon, T.congolense* savannah forest, Kilifi, *T. lewisi,* etc.	TRYP1S: CGT-CCC-TGC-CAT-TTG-TAC-ACA-C	Desquesnes *et al.*, 2002
	TRYP1R: GGA-AGC-CAA-GTC-ATC-CAT-CG	
Pan-tryp.: *T. vivax, Trypanozoon, T. congolense* savannah forest, Kilifi, *etc.*	ITS1 CF: CCG-GAA-GTT-CAC-CGA-TAT-TG	Njiru *et al.*, 2005
	ITS1 BR: TTG-CTG-CGT-TCT-TCA-ACG-AA	

*May also amplify other Trypanozoon (Holland *et al.,* 2001)
**May not amplify all *T. evansi* Type A (Njiru *et al.,* 2006), but may also amplify some *T. equiperdum* strains, while other primers such as Te664 may amplify some but not all strains of *T. evansi T. equiperdum* and *T. brucei* (Claes *et al.,* 2004).

False-negative results may occur when the parasitaemia is very low (< 1 trypanosome/ml of blood), which occurs frequently in chronic infections. False negative results may also occur when the specificity of the primers is too high, so that not all isolates of a particular trypanosome species are recognised. Sample collection has been simplified by adapting the test using blood or buffy coats spotted on to filter paper (Katakura *et al*., 1997); such methods are greatly favoured, nowadays, especially for international shipment of samples. A large number of samples can be processed at one time, making it potentially suitable for large-scale surveys.

Specific DNA reference samples for PCR can be obtained from the WOAH Reference Laboratory for animal trypanosomes of African origin (CIRAD, Montpellier, France) as well as from the WOAH Reference Laboratories for surra[3].

2. Serological tests

Several antibody detection techniques have been developed to detect trypanosomal antibodies for the diagnosis of animal trypanosomosis, with variable sensitivity and specificity. The methods of choice are the indirect fluorescent antibody test (IFAT) (Katende *et al*., 1987) and the trypanosomal antibody-detection ELISA (Hopkins *et al*., 1998; Luckins, 1977). The identification of major antigens of trypanosomes, and their production as recombinant molecules or synthetic peptides, should hopefully lead to the development of new tests based on the use of defined molecules. Thus, in the future, it may be possible to improve the specificity of serological tests to allow the detection of species-specific antibodies, and to reach a high level of standardisation that is currently not achieved by the use of total parasite extracts. Alternatively, improving techniques for *in-vitro* production of blood stages of various *Trypanosoma* spp. are highly promising as they will allow production of standardised whole cell lysate soluble antigens, which guaranties a high sensitivity because of the rich panel of native antigens they exhibit. In 2019, ELISA-*T. congolense* savannah, ELISA-*T. vivax* and ELISA-*T. b. brucei* are the recommended methods for detection of anti-trypanosome antibodies in most of the host species affected by nagana.

2.1 Rodent inoculation for antigen preparation

The laboratory animals are injected intraperitoneally with 0.1–0.5 ml (depending on the size of the rodent) of freshly collected infected blood. Artificial immunosuppression of recipient animals by irradiation or drug treatment (cyclophosphamide 200 mg/kg) will greatly increase the chances of isolating the parasite. A drop of blood is collected from the tip of the rodent's tail three times a week. The blood is examined using the wet blood film method. If an infection occurs, it generally shows after 3–10 days, however the rodents must be followed for at least 1 month.

3 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

2.2. Indirect fluorescent antibody test (IFAT)

The technique for the preparation of trypanosomal antigens (Katende *et al*., 1987) involves fixation of live trypanosomes using a mixture of 80% cold acetone and 0.25% formalin in normal saline.

2.2.1. Test procedure

i) Prepare thin smears from heavily parasitaemic blood or from a trypanosome suspension. Air-dry and fix in acetone for 5 minutes.

ii) Mark circles of 5 mm diameter on glass slides using nail varnish.

iii) Using a pipette, place a test serum, diluted 1/40, in each circle, ensuring that the area in each circle is completely covered.

iv) Incubate the antigen/test serum preparation at 37°C for 30 minutes in a humid chamber.

v) Wash the preparation three times in PBS for 5 minutes each time at 4°C, with gentle agitation. Air-dry the slides.

vi) Apply conjugate: rabbit or goat anti-bovine IgG (for tests on bovine sera) conjugated to fluorescein isothiocyanate.

vii) Incubate and wash as above. Rinse in distilled water. Air-dry the slides.

viii) Mount the slides in PBS or buffered glycerol and examine for fluorescence.

Interpretation of the fluorescence remains subjective and the procedure is not adapted to large-scale studies; therefore the IFAT is generally used for individual diagnosis as an alternative to ELISA.

2.3. Antibody-detection enzyme-linked immunosorbent assay (ELISA)

The original antibody ELISA (Luckins, 1977) has been further developed for use in large-scale surveys of bovine trypanosomosis (Desquesnes, 1997; Hopkins *et al*., 1998). Recommendations have been made that allow antigen production and standardisation of the test on a local basis (Desquesnes, 1997; 2004; Greiner *et al*., 1997; Wright *et al*., 1993).

2.3.1. Soluble antigens from whole cell lysate

The standard antigen for trypanosomosis antibody tests is derived from bloodstream-form trypanosomes produced in laboratory rats. Trypanosomes are separated by DEAE anion-exchange chromatography from whole blood of infected rats (Lanham & Godfrey 1970), washed by centrifugation and suspended in 1% PSG (phosphate saline glucose). The parasite suspension is treated with protease inhibitor cocktail. Antigens are prepared as a soluble fraction after lysis using five to seven freeze–thaw cycles and ultrasonicated three times for 2 minutes on ice to ensure complete disintegration of the organisms, and centrifuged at 4°C and 10,000 ***g*** for 10 minutes. The supernatant that contains the soluble antigens is collected and the protein concentration estimated by UV readings at 260 and 280 nm or by colorimetry, and stored at –80°C or –20°C for long and short periods, respectively. Alternatively, soluble antigens may be lyophilised for conservation at room temperature or international shipment of standardised reagents. ELISAs using *T. congolense* or *T. vivax* precoated microtitre plates have been developed that have the advantage of a standardised denatured antigen, which can be stored for long periods at room temperature (Rebeski *et al*., 2000), however sensitivity and specificity of the test are lower. A well standardised and performing method is expected in the near future with *in-vitro* produced blood-form trypanosomes.

2.3.2. Test procedure

i) *Sensitisation:* flat bottom, nonspecific binding 96-well polystyrene micro-plates[4] are used, with antigens diluted in a 0.01 M carbonate/bicarbonate coating buffer, pH 9.6.

4 The polystyrene plate of choice must not adsorb the proteins in a peculiar orientation so that all antigenic epitopes be available to bind the antibodies; hydrophobic microplates are preferred to hydrophilic plates where protein binding is structurally oriented, thus hiding some of the antigenic epitopes.

Trypanosome soluble antigen is diluted to a final concentration of 5 µg/ml, dispensed in 100 µl per well, incubated overnight at 4°C or for 2 hours at 37°C on a shaker at 300 rpm.

ii) *Blocking:* plates are emptied by inversion and 150 µl of blocking buffer (BB = 0.01 M PBS 5% skim milk, 0.1% Tween20) is added per well; plates are placed on a shaker-incubator at 300 rpm for 30 minutes at 37°C.

iii) *Serum predilution:* a predilution at 1/10 of serum or plasma is performed in a round-bottom polypropylene plate (U), using BB.

iv) *Transfer:* the plates are emptied by inversion, rinsed once with PBS, and 90 µl BB is added. 10 µl serum diluted 1/10 is transferred rapidly using an eight-channel pipette into two horizontally neighbouring wells (final dilution 1/100); the plates are placed on a shaker at 300 rpm for 30 minutes at 37°C.

v) *Washing:* the plates are emptied by inversion and the wells filled with washing buffer (WB = PBS with 0.1% Tween20); the plates are emptied and washed four additional times, and emptied and drained by banging the plates vigorously on absorbent paper towels.

vi) *Conjugate:* 100 µl of horseradish peroxidase-conjugate diluted in BB (dilutions adapted to the conjugate) is added to each well; the plates are placed on a shaker-incubator at 300 rpm for 30 minutes at 37°C.

vii) *Washing:* same washing and draining procedure as previously in step v).

viii) *Substrate-chromogen:* 100 µl of substrate-chromogen is added to each well (TMB, or a mixture of citric acid 25 ml + 125 ml ABTS + 100 µl H_2O_2). Plates are kept in the dark for 30 minutes at room temperature.

ix) *Reading:* the optical densities (absorbance) of the wells are measured, at the appropriate wavelength for the chromogen (TMB: 620 nm; ABTS: 405 nm).

Each ELISA-microplate is run with duplicate strong, medium and weak positive and negative reference sera (total of 6 control sera in duplicate), which are required to comply with pre-set values for quality assurance. The absorbance of each sample tested in duplicate is expressed as a relative percentage of positivity (RPP) of the positive and negative reference standards (Desquesnes, 1997); results are, therefore, quantifiable. The cut-off value is determined using known positive and negative field or experimental samples (Desquesnes, 1997; 2004). Both the IFAT and antibody-detection ELISA have been adapted for the analysis of blood samples collected on filter paper. 30–100 µl of serum or plasma sample is deposited on a filter paper. Samples are air-dried out of direct sunlight and placed in a plastic bag with self-indicating silica gel desiccant. The bag is sealed and should be kept as cool as possible until specimens are refrigerated or frozen. Further validation of this technique is being undertaken as such methods greatly facilitate the international shipment of samples.

ELISA performs better than IFAT in terms of sensitivity and specificity (Luckins, 1977), and can achieve a high degree of automation and standardisation; it is thus the recommended test. ELISA antibody-detection tests have high sensitivity (on average above 90%) and genus specificity (on average above 95%), but their subgenus and species specificity is generally low. Inter-species cross-reactions amongst *T. vivax*, *T. brucei* and *T. congolense* (and even *T. cruzi*) have been described and measured, but they do not allow a true species-specific diagnosis and may be improved by using a standardised set of the three species-specific tests (Desquesnes *et al*., 2011). However, animals infected with Megatrypanum, such as *T. theileri*, do not cross react in IFATs and ELISAs for *T. vivax*, *T. brucei*, *T. evansi* and *T. congolense* (Desquesnes 2004; Luckins, 1977).

In areas where several species of trypanosomes are present (including *T. cruzi*, *T. evansi* and *T. equiperdum*), mixed infections may not always be detected because cross-reactions among pathogenic trypanosomes may occur with any serological test employed, and agent detection tests may provide false negative results (due to low parasitaemia). Thus, once an animal is seropositive, it is not possible to ascertain whether it harbours one or several trypanosome species.

Serological tests detect immune responses to current and past infections and can, therefore, only provide a presumptive diagnosis of active infection. However, persistence of antibodies after a curative treatment or a self-cure is estimated to be on the average of 3–4 months in young and adult cattle infections (Desquesnes, 2004); although it might take up to 13 months before all antibodies have disappeared in some animals (Van den Bossche

et al., 2000) consequently, proper sampling and knowledge of trypanocidal use will give more accurate information. Positive seroconversion in ELISA generally occurs within 1 to 6 weeks after infection, thus, a negative results in a field sample must be interpreted in the light of other information or test results.

Immunodiagnosis requires the production of native antigens, needs expensive and sophisticated equipment and expertise, which are not always available. It has to be performed in specialised laboratories and there is a substantial delay between the actual sampling and the availability of the results. Nevertheless, the antibody ELISA lends itself to a high degree of automation and standardisation. Sample collection and storage is made easy through the use of filter papers. All of these factors make the antibody ELISA a very useful test for large-scale surveys to determine the distribution of animal trypanosomosis, as well as for post-treatment follow-up or elimination campaigns.

Specific antigens for ELISA and reference samples (positive and negative reference serum samples) can be obtained from the WOAH Reference Laboratory for animal trypanosomes of African origin; lyophilised reagents are now available, that considerably ease the shipment from the Reference laboratory to regional laboratories.

3. Test applications

Clinical signs including fever, anaemia, oedema, loss of weight, abortion and nervous signs can only lead to a suspicion of animal infection with salivarian trypanosomes; to complete the clinical diagnosis, recommended tests are (alternative tests into brackets): GSBS (/TGSBF), HCT (/BCT), PCR (for primers, see below) and ELISA (/IFAT) (for species antigens used in serology, *T. vivax, T. brucei* and *T. congolense*, see below). Rather than a single test, a combination of several recommended tests must be implemented, according to the context, as indicated below, and in line with the *Terrestrial Code* recommendations.

3.1. Characteristics and performances of recommended tests for diagnosis of animal trypanosomosis

Parasitaemia being highly variable, the sensitivity of the agent detection tests is highly variable.

i) GSBS: low sensitivity (10^5–10^6 trypanosomes/ml of blood); subgenus and sometimes species specific; the species can also be deduced from epizootiological information. When positive, the GSBS brings diagnostic certainty. Parasitaemia being highly variable, the sensitivity of the test is highly variable and generally considered as low.

ii) HCT: medium sensitivity (10^2–10^3 trypanosomes/ml of blood); genus and sometimes subgenus specific; examinations must be carried out within a short time after blood sampling (preferably 1–2 hours). When positive, HCT must be complemented with GSBS and/or PCR (+/- ELISA), to confirm the diagnosis.

iii) PCR: Sample preparation for PCR must be done on blood, or preferably on buffy coat, after blood centrifugation, using a commercial DNA purification kit or a Chelex resin preparation method (Penchenier *et al.*, 1996). Recommended primers (Gold standard) are those targeting the satellite DNA (Masiga *et al.,* 1992); primers predominantly used are: TVW (*T. vivax*), TBR (Trypanozoon) and TCS (*T. congolense* type savannah); PCRs using these primers are highly sensitive and species-, sub-genus- or type-specific, respectively. When positive, PCR alone is not a diagnostic certainty; it must be complemented by other tests or information (see below), because it may give false positive results due to sample contaminations. When negative, PCR alone cannot ascertain the absence of infection due to false negative results obtained from animals with low parasitaemia.

Antibody-detection tests (ELISA and IFAT) become positive 1–6 weeks after infection, thus with an incubation period of 2 weeks on average, and the persistence of antibodies after parasite elimination can last from 1 to 13 months, and is 3 months on average.

iv) ELISA: Three ELISAs using soluble antigens from whole cell lysates of animal trypanosomes are recommended: ELISA *T. vivax,* ELISA *T. brucei brucei* and ELISA *T. congolense* type savannah; depending on the context (America/Africa), 1 to 3 tests can be recommended; they all cross react. ELISAs exhibit high sensitivity (> 90%) and genus specificity (>95%), but subgenus specificity is not consistent, and none of them is species-specific. A positive sample reveals an immune response in the host to the parasite, but it does not indicate an active infection due to the persistence of antibodies after parasite elimination (as stated above), thus: (i) it must be complemented by other

tests or information (see below) if active infection is to be confirmed, and (ii) once an animal is seropositive to one or several ELISAs (or IFATs), it is not possible to determine whether it is or has been harbouring one or several trypanosome species. Even when a positive serology is associated with a positive species-specific agent detection test, other *Trypanosoma* species may be suspected in a mixed infection.

Providing it is associated with negative results to sensitive agent detection tests, when negative, ELISA is reliable, due to its high genus specificity. Both tests must be repeated at 30-day intervals due to the delay in seroconversion; however, longer delays may be observed occasionally.

Alternatively, IFATs can be used in the same conditions as ELISAs.

3.2. Association of recommended tests for the diagnosis of animal trypanosomosis

3.2.1. Recommended method for sensitive agent detection:

A combination of GSBS, HCT and PCR is recommended for agent detection.

i) GSBS: when positive, it brings diagnostic certainty, but when negative, it must be complemented with more sensitive test: HCT and/or PCR.

ii) HCT: when positive, HCT brings genus- or subgenus-specific diagnostic certainty, but it must be complemented with GSBS and PCR for agent identification. When negative, it should be complemented with the more sensitive PCR.

iii) PCR: routine diagnosis must use the satellite DNA primers that detect the most prevalent species responsible for Nagana: TVW, TBR and TCS in Africa, and TVW and TBR in America. When positive, PCR must be complemented with a positive GSBS, HCT, ELISA, or clinical examination to ascertain the infection.

In Africa, if PCR with the above primers is negative, complementary primers must be added: TCF, TCK and TSIM.

If TBR primers give positive response, and *T. evansi* might be suspected, complementary primers must be used to ascertain the species: RoTat1.2 & EVAB; however, their sensitivity is lower, which may lead to inconclusive outcome when they provide negative results.

In Africa, if TBR primers give positive response, and human *Trypanosoma* sspp. are suspected, complementary primers must be used: Tgs-GP and Tbr F/R however, their sensitivity is lower, which may lead to inconclusive situation when they provide negative results.

If PCR with the above primers are negative in a context of trypanosomosis suspicion (clinical signs, ELISA or HCT positive), other complementary primers must be used for parasite identification: TRYP1 or ITS1-CF/BR, and, in America and Latin America, TCZ (for *T. cruzi* DNA detection).

Due to possible low or even no parasitaemia, agent detection tests can confirm an infection when they are positive, but they cannot confirm the absence of infection when they are negative; they need to be associated with negative antibody detection test for this purpose.

3.2.2. Recommended method for antibody detection

ELISAs using soluble antigens extracted from whole trypanosome lysate are recommended tests, to be applied according to the epizootiological context. In Africa the recommended method to get high sensitivity and cover the most prevalent *Trypanosoma* spp. is to use all three: ELISA *T. vivax*, ELISA *T. brucei brucei* and ELISA *T. congolense* type savannah. In Latin America, the recommended method is to use only ELISA *T. vivax*, preferably in association with ELISA *T. evansi* (or ELISA *T. brucei brucei*) when Trypanozoon parasites are also investigated.

A seropositive sample is “suspect for active infection” but it is not a confirmed diagnosis; it must be complemented with positive results to GSBS or PCR, or associated with clinical signs of trypanosomosis or epidemiologically linked to a confirmed trypanosomosis case. In Africa, infections with *T. evansi* and *T. equiperdum* may interfere in the serodiagnosis of nagana. In

America, infections with *T. evansi*, *T. equiperdum* and *T. cruzi*, as well as *Leishmania* spp., may interfere in the serodiagnosis of *T. vivax*. Appropriate primers must be used in PCR to ascertain the infection and identify the parasite.

Finally, in areas where several species of trypanosomes are present (including T. *cruzi, T. evansi* and *T. equiperdum*), since cross-reactions may occur with any serological test employed, the exact status of a seropositive animal regarding active infection with one or several *Trypanosoma* species cannot be established.

Alternatively, IFATs can be used, in the same conditions described above for ELISAs.

C. REQUIREMENTS FOR VACCINES

No vaccines are in use at the present time.

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NB: There is a WOAH Reference Laboratory for Nagana (infections with salivarian trypanosomoses) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Nagana.

NB: FIRST ADOPTED IN 1991 AS TRYPANOSOMIASIS. MOST RECENT UPDATES ADOPTED IN 2021

CHAPTER 3.4.15.

THEILERIOSIS IN CATTLE (INFECTION WITH *THEILERIA ANNULATA*, *T. ORIENTALIS* AND *T. PARVA*)

SUMMARY

Description and importance of the disease: *Tick-transmitted* Theileria *parasites of cattle are a major constraint to the improvement of the livestock industry in large parts of Europe, Africa and Asia.* Theileria annulata *and* T. parva*, the most economically important species, are responsible for mortality and losses in production. Bovine theileriosis is generally controlled by the use of acaricides to kill ticks, but this method is not sustainable. Acaricides are expensive, they cause environmental damage, and over time ticks develop resistance to them requiring newer acaricides to be developed. More sustainable and reliable methods for the control of theileriosis that deploy a combination of strategic tick control and vaccination are desirable. However, these are yet to be successfully applied on a large scale in endemic areas.*

Identification of the agent: *Diagnosis of a variety of disease syndromes caused by the parasites is principally based on clinical signs, knowledge of disease and vector distribution, and identification of parasites in Giemsa-stained blood and lymph node smears. The presence of multinucleate intra-cytoplasmic and free schizonts, in lymph node biopsy smears, is a characteristic diagnostic feature of acute infections with* T. parva *and* T. annulata*. Animals infected with* T. parva *show enlarged lymph nodes, starting with the parotid lymph node, fever, a gradually increasing respiratory rate, dyspnoea and occasional diarrhoea. Post-mortem lesions observed are pulmonary oedema with froth in the trachea, enlargement of lymph nodes and spleen, haemorrhages in internal organs, abomasal erosions, the presence of parasitised lymphocytes and lympho-proliferative infiltrations in visceral tissues. The gross pathology caused by schizonts of* T. annulata *resembles that of* T. parva*, while the piroplasm stages may also be pathogenic, causing anaemia and jaundice. For* T. annulata*, the first lymph nodes involved are the pre-crural ones as a result of the predilection sites of the vector ticks.*

Molecular-based tests: *In addition, molecular diagnostic tests, particularly those based on the polymerase chain reaction and reverse line blot hybridisation are proving to be powerful tools for detecting theileria parasites in the vertebrate and invertebrate hosts, characterising species and parasite polymorphisms, defining population genetics and generating epidemiological data.*

Serological tests: *The most widely used diagnostic test for* Theileria *species has been the indirect fluorescent antibody (IFA) test. For the IFA test, both schizont and piroplasm antigens may be prepared on slides or in suspension and preserved by freezing at ≤ –20°C, except in the case of the piroplasm suspension, which is stored at 4°C. Test sera are diluted with phosphate-buffered saline and incubated with the antigen in suspension, and anti-bovine immunoglobulin conjugate is then added. Using the test as described, the fluorescence is specific to the causative agent. The IFA test is sensitive, fairly specific, and usually easy to perform. However, because of the problems of cross-reactivity among some* Theileria *species, the test has limitations for large-scale surveys in areas where species distribution overlaps. The IFA test for* T. parva *does not distinguish among the different immunogenic stocks. An indirect enzyme-linked immunosorbent assay for* T. parva *and* T. mutans*, based on recombinant parasite-specific antigens, has demonstrated higher sensitivity and specificity.*

Requirements for vaccines: *Reliable vaccines of known efficacy have been developed for* T. parva *and* T. annulata*. For* T. annulata*, the vaccine is prepared from schizont-infected cell lines that have been isolated from cattle and attenuated during* in vitro *culture. The vaccine must remain frozen until*

shortly before administration. Vaccination against T. parva *is based on a method of infection and treatment in which cattle are given a subcutaneous dose of tick-derived sporozoites and a simultaneous treatment with a long-acting tetracycline formulation. This treatment results in a mild or inapparent East Coast fever reaction followed by recovery. Recovered animals demonstrate a robust immunity to homologous and, to varying degrees, heterologous challenge, which usually lasts for the lifetime of an animal. In endemic areas characterised by high transmission intensities, immunisation of animals with a stock(s) engendering a broad-spectrum immunity is desirable to cover a range of immunologically distinct* T. parva *strains that exist in the field. Immunised animals may become carriers of the immunising parasite stock. Safety precautions must be taken in the preparation and handling of* T. parva *vaccines to protect the workers and to avoid contamination of the stabilates. Consideration should also be given to the risk of introducing new isolates into an area where they may then become established through a carrier state.*

A. INTRODUCTION

Theileriae are obligate intracellular protozoan parasites that infect both wild and domestic *Bovidae* throughout much of the world. They are transmitted by ixodid ticks, and have complex life cycles in both vertebrate and invertebrate hosts. There are a number of species of *Theileria* spp. that infect cattle; the two most pathogenic and economically important are *T. parva* and *T. annulata*. *Theileria parva* occurs in 13 countries in sub-Saharan Africa causing east coast fever (ECF), whilst *T. annulata* (tropical/Mediterranean theileriosis) occurs in southern Europe as well as North Africa and Asia. Endemic regions of *T. annulata* and *T. parva* do not overlap. *Theileria annulata* can occur in cattle, yaks, water buffalo and camels and is transmitted by ticks of the genus *Hyalomma.* Tropical theileriosis is more severe in European breeds, with a mortality rate of 40–90%, while the mortality rate in indigenous breeds of cattle from endemic areas can be as low as 3%. In Spain, *T. annulata* infections are mainly restricted to the southern and Mediterranean areas such as Menorca island, where the tick vector (*Hyalomma* sp.) is present. In northern Spain, reports of the presence of *Hyalomma* ticks are sporadic, as are associated *T. annulata* infections. However, tick distribution might change because of changes in climatic conditions. *Theileria orientalis/buffeli* complex is now thought to consist of two species – *T. orientalis*, occurring in the far east, and *T. buffeli* having a global distribution (Gubbels *et al*., 2000; Jeong *et al.,* 2010). Infection is generally subclinical; however, disease can occur in cattle depending on a number of epidemiological factors (including previous exposure to theileriae, stress or health status, and variations in the species pathogenicity, as reported recently in Australia and New Zealand (Gebrekidan *et al.,* 2015; McFadden *et al*., 2011). *Theileria taurotragi* and *T. mutans* generally cause no disease or mild disease, and *T. velifera* is nonpathogenic. These last three parasites are found mainly in Africa, and overlap in their distributions, complicating the epidemiology of theileriosis in cattle.

Theileria lestoquardi, also transmitted by *Hyalomma* ticks, is the only species of economic significance infecting small ruminants and it also occurs in north Africa, the Mediterranean basin and Asia. In sheep and goats, the morbidity rate from *T. lestoquardi* can approach 100% with a mortality rate of 46–100% in the most susceptible breeds.

Theileria uilenbergi and *T. luwenshuni* are pathogenic ovine piroplasms described in north-western China (People's Rep. of), though *Theileria* parasites with similar sequences have been found in sheep in northern Spain and Turkey, but apparently with a low pathogenicity. *Theileria luwenshuni* has also been detected in sheep in the United Kingdom associated with clinical signs (Phipps *et al*., 2016).

Some *T. parva* stocks produce a carrier state in recovered cattle, and studies using DNA markers for parasite strains have shown that *T. parva* carrier animals are a source of infection that can be transmitted naturally by ticks in the field (Bishop *et al*., 1992; Kariuki *et al*., 1995; Marcotty *et al*., 2002; Maritim *et al*., 1989). The severity of ECF may vary depending on factors such as the virulence of the parasite strain, sporozoite infection rates in ticks and genetic background of infected animals. Indigenous cattle in ECF-endemic areas are often observed to experience mild disease or subclinical infection, while introduced indigenous or exotic cattle usually develop severe disease.

The most practical and widely used method for the control of theileriosis is the chemical control of ticks with acaricides. However, tick control practices are not always fully effective for a number of reasons, including development of acaricide resistance, the high cost of acaricides, poor management of tick control, and illegal cattle movement in many countries. Vaccination using attenuated schizont-infected cell lines has been widely used for *T. annulata*, while for *T. parva* control, infection and treatment using tick-derived sporozoites and tetracycline is being implemented in a number of countries in eastern, central and southern Africa.

Chemotherapeutic agents such as parvaquone, buparvaquone and halofuginone are available to treat *T. parva* and *T. annulata* infections. Treatments with these agents rely on early detection of clinically affected animals and do not completely bring about eradication of theilerial infections, leading to the development of carrier states in their hosts.

The immune response to theileriae parasites is complicated. Cell-mediated immunity is thought to be the most important protective response in *T. parva* and *T. annulata*. In *T. parva,* the principal protective responses are mediated through killing of infected cells by bovine major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes. *Theileria annulata* schizonts inhabit macrophages and B cells. Innate and adaptive immune responses cooperate to protect cattle against *T. annulata* theileriosis. Intracellular parasites are mostly affected by cell-mediated immunity. Infection of leukocytes with *T. annulata* activates the release of cytokines, initiating an immune response and helping to present parasite antigen to $CD4^+$ T cells. These cells produce interferon-γ (IFN-γ), which activates non-infected macrophages to synthesise tumour necrosis factor α (TNF-α) and nitric oxide (NO), which destroy schizont- and piroplasm-infected cells. $CD8^+$ T cells have recently been shown to recognise parasite antigens presented by the MHC and to kill infected leukocytes. B cells produce antibody that along with NO kill extracellular merozoites and intracellular piroplasms. On the other hand overproduction of cytokines, in particular TNF-α, by macrophages generates many of the clinical signs and pathological lesions that characterise *T. annulata* theileriosis and the outcome of the infection depends upon the fine balance between protective and pathological properties of the immune system.

B. DIAGNOSTIC TECHNIQUES

Diagnosis of acute theileriosis is based on clinical signs, knowledge of disease, and vector distribution as well as examination of Giemsa-stained blood, lymph node and tissue impression smears. *Theileria parva* and *T. annulata* are diagnosed by the detection of schizonts in white blood cells or piroplasms in erythrocytes. The piroplasmic stage follows the schizont stage and, in both *T. parva* and *T. annulata*, it is usually less pathogenic and is thus often found in recovering or less acute cases. It is hoped that a combination of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) will greatly enhance our present capacity to identify infected animals, thus making possible accurate surveys of *Theileria* species. Eventually, the aim would be to develop these technologies for the diagnosis of all the vector-borne diseases.

Table 1. Test methods available for the diagnosis of theileriosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[(a)]						
Microscopic examination	–	+++	–	+++	–	–
PCR	+	++	++	+++	+	–
Detection of immune response						
IFAT	+	+++	++	–	+++	–
ELISA	+	+	++	–	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.
[(a)] A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Microscopic examination

Multinucleate intralymphocytic and extracellular schizonts can be found in Giemsa-stained biopsy smears of lymph nodes, and are a characteristic diagnostic feature of acute infections with *T. parva* and *T. annulata.* Both intracellular and free-lying schizonts may be detected, the latter having been released from parasitised cells during preparation of the smears. Schizonts are transitory in *T. mutans* and the *T. orientalis/buffeli* group, in which the piroplasm stage may be pathogenic. *Theileria taurotragi* schizonts are not readily detected in Giemsa-stained blood smears. A veil to the side of the piroplasm may distinguish *T. velifera*. The schizonts of *T. mutans*, if detected, are distinct from *T. parva,* having larger, flattened, and irregular nuclear particles. The piroplasms (intra-erythrocytic stage) of *T. parva, T. annulata* and *T. mutans* are similar, but those of *T. annulata* and *T. mutans* are generally larger and may be seen to divide. However, for practical purposes schizonts and piroplasms of different theilerias are very difficult to discriminate in Giemsa-stained smears.

The schizont is the pathogenic stage of *T. parva* and *T. annulata.* It initially causes a lymphoproliferative, and later a lymphodestructive disease. The infected animal shows enlargement of the lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea and/or diarrhoea. The most common post-mortem lesions are enlarged lymph nodes, a markedly enlarged spleen, pulmonary oedema, froth in the trachea, erosions and ulceration of the abomasum, and enteritis with necrosis of Peyer's patches. Lymphoid tissues become enlarged in the initial stages of the disease, but then atrophy if the animal survives into the chronic stages of the disease. When examined histologically, infiltrations of immature lymphocytes are present in lung, kidney, brain, liver, spleen, and lymph nodes. Schizont-parasitised cells may be found in impression smears from all tissues: lung, spleen, kidney and lymph node smears are particularly useful for demonstrating schizonts. In longer standing cases, foci of lymphocytic infiltrations in kidneys appear as white infarcts. In animals that recover, occasional relapses may occur. A nervous syndrome called 'turning sickness' is sometimes observed in *T. parva*-endemic areas, and is considered to be associated with the presence of intravascular and extravascular aggregations of schizont-infected lymphocytes, causing thrombosis and ischaemic necrosis throughout the brain.

In *T. annulata,* both the schizont and piroplasm stages may be pathogenic. Schizonts are scarce in the peripheral blood of acutely sick animals and their presence in blood smears indicates a poor prognosis. However, schizonts can be easily detected in smears from lymph nodes, spleen and liver tissues obtained by needle biopsy of these organs. The gross pathology caused by schizonts of *T. annulata* resembles that of *T. parva*, while anaemia and jaundice are features of both schizont and piroplasm pathology. Pathogenic strains of *T. mutans* also cause anaemia, as can strains from Japan and Korea referred to as *T. sergenti*.

Piroplasms of most species of *Theileria* may persist for months or years in recovered animals, and may be detected intermittently in subsequent examinations. However, negative results of microscopic examination of blood films do not exclude latent infection. Relapse parasitaemia can be induced with some *Theileria* species by splenectomy. Piroplasms are also seen in impression smears prepared at post-mortem, but the parasites appear shrunken and their cytoplasm is barely visible.

1.2. Molecular methods

The early DNA-based molecular methods to detect *Theileria* species were based on Southern blotting using a range of probes, derived from ribosomal RNA gene sequences, to detect all the *Theileria* species that are known to infect cattle (Allsopp *et al*., 1993; Bishop *et al.,* 1995). DNA probes specific for *T. parva* (Allsopp & Allsopp, 1988; Conrad *et al*., 1987; Morzaria *et al.,* 1999a) and *T. mutans* (Morzaria *et al.,* 1989) were also developed. The Southern blotting methods have largely been supplanted by species-specific PCR protocols designed to detect *T. annulata* or *T. parva* carrier cattle (Bishop *et al.,* 1992; D'Oliveira *et al.,* 1995; Odongo *et al.,* 2010; Skilton *et al.,* 2002). Several PCR assays have also been developed using specific genes or satellite sequences for characterisation of different isolates/strains/clones of *T. parva* (Geysen *et al.,* 1999; Oura *et al.,* 2003; Patel *et al.,* 2011).

A reverse line blot (RLB) assay based on hybridisation of PCR products to specific oligonucleotide probes immobilised on a membrane for simultaneous detection of different *Theileria* species has been

introduced (Gubbels *et al.,* 1999), and fluorescence resonance energy transfer (FRET)-based real-time assays have been developed for specific diagnosis of *T. parva* (Sibeko *et al.,* 2008).

The primer and probe sequences for several of these assays are presented in Table 2, together with the cycling conditions for the commonly used p104 nested PCR for *T. parva*.

PCR amplification of the p33/34 genes of the *T. orientalis/buffeli* complex followed by restriction enzyme analysis can be used to characterise the various types (Kawazu *et al.,* 1992; Kubota *et al.,* 1995).

***Table 2. PCR primers amd probes for the detection of* T. annulata *or* T. parva**

Target gene	Primer sequences (5'–3')	Probe sequences	Reference
T. annulata			
30 kDa protein	GTA-ACC-TTT-AAA-AAC-GT GTT-ACG-AAC-ATG-GGT-TT	n/a	D'Oliviera *et al.,* 1995
T. parva			
p104	ATT-TAA-GGA-ACC-TGA-CGT-GAC-TGC TAA-GAT-GCC-GAC-TAT-TAA-TGA-CAC-C	n/a	Skilton *et al.,* 2002
p104 (nested)	Primary PCR: ATT-TAA-GGA-ACC-TGA-CGT-GAC-TGC TAA-GAT-GCC-GAC-TAT-TAA-TGA-CAC-C Secondary PCR: GGC-CAA-GGT-CTC-CTT-CAG-ATT-ACG TGG-GTG-TGT-TTC-CTC-GTC-ATC-TGC Cycling conditions: Primary: • 94°C for 1 minute • 40 cycles of 94°C/1 minute, 60°C/1 minute, 72°C/1 minute • 72°C for 9 minutes after the last cycle. Secondary: • 94°C for 1 minute • 30 cycles of 94°C/1 minute, 55°C/1 minute, 72°C/1 minute • 72°C for 9 minute after the last cycle	n/a	Odongo *et al.,* 2010.
18S RNA (RLB)	GAG-GTA-GTG-ACA-AGA-AAT-AAC-AAT-A TCT-TCG-ATC-CCC-TAA-CTT-TC	TTC-GGG-GTC-TCT-GCA-TGT	Gubbels *et al.,* 1999
18S RNA (FRET)	CTG-CAT-CGC-TGT-GTC-CCT-T ACC-AAC-AAA-ATA-GAA-CCA-AAG-TC	GGG-TCT-CTG-CAT-GTG-GCT TAT–F LCRed640-TCG-GAC-GGA-G TTC-GCT-PH	Sibeko *et al.,* 2008

2. Serological tests

2.1. The indirect fluorescent antibody test

The indirect fluorescent antibody (IFA) test is the most widely used diagnostic test for *Theileria* spp. The IFA test is robust, easy to perform and provides adequate sensitivity and specificity for use in the field for detection of prior infection with *T. parva* and *T. annulata* under experimental situations and in a defined epidemiological environment where only one theilerial species is present. The IFA test has limitations for large-scale serological surveys due to its reduced specificity in field situations where several *Theileria* species co-exist. There is a need for tests that are more specific, easy to interpret, and robust enough to be used in field conditions.

2.1.1. Preparation of schizont antigen

i) Schizont antigen slides

The antigens used for the IFA test are intracytoplasmic schizonts derived from infected lymphoblastoid cell lines for *T. parva* and from infected macrophage cell lines for *T. annulata.*

Cultures of 200 ml to 1 litre of either *T. parva* or *T. annulata* schizont-infected cells containing 10^6 cells/ml, of which at least 90% of the cells are infected, are centrifuged at 200 ***g*** for 20 minutes at 4°C. The supernatant fluid is removed and the cell pellet is resuspended in 100 ml of cold (4°C) phosphate-buffered saline (PBS), pH 7.2–7.4, and centrifuged as before. This washing procedure is repeated three times, and after the final wash the cell pellet is resuspended in PBS (approximately 20–100 ml) to give a final concentration of 10^7 cells/ml.

Using a template or pipette tip, thin layers of the cell suspension are placed on Teflon-coated multispot slides, or on ordinary slides using nail varnish for separation. The smears should give between 50 and 80 intact cells per field view when examined under a ×40 objective lens. The antigens are distributed on to the slides using multichannel or a 100 µl pipette. By dispensing and immediately sucking up the schizont suspension, a monolayer of schizonts remains in each well. This is performed for each enclosure until the volume is exhausted. With this method, approximately 600 good quality slides containing a total of 6000 individual antigen spots can be obtained. The slides are air-dried, fixed in acetone for 10 minutes, individually wrapped in tissue paper and then in groups of five in aluminium foil, and stored in airtight, waterproof plastic containers at either –20°C or –70°C. The antigens keep for at least 1 year at –20°C and longer at –70°C.

ii) Schizont antigen in suspension

First, 500 ml of *T. parva*- or *T. annulata*-infected cells containing 10^6 cells/ml are centrifuged at 200 ***g*** for 10 minutes at 4°C, and the cell pellet obtained is washed twice in 100 ml of cold PBS. The viability of the cells is determined by eosin or trypan blue exclusion (it should be greater than 90%). The cells are resuspended at 10^7/ml in cold PBS. To this volume, two volumes of a cold fixative solution containing 80% acetone and 0.1% formaldehyde (0.25% formalin) in PBS are added drop by drop while the cell suspension is stirred gently and continuously in an ice bath. The cell suspension is kept at –20°C and allowed to fix for 24 hours. Siphon about 2/3 of the volume off, centrifuge and decant. The fixed cells are then washed three times in cold saline and centrifuged at 200–400 ***g*** for 20 minutes at 4°C. After the last wash, the cells are resuspended into 5 ml PBS + 0.2% BSA (bovine serum albumin) at 10^7/ml. The fixed cells are distributed in aliquots of 0.5 ml. The antigen is stable at 4°C with 0.2% sodium azide as preservative for 2 weeks, and keeps indefinitely at –20°C.This method can also be used to prepare schizont antigen for *T. taurotragi*.

2.1.2. Preparation of piroplasm antigen

The piroplasm stage of *Theileria* spp. cannot be maintained in culture, therefore the piroplasm antigen must be prepared from the blood of infected animals. Due regard should be paid to the principle of 'The Three Rs' as set out in the WOAH *Terrestrial Code*, Chapter 7.8 *Use of animals in research and education.*

i) Piroplasm antigen slides

Experimental infections are induced by infecting cattle subcutaneously with sporozoites, or applying ticks infected with *T. parva*, *T. annulata* or *T. taurotragi*. Infection with *T. annulata* is invariably produced by inoculation of blood drawn from cattle with acute theileriosis. Splenectomy of the recipient cattle prior to the infection considerably increases the piroplasm parasitaemia in red blood cells (RBC). Peak parasitaemias are of short duration and if animals survive the disease the percentage of infected RBC decreases considerably in a few days. Infections with the parasite group referred to as *T. orientalis/buffeli, T. mutans* or *T. velifera* are usually induced by inoculating splenectomised cattle intravenously with blood from a carrier animal, or with a blood stabilate, or by application of infected ticks. When the piroplasm parasitaemia is 10% or higher, 100 ml of the infected blood is collected from the jugular vein in a heparinised or ethylene diamine tetra-acetic acid (EDTA) vacutainer, and gently mixed with 2 litres of PBS. The mixture is centrifuged at 500 ***g*** for 10 minutes at 4°C; the plasma and buffy coat are removed, the RBC are again resuspended in 2 litres of PBS, and the centrifugation step is repeated. It is important to remove the buffy coat after each wash. This washing procedure is repeated four times. After the final wash, an aliquot of the packed RBC is used to make doubling dilutions in PBS, and a 5 µl drop of each dilution is placed on slides. The dried spots are fixed in methanol and stained with Giemsa's stain, and the concentration of RBC is examined using a light microscope. The dilution that gives a single layer of RBC spread uniformly on the spot is then selected for large-scale preparation of piroplasm antigen slides. Approximately 10,000 antigen slides (100,000 antigen spots) can be prepared from 100 ml of infected blood. The antigen smears are allowed to dry at room temperature before fixing in cold (4°C) acetone for 10 minutes. The fixed smears can be stored as for the schizont antigen slides, and kept for similar periods.

ii) Piroplasm antigen suspension

An alternative method of preparing antigens to that described above is available, and has been tested for *T. parva*. In this procedure, 100 ml of blood are taken from an animal with a high piroplasm parasitaemia and prepared as described previously, and the packed cell volume is adjusted to 5% in PBS.

One volume of the RBC suspension is added to two volumes of the fixative (see Section B.2.1.1.ii above) while stirring. The cells are allowed to fix at –20°C for 24 hours. The fixed cells are then washed three times with PBS and centrifuged at 1000 ***g*** for 30 minutes. The deposit is resuspended to the original volume of blood with PBS containing 0.2% sodium azide, and distributed in aliquots of 0.5 ml.

The piroplasm antigen is stable at 4°C when preserved with 0.2% sodium azide for a period of at least 3 years.

2.1.3. Standardisation of antigen

Schizont or piroplasm antigen suspensions are mixed on a rotor mixer and titrated in PBS by doubling dilution starting from undiluted through to 1/16. The dilution giving a cell distribution of approximately 50–80 schizont-infected cells or 150–200 infected RBC per field view when examined under a ×40 objective lens is recommended for use for that batch of antigen. Using this dilution, test antigen smears are prepared on slides. These antigen smears plus the antigen slides previously frozen (and thawed before use) are tested against a range of dilutions of a panel of known strong, intermediate and weak positive and negative control sera. If the positive control sera titrate to their known titres and the negative control sera give no fluorescence, the antigen is used in the routine IFA test.

Both types of antigen preparations, acetone-fixed smears stored at either –20°C or –70°C, and antigens fixed in suspension and stored at either 4°C or –20°C, are used routinely in many laboratories. The sensitivity of both types of antigen is comparable. In laboratories where adequate low temperature storage facilities and a reliable supply of electricity are available, the antigen slides can be used. However, such antigens can only be transported on dry ice or in liquid nitrogen. Antigens fixed in suspension have the advantage over antigen slides in that the initial method of preparation is simpler and quicker. A large batch of this antigen can be stored in one

container, and aliquots may be taken out as necessary from which fresh smears are prepared for the IFA test. The need for a large storage facility is thereby avoided. The antigens fixed in suspension can also be stored at 4°C and can be safely transported at room temperature without loss of antigenicity.

2.1.3.1. Preparation of bovine lymphocyte lysate

A lymphocyte lysate is prepared according to the method described by Goddeeris *et al*. (1982), for use in tests with antigens of *T. parva* in suspension. Briefly, a 3-month-old calf is splenectomised and maintained in a tick-free environment. To exclude the possibility of latent theilerial infections, Giemsa-stained blood smears are examined daily for a period of 4 weeks for parasites. The parasite-free animal is killed and the thymus and all the accessible lymph nodes are removed. These tissues are sliced into small pieces in cold PBS containing 0.45% EDTA as anticoagulant. Cells are teased out of the tissue, separated from the debris by passing through a muslin cloth, and washed three times with PBS/EDTA by centrifugation at 200 ***g*** for 20 minutes at 4°C. The washed lymphocytes are resuspended in PBS without EDTA, to give a final concentration of 5×10^7 cells/ml. The cells are disrupted by sonication in 100-ml aliquots on ice for 5 minutes using the 3/8 probe. The sonicated material is centrifuged at 1000 ***g*** for 30 minutes at 4°C, and the supernatant, adjusted to 10 mg protein/ml, is stored at –20°C in 4-ml aliquots.

2.1.3.2. Test procedure

i) With schizont or piroplasm slide antigen

a) Remove antigen slides from freezer and allow to thaw for 30 minutes at 4°C and then for 30 minutes at room temperature.

b) Inactivate the sera to be tested for 30 minutes in a water bath at 56°C.

c) Unpack the slides and label the numbers of the sera tested.

d) Prepare 1/ 40 and 1/80 dilutions of sera to be tested. Validated positive and negative sera are included with each test as controls. Further doubling dilutions can be made if end-point antibody titres are desired.

e) Transfer 25 µl of each serum dilution to a spot of antigen.

f) Incubate in a humid chamber for 30 minutes at room temperature.

g) Remove the serum samples from the antigen wells by washing with PBS and rinse by immersing in two consecutive staining jars containing PBS for 10 minutes each time.

h) Distribute to each well 20 µl of diluted anti-bovine immunoglobulin fluorescein isothiocyanate conjugate at appropriate dilution (generally, dilutions recommended by manufacturers are suitable; however, minor adjustments may be necessary for optimal results). Incorporate Evans blue into the conjugate at a final dilution of 1/10,000 as a counterstain and incubate in a humid chamber for 30 minutes at room temperature.

i) Repeat step g and mount with a cover-slip in a drop of PBS/glycerol (50% volumes of each).

j) Read the slides under a fluorescent microscope equipped with epi-Koem illumination (100 W mercury lamp), UV filter block, ×6.3 eyepieces and Phaco FL 40/1.3 oil objective lens.

ii) With schizont antigen stored in suspension

a) Thaw frozen antigen at room temperature.

b) Distribute the antigen suspension on the spots of multispot slides, using multichannel or a 100-µl pipette. By dispensing and immediately sucking up the suspension a monolayer of schizont-infected cells remains on each well.

c) Allow slides to dry at room temperature or 37°C.

d) Dilute test and control sera 1/40 in lymphocyte lysate (195 µl lymphocyte lysate + 5 µl serum).

e) Proceed as described in steps e to j (Section B.2.1.3.2.i).

iii) With piroplasm antigen stored in suspension

a) Resuspend piroplasm antigen (stored at 4°C) by agitation and disperse RBC by passing the suspension through a 25-gauge needle to break the clumps.

b) Dilute the antigen to previously standardised dilutions (see preparation of piroplasm antigen).

c) Allow slides to dry at room temperature or 37°C.

d) Proceed as described in steps d and e (Section B.2.1.3.2.ii).

2.1.4. Characteristics of the indirect fluorescent test

The incorporation of Evans blue provides a good contrast, enabling good differentiation of non-infected cells from the infected ones under the fluorescent microscope. Mounting the slides in 50% glycerol, at pH 8.0, reduces the rapid fading of fluorescein isothiocyanate and makes photography of the preparation possible. Once prepared, slides are stable and can be read for up to 72 hours after preparation when kept at 4°C in the dark.

The sensitivity of the IFA test depends on the period of time that has elapsed since the onset of infection. Following infection with sporozoites, antibodies to *T. parva* and *T. annulata* are first detected between days 10 and 14 using the schizont antigen. Using the piroplasma antigen, antibodies are first detected between days 15 and 21. Antibodies last for a variable period of time after recovery, depending on such factors as the establishment of a carrier state, chemotherapeutic intervention, and presence or absence of a rechallenge. Following recovery from infection with *T. parva* or *T. annulata*, high levels of antibody are generally detected for 30–60 days. The antibody levels gradually decline and low antibody titres are still detectable 4–6 months after recovery. Later, antibody may become undetectable at a serum dilution of 1/40, but may persist for more than 1 year following a single challenge. In ECF endemic regions, the seroprevalence in cattle population fluctuates considerably depending on the level and regularity of challenge. In an endemic area where a seasonal transmission cycle of ECF occurs, IFA has been shown to lack sensitivity. The overall diagnostic sensitivity of the IFA test has been evaluated as 55% at a cut off titre 1/40 and 28% at cut off 1/160. The specificity of the test for the two cut off points was 86% and 95% respectively (Billiouw *et al*., 2005).

The IFA test is useful for identifying herds that contain carriers of *T. annulata*, but is not always sufficiently sensitive to detect all infected individuals. Both schizont and merozoite (piroplasm) IFA antigens have failed to detect antibody in some animals despite carrying patent infection with piroplasms (Darghouth *et al*., 1996).

In *T. mutans* infections induced by sporozoite inoculation, antibodies are first detected between days 10 and 15 after the appearance of piroplasms. Low titres are detectable for at least 12–24 months.

The *T. parva* IFA test is highly sensitive for detection of antibodies in an epidemiological situation where only one species of *Theileria* exists. However, if the test is used to detect antibodies where mixed infections of *Theileria* occur, the specificity of the test needs to be carefully evaluated. For example, *T. annulata* and *T. parva* cross-react, although these cross-reactions are four- to six-fold lower than with the homologous sera. The cross-reactivity between the two species has little practical significanace as the geographical distribution of these two parasites does not overlap. In the IFA test such cross-reactivity does not occur between *T. parva* and *T. mutans* or between *T. annulata* and *T. mutans*. There is a low level of cross-reactivity between *T. parva* and *T. taurotragi*, reducing the specificity of these two tests in many parts of sub-Saharan Africa where their distribution overlaps.

A panel of monoclonal antibodies (MAbs) detecting various epitopes on the polymorphic immunodominant antigen of the *T. parva* schizont stage has been generated. This panel can be used in the IFA test using the schizont-infected lymphoblastoid cells to detect differences between certain stocks of *T. parva* and between *T. parva* and other theilerial species. This test has been deployed as one of the several characterisation tools to differentiate various stocks of *T. parva*, and for quality control during sporozoite stabilate preparation (Bishop *et al*., 1994).

2.2. Enzyme-linked immunosorbent assays

Serological tests based on the ELISA have been developed for the detection of antibodies to *T. annulata* (Gray *et al*., 1980). Tests used for *T. parva* and *T. mutans* are indirect ELISAs based on parasite-specific antigens, PIM and p32, respectively (Katende *et al*., 1998; Morzaria *et al*., 1999a). These ELISAs provide higher (over 95%) sensitivity than the IFA tests. The ELISA reagents are available from the International Livestock Research Institute, Nairobi, Kenya.

C. REQUIREMENTS FOR VACCINES

C1. Cell culture live vaccines for *Theileria annulata*

1. Background

Vaccination against *T. parva* and *T. annulata* has been attempted since the causal organisms were first recognised early in the last century. However, reliable live vaccines of known potency are a more recent development. The most widely used are attenuated schizont cell culture vaccines against *T. annulata*. The procedures for production and safety testing have been described (Food and Agriculture Organization of the United Nations (FAO), 1984; Hashemi-Fesharki, 1988; Pipano, 1989b), and the vaccine is used in Israel, Iran, Turkey, Spain, India, northern Africa, central Asia and the People's Republic of China.

Despite the fact that vaccination with the cell culture vaccine against *T. annulata* has been available for more than three decades and has shown to be effective under field conditions, the use of this vaccine has been limited. The concern about the introduction of vaccine-derived parasites into the field tick population has led to individual countries developing vaccines from local isolates (Morisson & Mc Keever, 2006). Some attenuated cell lines have lost the ability to differentiate to erythrocytic merozoites (piroplasms) when inoculated to cattle and in one instance, *Hyalomma* nymphs fed on vaccinated cattle did not become infected (Kachani *et al*., 2004a). However in most cases the loss of differentiation is based on macroscopic examination of blood films from vaccine inoculated cattle. This drawback, the difficulties in standardisation of the antigenic composition of the cultured parasites and the need of a cold chain for distribution of the vaccine to the field are limiting factors in commercialisation of this vaccine (Morisson & Mc Keever, 2006).

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Primary cultures of *T. annulata*-infected cells may be established from trypsinised lymph nodes, liver, or spleen taken aseptically from an infected animal after death, or from the buffy coat of heparinised peripheral blood separated on a density gradient (Ficoll Hypaque), or by lymphocytes harvested from lymph node biopsy material using a plastic syringe method (Brown, 1979; FAO, 1984).

Seed cultures are prepared from cryopreserved cell lines that have been isolated from cattle and attenuated as described below. Vaccines should be produced from a seed culture (master seed) that has been passed less than 30 times, because there is some uncertainty about the immunogenic stability of these cultures in long-term passage.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. As cell culture material is derived from field animals, their cells might be potential sources of contamination of the vaccine with extraneous pathogens. Potential contaminants include bovine leukosis, mycoplasma, bovine viral diarrhoea virus, bovine spongiform encephalopathy (BSE) and other bacteria and viruses.

Cell cross-contamination in cell cultures is a common problem during cell culturing and use. The problem can be solved by increasing the awareness and by introducing regular quality control of cell cross-contamination.

2.1.3. Validation as a vaccine strain

Attenuation of *T. annulata* schizonts is achieved by prolonged growth and passage in culture (Pipano, 1989b). The loss of parasite virulence appears to be due to a change in parasite gene expression. Attenuation is assessed by the inoculation of the culture into susceptible calves every 20–30 passages. A sample of culture should be cryopreserved every ten passages in case of accidental loss or contamination. Complete attenuation is achieved when cultures do not cause fever or detectable schizonts and piroplasms in susceptible cattle but could take up to 300 passages. An attenuated culture will reliably infect cattle at 10^5 cells and induce a serological reaction, and will not produce disease at 10^9 cells. Cultures may be cryopreserved using either dimethyl sulphoxide (DMSO) or glycerol. Two methods of storing and delivering the vaccine are described below.

2.2. Method of manufacture

2.2.1. Procedure

The infected cells are cultured initially in Eagle's minimal essential medium (MEM) or Leibovitz L15 medium supplemented with 20% calf serum and containing penicillin (100 units/ml), streptomycin (50 μg/ml), and mycostatin (75 units/ml) in 25-ml plastic screw-cap tissue-culture flasks. An alternative medium is RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin, and is usually used with established cultures. Medium is replenished every 3–4 days. The presence of bright refractile cells free in the medium (on examination using a phase-contrast or inverted microscope) is indicative of infected cell growth. The cultures may establish as a monolayer or in suspension. Passage is effected by decanting the medium, adding 0.025% EDTA (versene) for 15 minutes to monolayer cultures, dispersing the cells, then counting and dispensing according to flask size. Approximately 10^6 cells are introduced into a 25 cm^2 flask, and the same seed rate in 100–200 ml is used in larger flasks. The general culture technique is as described by Brown (1979).

Serum is essential for maintenance of these cultures, and is obtained either from calves up to the age of 6 months, or from commercial sources, and is tested for toxicity through three passages in an established cell line before use.

2.2.2. Requirements for ingredients

Before starting to produce vaccine, seed material with known characteristics is required (Pipano, 1997). Three types of seed material are distinguished:

i) Master seed

Schizont-infected cells from a specific passage that have been selected and permanently stored and from which all other passages are derived. The master seed should consist of a single uniform batch of seed that has been mixed and filled into containers as one batch. As *T. annulata* schizont-infected cells are used for the manufacturing process, the master seed also represents the master cell stock (see Chapter 1.1.8 *Principles of veterinary vaccine production*). To prepare a master seed, schizont-infected cells that have proved to be safe for cattle are propagated to obtain in a single culture passage approximately 5×10^8 cells. The cells are cryopreserved in about 100 cryotubes each containing 5×10^6 cells. A viability check of the master seed should be performed once the master seed has been cryopreserved for at least 24 hours by reviving one of the cryotubes.

ii) Working seed

Schizont-infected cells at a passage level between the master seed and the production seed. To prepare a working seed, the contents of a single cryotube of master seed are transferred to a 10 ml centrifuge tube containing 8 ml complete medium. The tube is centrifuged at 600 ***g*** for 15 minutes at 4°C and the pellet is transferred into a 75 cm^2 culture

flask containing 15–20 ml medium. The medium is replaced the next day, and 4 days later the cells are dispersed and subcultured in larger vessels. After 5–6 subcultivations, a sufficient number of infected cells is available to start the production run.

iii) Production seed

Schizont-infected cells from a specific passage level are used without further propagation for the preparation of a batch of vaccine. The production seed is obtained by propagating large numbers of cells in monolayer or suspension cultures. Monolayer cultures are grown in flasks, 150 cm^2 to 175 cm^2, which usually provide an average of from 7×10^7 to 8×10^7 cells per vessel. About 80 ml of complete medium per flask is required. In a roller bottle culture system, $1.2–1.5 \times 10^8$ cells can be obtained in a conventional roller bottle (700 cm^2) containing 100–120 ml of medium. To obtain optimal yield of cells, stationary cultures or roller bottles cultures are incubated for 6–7 days with culture media as described previously, see Section C1.2.2.1.

The schizont-infected cells from all vessels are harvested and pooled together and the total number is computed. Alternatively, about 20% of the cells may be seeded again to prepare another batch of vaccine. Several batches of vaccine can be produced using a portion of the production seed as working seed. As prolonged cultivation may generate alteration in the futures of the schizonts, such as immunogenic capacity, after several batches, subsequent vaccine is produced by making a fresh production seed from the master seed.

Schizont-infected cells are mixed with DMSO at a final concentration of 7% or glycerol at a final concentration of 10%, and dispensed in 1.8-ml aliquots into 2-ml plastic vials, each vial containing ten doses of concentrated vaccine. As DMSO immediately penetrates the cell membranes, the time spent in dispensing the vaccine into the vials should be as short as possible. When glycerol is used, an equilibration time of 30–40 minutes is required before freezing the vaccine. There is no consensus on how many schizont-infected cells should constitute one dose of the vaccine. A recommended practical approach is to prepare doses of $10^6–10^7$ infected cells in order to counteract variable environmental conditions in the field. However, considerable protection against sporozoite-induced infection has been achieved by vaccination with 10^5 infected cells (Kachani *et al*., 2004b).

The vaccine is frozen by introducing the vials in an ultracold deep freezer (–70°C) and transferring them 24 hours later to liquid nitrogen containers. Alternatively vials can be introduced in gas phase liquid nitrogen for 3 hours and then immersed in the liquid nitrogen for storage (Pipano, 1989b). Vaccine is transported to the field in liquid nitrogen, and diluted 1/10 in isotonic buffered saline in a screw-cap bottle with a rubber or silicone septum for aseptic withdrawal. For dilution of vaccine frozen with glycerol, isotonic buffered saline should also contain 10% glycerol in order to avoid osmotic damage to the schizonts. The vaccine is administered subcutaneously within 30 minutes of thawing (Pipano, 1977).

The vaccination regimen in Iran up to 1990 was to inoculate two different mild strains 1 month apart. But, in order to reduce cost and save time, a new method was implemented involving only a single dose of a local live attenuated vaccine strain (Hashemi-Fesharki, 1998). A fresh culture vaccine is used in Morocco, usually at a tenfold lower dose (10^4 schizont-infected cells) (Kachani *et al*., 2004b). However there are problems with quality control of vaccines with short shelf life.

2.2.3. In-process controls

Records of the source and passages of the working seed material should be maintained. Seeds should be free of infective agents such as enzootic bovine leukosis, bovine immunodeficiency virus, bovine pestivirus, bovine syncytial virus, Rift Valley fever, etc. Test procedures will depend on availability preferably using DNA analysis.

pH, temperature and coloration of the solutions should be checked during the process and must have been shown to be free from contaminants. Numbers and contamination in growing cell cultures should be checked on a daily basis by examination using an inverted microscope.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety precautions

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

Theileria annulata schizonts are not hazardous for humans or contagious for animals, therefore the main purpose in designing a vaccine production facility is to prevent contamination of the product by extraneous organisms.

a) Freedom from properties causing undue local or systemic reactions

For testing the safety of the master seed, two to four susceptible calves, of the most sensitive stock available, are inoculated with a tenfold greater dose than is recommended for immunisation. This dose should not produce clinical signs beyond a transient rise in temperature. With completely attenuated master seed, no schizonts or piroplasms will be seen in lymph node and liver smears or in blood films. However, different breeds of cattle may show different sensitivities to the vaccine. This should be borne in mind when vaccine from a partially attenuated master seed is to be administered to high-grade cattle stocks.

Following a successful test for safety of a sample, all subsequent batches produced from the same master seed can be released without further testing for safety. However, if parasites are detected in the blood or tissues of vaccinated field animals, or if clinical signs develop following the inoculation of the vaccine, the batch or a parallel batch, from the same master seed, should be retested for safety.

iii) Batch potency

In Israel the schizont vaccines are tested using a documented procedure (Pipano, 1989a) before release.

Usually, the schizont vaccine is produced in small individual batches (3–5 thousand doses), which makes the full testing of each batch impractical for economic reasons. It is recommended therefore that the first batch of vaccine produced from a master seed be tested for safety, efficacy, potency and sterility, while each subsequent batch be tested for sterility and potency only. This recommendation is based on the fact that once the cultured schizonts become attenuated, no reversion to virulence has ever been observed during further cultivation. As far as efficacy is concerned, no obvious alteration of the immunogenic properties has been observed during the limited number (20–30) of passages involved in producing the actual vaccine.

a) Viability of schizont-infected cells

The potency test is conducted by quantitative *in-vitro* methods. Frozen vaccine remains stable during the storage period, even for long periods, but some loss of viability occurs during the freezing and thawing processes. Viability should be tested under conditions as similar as possible to those obtained when the vaccine is used in the field. For this reason, vaccine should be thawed and the diluted suspension of schizont-infected cells should be left at ambient temperature for 60 minutes before performing the viability tests. A simple test for evaluating viability of the infected cells is nigrosin dye exclusion counting (Wathanga *et al.,* 1986). Vaccine that, after being thawed and diluted and left at room temperature for 1 hour, still contains 50% or more live cells can be released for use although in most cases 80–90% of live cells are found.

Viability of the schizonts is also reflected by the plating efficiency of the schizont-infected cells (Wathanga *et al.,* 1986), as only cells containing viable schizonts multiply in culture. For

this purpose, the thawed, diluted vaccine is transferred from the bottle to a centrifuge tube. A sample for counting is taken and the suspension is centrifuged for 15 minutes at 600 ***g***. Meanwhile, the total number of cells (live and dead) is determined in order to ascertain that the frozen vaccine had the necessary initial concentration of cells. After centrifugation, the supernatant is discarded and the cells are resuspended to the original volume using complete culture medium. Serial tenfold dilutions of cells in complete medium are performed in sterile 10 ml tubes so that the last two dilutions contain 50, and 5 cells per ml, respectively. Twelve replicates of 200 µl from each of the last two dilutions are introduced into a 96-well culture plate. The plates are incubated at 37°C in a 5% CO_2 atmosphere and cultures are checked with an inverted microscope 6 and 9 days after seeding. The number of wells theoretically containing 1 cell each in which growth is observed is counted. Vaccine showing a plating efficiency <2 (cells) are adequate for field use.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C2.2.1 and C2.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Theileria annulata shizonts are not contagious for animals. These vaccines produce no adverse effects in healthy cattle. However, animals with existing infections, particularly viral infections, may not tolerate vaccination well. The administration of a viral vaccine, such as for foot and mouth disease, during the immunisation period (reaction period) is not recommended as the immune response may be compromised (Hashemi-Fesharki, 1988). In Iran, it is not recommended to vaccinate cows that are over 5 months pregnant, although studies in pregnant cattle with the vaccine stocks used in Israel found no effect on pregnancy (Pipano, 1989a). The immunity engendered is long lasting.

In general, cattle should be immunised in the first few months of life, and tick challenge under natural conditions reinforces the immunity. Although antigenically different strains of *T. annulata* have been identified (Pipano, 1977), it is generally considered that there is sufficient cross-protection among strains to provide adequate protection against field challenge as observed in Israel. In the vast infected areas of central Asia, a single stock has proved immunologically effective in 1.5 million cattle (Dolan, 1989; Wathanga *et al*., 1986). However, as described previously, two stocks are used routinely in Iran (Hashemi-Fesharki, 1988).

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Once the cultured schizonts become attenuated, no reversion-to-virulence has ever been observed during further cultivation.

iii) Precautions (hazards)

Different breeds of cattle may show different sensitivities to the vaccine. This should be borne in mind when vaccine from a partially attenuated master seed is to be administered to high-grade cattle stocks.

2.3.3. Efficacy requirements

i) Capacity to protect against naturally transmitted theileriosis

The batch of experimental vaccine used for the safety test can also be used for testing efficacy of the culture-derived anti-theilerial vaccine. Three or four calves are vaccinated with a conventional dose of vaccine and 6 weeks later, the vaccinated calves and the same number of unvaccinated calves are infected with sporozoites of *T. annulata*. Infection can be

induced by live adult ticks issued from *T. annulata*-infected pre-imaginal stages or by inoculation of stabilate prepared from macerated infected ticks (for techniques see Section C2.2) Experience shows that inoculation of stabilate (macerated ticks) generally induces a more severe response than an equivalent number of live, infected ticks allowed to feed on the cattle. However in the long run, the results obtained by challenge with stabilate appear to be more reproducible than those obtained with different batches of live ticks.

There are no internationally agreed standards for the size of a challenge dose used in testing the efficacy of *T. annulata* culture-derived vaccine. Five to ten female and the same number of infected, unfed male *Hyalomma* ticks have been used for infection of cattle. Alternatively, stabilate equivalent to 2–4 macerated ticks inoculated subcutaneusly in the neck area will invariably produce acute theileriosis. The responses to the challenge infection of the vaccinated and unvaccinated control calves are monitored using the following parameters: duration and severity of pyrexia, rate of schizont-infected cells in lymph node or liver biopsy smears, rate of piroplasm infected erythrocytes in the blood films, decrease in white and red blood cell counts, and severity of clinical manifestations such us anorexia, depression and recumbency.

The results of the efficacy test depends on factors such us immunological characteristics of the *T. annulata* isolate grown and attenuated in culture, the virulence and dose of the field isolate used for challenge, the species of infected ticks used to produce sporozoites. Research studies (Pipano, 1989b) show that calves vaccinated with schizont vaccine may exhibit an apparently near total protection or show a low level parasitaemia, accompanied by mild fever and insignificant alteration of the remaining parameters from their pre-vaccination values following a potentially lethal homologous challenge. A lesser degree of protection has been exhibited when cattle vaccinated with schizont vaccine were challenged with tick-derived parasites from a geographically remote area. In contrast, in most of the trials the non-vaccinated control calves have exhibited a high level of parasitaemia and pancytopenia accompanied by severe clinical manifestations. In the absence of specific medication, the majority of the control animals have succumbed to the infection (Pipano, 1989b).

Field observations have also been used for evaluation of the efficacy of anti-theilerial vaccines (Pipano, 1989a; Stepanova & Zablotskii, 1989). Susceptible indigenous cattle as well as high-grade exotic breeds were protected against clinical theileriosis and death in pastures on which nonvaccinated cattle succumbed to theileriosis. As completely attenuated schizont vaccine does not yield piroplasms, the presence of this theilerial stage in vaccinated cattle showing no clinical signs is considered to be the result of unapparent tick-induced infection.

The frozen vaccine is viably preserved in large liquid nitrogen refrigerators at production facility and transported to farms in smaller liquid nitrogen containers. Field centres for storage and supply of vaccine can be set up in theileriosis-enzootic areas. The basic equipment required for field application of frozen vaccine includes a wide mouthed jar for preparing a 40°C water bath, a thermometer for measuring the temperature of water, long forceps, face shield and temperature-resistant gloves. Application of the frozen vaccine to field cattle begins by donning the face shield and temperature-resistant gloves. The required numbers of vials are withdrawn with the forceps from the canister of the liquid nitrogen refrigerator. When withdrawing the vials, the canister should be kept as deep as possible in the neck of the refrigerator to avoid quick warming of the remaining vials. Each withdrawn vial should be checked in order to ascertain that liquid nitrogen has not leaked inside. The liquid nitrogen does not alter the vaccine, but may cause the vial to explode when introduced in the water bath. Such a vial should be held at ambient temperature for 1–2minutes to allow the nitrogen to escape and then processed in the usual way. Leaking of liquid nitrogen into a vial containing frozen vaccine has raised questions to about the sterility of the frozen vaccine. However the system has been used for decades with no significant problem observed. The vaccine is administered subcutaneously within 30 minutes of thawing (Pipano, 1977).

2.3.4. Duration of immunity

Controversial results about the length of immunity engendered by vaccination with the cell culture vaccine have been obtained. Periods of from more than 48 months (Stepanova & Zablotskii, 1989) to less than 13 months (Ouelli *et al*., 2004) have been reported.

2.3.5. Stability

The frozen vaccine has a practically unlimited shelf life.

C2. Immunisation of cattle against *Theileria parva* by the infection and treatment method (live vaccine)

1. Background

Vaccination against *T. parva* is based on a method of infection and treatment in which an aliquot of viable sporozoites is inoculated subcutaneously, and the animals are simultaneously treated with a formulation of a long-acting tetracycline, the so-called infection and treatment method (ITM) (Radley, 1981). Tetracyclines reduce the severity of the infection, and the resulting mild infection is usually controlled by the host's immune response, so that a carrier state is achieved. There are always risks associated with the use of live parasites for immunisation, however, with appropriate quality control and careful determination of a safe and effective immunising dose, the method can and is being used successfully in the field. Some *T. parva* stocks have been shown to infect cattle reliably without inducing disease, and these can be used without tetracycline treatment. One such stabilate is being applied in the field and offers considerable advantages over potentially lethal stabilate infections and savings in the cost of vaccination. However, different stabilates of these stocks can produce severe disease in cattle, emphasising the importance of a carefully controlled immunising dose.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Theileria parva infected ticks can be produced by feeding nymphal *Rhipicephalus appendiculatus* ticks on the ears of an animal undergoing an active ECF infection. After moulting, these ticks, when prefed for 4 days on rabbits will have infective sporozoites in their salivary glands. By grinding these prefed ticks in a specific medium, sporozoites will be released in the supernatants and a stabilate can be produced (FAO, 1984) that can be cryopreserved and when in sufficient quantity be earmarked as a master seed.

If needed, working seed stabilates are prepared by injecting cryopreserved sporozoites from a master seed into experimental cattle and producing a working seed stabilate as described below. Vaccines should be produced from a seed (working seed) that has not undergone further tick passages after its immunological characterisation because this might change after passage,

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

i) Field collection of ticks

It is important that well characterised laboratory strains of *R. appendiculatus* be used during preparation of immunising stabilates.

If field ticks are collected for experimental purposes, then consideration should be given to the possible hazard to humans from pathogens present in these ticks. The most important pathogen that has been recognised is Crimean–Congo haemorrhagic fever virus, usually associated with ticks of the genus *Hyalomma* and widely prevalent within the geographical distribution of *R. appendiculatus*. Those handling field tick collections should, therefore, be made aware of potential hazards. Ticks of *Hyalomma* species generally should not be removed from hosts; engorged or partially engorged ticks should not be crushed between the fingers. If removed, ticks should be handled with a forceps.

ii) Tick-handling facilities

The handling of field-collected ticks in the laboratory must be controlled so as to avoid accidental attachment to personnel. Field-collected ticks should be fed on rabbits and cattle in isolation facilities. Animals on which laboratory-infected or field-collected ticks have fed should be destroyed. Following engorgement of field-collected ticks on laboratory animals, aliquots should be homogenised and tested for extraneous human pathogens by inoculation in baby hamster kidney (BHK) and Vero cells. The effects of these inoculations should be studied through three passages. Any unused ticks should be destroyed by chemical means or by incineration.

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. As stabilate material is derived from field animals and rabbits, their cells might be potential sources of contamination of the vaccine with extraneous pathogens. Potential contaminants include bovine leukosis, mycoplasma, bovine viral diarrhoea virus, BSE or other *Theileria* species transmitted by *R. appendiculatus* and alongside other bacteria and viruses.

In the case of different stocks being used on the same premises, problems with labels could be minimised by using appropriate pencils and clear codes. Preparation of different stabiles should be done sequentially to avoid cross-contamination and mislabelling. Regular quality control should be introduced to ensure the right stock(s) are used.

2.1.3. Validation as a vaccine strain

A vaccine strain should be identified in cross-immunity trials. These are set up between a vaccine strain and stabilates from *T. parva* field isolates from the area where the protection of the vaccine strain is required. Ideally, five animals and two controls should be used per test, taking into account that these should be set up in two ways. First using the vaccine as a challenge in animals immunised with the local isolates and confirmed by subsequent homologous challenge. Secondly, using the local strain(s) as challenge in animals immunised with the vaccine and confirmed by subsequent homologous challenge. This will give information as to what extent a vaccine strain will give protection. On the other hand, results will indicate if a breakthrough might occur from the local *T. parva* population, present in carrier animals in the region where the vaccine will be deployed. A second test consists in testing if infection with the vaccine strain can be controlled by the intended tetracycline treatment during the vaccination process.

2.2. Method of manufacture

2.2.1. Procedure

Vaccine batches are produced in several institutes in eastern Africa, using different strains, possibly requiring different parameters than the ones described here. For consistency in immunisation in field, it is essential that tick-derived sporozoite stabilates of an immunising stock are prepared from a fully characterised 'working seed stabilate'. The 'working seed stabilate' should be derived directly from the reference 'master seed stabilate', which is available in suitable quantity for future preparation of immunising stabilates. Immunising stabilates can be prepared according to a proposed set of standards (Morzaria *et al*., 1999b).

2.2.2. Requirements for ingredients

Before starting to produce vaccine, seed material with known characteristics is required. Three types of seed material are distinguished:

i) Master seed

The master seed is a cryopreserved sporozoite stabilate from a specific stock that has been selected and permanently stored and from which all other seeds are derived. The master seed should consist of a single uniform batch of seed that has been mixed and filled into containers as one batch. As *T. parva*-infective sporozoites are used for the manufacturing process, the master seed also represents the master stock (see Chapter 1.1.8). To prepare a master seed, *T. parva*-infected adult *R. appendiculatus* ticks are used that have fed as

nymphs on the ears of an animal going through an active ECF infection. After moulting, these ticks, when prefed during 4 days on rabbits will have infective sporozoites in their salivary glands. These can be quantified by dissecting prefed ticks and determining infection rates in the dissected salivary glands by coloration (Walker *et al*., 1981). By grinding these prefed ticks in a specific medium, sporozoites will be released in the supernatants and a stabilate can be produced (FAO, 1984) that can be cryopreserved in minimum 100 cryotubes each containing preferably a set of infected acinus equivalent per vial or in case of a new stock an equivalent of 10 infected ticks per vial. A viability check of the master seed should be performed once the master seed has been cryopreserved for at least 24 hours by reviving one of the cryotubes.

ii) Working seed

The working seed is derived from infective sporozoites at a passage level between the master seed and the production seed. To prepare a working seed, the contents of a single cryotube of master seed are injected into a naive healthy experimental animal to produce an acute ECF infection.

iii) Production seed

To prepare a vaccine batch, the contents of sufficient cryotubes of working seed is mixed and the appropriate dose is injected into the required number of naive experimental cattle to produce an acute ECF infection.

Infection is established, with the working seed stabilate of *T. parva,* by inoculation of healthy cattle serologically and, ideally, PCR-negative for tick-borne diseases. During the parasitaemic phase of the ensuing disease reaction, clean laboratory-raised nymphs of *R. appendiculatus* are fed on the animals, and the engorged infected ticks are collected. The resultant adult ticks, within 3 weeks to 4 months after moulting, are applied in ear-bags to healthy rabbits. About 600 ticks are applied to each ear and unattached ticks are removed after 24 hours. After 4 days, the ticks are removed and samples (usually 60 ticks) taken to determine infection rates in dissected salivary glands. The remaining ticks are counted into batches of approximately 1000. An estimate of the total number of ticks can be obtained by counting and weighing a given number of ticks and then weighing the total number of ticks. The ticks are washed in a sieve under fast flowing tap water and may be surface disinfected in 1% benzalkonium chloride, or in 70% alcohol, and then rinsed again in distilled water.

The ticks are placed (~1000) in heavy glass specimen jars or plastic beakers, and 50 ml MEM with Hank's or Earle's salts and 3.5% bovine plasma albumin (BPA) is added. The jars are kept on ice, and the ticks are ground using a tissue homogeniser (for instance Silverson LR2) for 2 minutes using a large aperture disintegrating head, and for 3 minutes using a small aperture head (emulsor screen). For smaller batches, an alternative method may consist of grinding the ticks, in batches of 1000 ticks using a mortar and pestle. Ticks are then crushed continuously by teams of two people for 15–30 minutes in a mortar; 30–35 ml cooled MEM/3.5% BSA medium, without glycerine with 50–100 g glass is initially used. The difference (from 50 ml) MEM/BSA without glycerine is used to rinse the mortar and pestle and glass material used in crushing the ticks. Note that most of the crushing is done at the sidewalks of the mortar. Check for good crushing under a stereoscopic microscope and otherwise add glass. ALWAYS KEEP MEDIA AT 4°C.

The ground-up tick material is made up to 50 ml for every 1000 ticks, then centrifuged at 50 ***g*** for 5 minutes, and the supernatant is harvested. An equal volume of cold 15% glycerol in MEM/BSA is added dropwise while the tick material is maintained chilled on ice and stirred by a magnetic stirrer. The final volume will contain sporozoites from the equivalent of ten ticks/ml. The number of tick-equivalents/ml can be adjusted if parasite infection rates in a particular tick batch were either very high or very low. The final concentration of glycerol in the sporozoite stabilate is 7.5%.

The bottle containing the ground up tick material (gut) is fitted with a dispenser. Cryotubes of 1 ml are filled with the stabilate (1 ml per vial) while constantly stirring on an ice bath. Aliquots are kept at 4°C. Alternatively, artificial insemination equipment, as used to dispense semen, has been used with pre-labelled plastic straws. This latter system is ideal for large volume stabilates, and colour

coding and labelling provide additional check on the identity of the immunising stabilate. An equilibration time of 30–45 minutes should be allowed

The aliquots are then stored in insulation trays and moved to a –80°C deep-freezer as soon as possible. They are kept there for 24 hours, to allow a gradual cooling down (step-freezing) of the stabilate. On the second day, the aliquots are transferred into liquid nitrogen until use. Alternatively vials can be introduced in gas-phase liquid nitrogen for 3 hours and then immersed in the liquid nitrogen for storage (Pipano, 1989b). Vaccine is transported to the field in liquid nitrogen. Vials are taken out at the place of vaccination, by immersing the liquid nitrogen vials in lukewarm water (38°C) for 30 minutes to allow for good regeneration.It should be administered within 60 minutes after withdrawal from the liquid nitrogen container. Once unfrozen it can be kept alive on ice (+4°C) for another 6 hours (Marcotty *et al.,* 2001; Mbao *et al.,* 2007).The infection and treatment method is usually applied using long-acting tetracycline intramuscularly, and it is recommended that the tetracyline be administered first, in case an animal escapes having received stabilate only. Thereafter the stabilate is inoculated subcutaneoulsy over the parotid lymph node at the base of the ear.

The procedure for the preparation and testing of a multi-valent ITM vaccine (the Muguga cocktail) has been described in detail (Patel *et al.,* 2016). It is important to note that each of the component stabilates is produced before combining the infected and fed ticks immediately before homogenisation. The number of ticks from each component is calculated to produce a final vaccine stabilate containing equal numbers of infected acini from each component.

2.2.3. In-process controls

Records of the source and passages of the working seed material should be maintained. Seeds should be free of infective agents like enzootic bovine leukosis, bovine immunodeficiency virus, bovine pestivirus, bovine syncytial virus, Rift Valley fever, etc. Test procedures will depend on availability preferably using DNA analysis.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials for veterinary use may be found in chapter 1.1.9.

ii) Safety

Both ticks and experimental mammals are potential sources of contamination of stabilates with extraneous pathogens. In both cases, potential contaminants include *Ehrlichia bovis*, bovine *Borrelia* sp., orbiviruses, bunyaviruses, and others. Field-collected ticks should therefore not be used for the preparation of stabilates to be used for immunisation. Well characterised and pathogen-free laboratory colonies of ticks should be used for this purpose. Only healthy cattle and rabbits, free from tick-borne parasites, should be used for tick feeding. Stabilates should be prepared under aseptic conditions. In some circumstances, the use of antibiotics at concentrations appropriate for tissue culture may be indicated. Prepared stabilates should be subjected to routine tests for any viral infections in BHK and Vero cells (as above). Stabilates should be subjected to routine characterisation *in vivo*, which should involve infectivity testing in intact susceptible cattle, sensitivity to tetracyclines and other anti-theilerial drugs, and cross-immunity studies. A characterised 'working seed stabilate' should be prepared to ensure the purity of the *T. parva* stocks in the daughter immunising stabilate.

During stabilate preparation care must also be taken to avoid extraneous contamination of the stock being used with other *T. parva* stocks. Quality assurance procedures must be enforced, for example for the handling of infected ticks, and the rules should be adhered to rigidly. Tick unit facilities should allow for strict separation of infected and uninfected ticks. Tick unit personnel should use separate overalls for each batch of ticks used in stabilate preparation, and the overalls should be sterilised daily. Simultaneous work on many different stocks should be avoided. Stabilate storage systems should incorporate clear

labelling of each stabilate tube or straw also mentioning the preferred number of doses per vial or straw. This will vary according to use in small holder dairy or pastoral herds etc.

Quality control checks on the stabilate should determine the similarity to the parent seed stock and also detect any extraneous *T. parva* contamination.

iii) Batch potency

The evaluation of the number of acini infected with *T. parva* in dissected tick salivary glands, before grinding, is a useful indicator of the level of infection but does not take into account the variable loss of viability during stabilate preparation caused by the intensity of grinding and the freeze–thaw processes. Furthermore, the state of maturation of the sporozoites is difficult to estimate by histological examination of the tick salivary glands. Therefore, the infectivity of the stabilate is determined by inoculation of a standard dose of 1.0 ml into susceptible cattle. The contents of 2–4 randomly selected tubes are mixed and then titrated in cattle, and its infectivity and lethality at different dilutions are established for use in immunisation. As the response of cattle to the infection and treatment method is dependent upon their susceptibility to the infection, it is important to titrate stabilates in cattle of the same type as those to be immunised. Titration of vaccine stabilates remains a highly controversial matter. Ideally, a median infectious dose (ID_{50}) and median lethal dose (LD_{50}) should be determined by titration of the stabilate using a tenfold dilution range (Duchateau *et al.,* 1998; 1999). The ID_{99+} (corresponding to close to 100% infectivity and having minimal lethality) should then be quantified by means of a finer titration, using dilutions around the LD_{50}. With respect to compound vaccines, quantification of the vaccine dose is complex as different strains need to be put together, changing the total lethality of the vaccine (Speybroeck *et al.,* 2008). The sensitivity to tetracyclines is also determined, essentially to provide a dose of stabilate that is controlled, preferably by a single dose of long-acting tetracycline administered at the same time as inoculation. The immunising dose should induce a very mild or inapparent infection, and the animal should develop a serological titre and be immune to lethal homologous challenge. Should a single treatment with tetracycline fail to suppress the infection in all cattle, then either a lower dose of the immunising stabilate or two treatments of tetracycline (on days 0 and 4) may be used. A single dose of 30 mg/kg long-acting oxytetracycline has been found to be effective in field immunisations, when used with an appropriate stabilate dilution. An alternative method that has been used involves stabilate infection and treatment with parvaquone at 20 mg/kg on day 8 (depending on the stabilate). This method can be applied where tetracyclines are not reliable, but it requires that the animal be handled more than once. A single treatment with buparvaquone at 2.5 mg/kg at the time of infection has also been shown to be effective with stabilate infections that were not controlled with a single treatment at 20 mg/kg of a long-acting formulation of tetracycline.

Once the procedure that results in a safe and effective immunising dose is established, it must be adhered to strictly in the field, or breakdown of immunisation may occur. It is also important that the stabilate dilution and drug/dose regimen be determined in the most susceptible cattle in which it is likely to be used.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C2.2.2.1 and C2.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Theileria parva sporozoites are not hazardous to humans, but they are infectious to bovines, and infection of naive animals for production of vaccine batches, as well as the titration experiments for dose quantification should be done in tick-free facilities. Tests for sterility

and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. At a meeting in Malawi in 1988, the following recommendations on safety in the preparation, handling and delivery of *T. parva* infection and treatment vaccines were adopted (Anon, 1989).

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

The introduction of an immunising stock into an area/country from which it does not originate may result in that parasite, or a component parasite(s) of that stock, becoming established through a carrier state in cattle and transmission by ticks. The long-term effect of the introduction of new (and potentially lethal) parasites on the disease epidemiology should be considered before introduction, and should be monitored carefully following immunisation.

The characterisation of parasites in target populations should be carried out before immunisation, and at intervals following immunisation. At present the characterisation of parasite stocks with reference to vaccination relies primarily on immunisation and cross-challenge experiments in cattle. However a number of methods for characterising parasite stocks *in vitro* have been attempted in laboratories possessing a high degree of expertise. Early studies have shown that parasite stocks that differ in MAb profile may not cross-protect, whereas stocks showing similar profiles give cross-protection (Irvin & Morrison, 1987). However, in more recent experiments using other *T. parva* stocks, this observation has been proven to be wrong. Another method to detect antigenic differences used T cell clones specific for parasitised cell lines, as T cell responses are believed to be important in mediating immunity against *T. parva* and the strain specificity observed in *in-vitro* killing assays reflects the *in-vivo* challenge results. (Irvin & Morrison, 1987; Taracha *et al.,* 1995). Apart from this, there are no other, simpler *in vitro* assays that correlate with protection *in vivo*. Statistically derived disease reaction index, based on parasitological, clinical and haematological measurements, was proposed for characterising levels of infectivity and virulence of different parasite stocks and assessing the impact of control intervention against theileriosis (Rowlands *et al*., 2000; Schetters *et al*., 2010). Recently, DNA typing for characterisation of vaccine stabilates has been used and could be based on multi-locus genotyping using polymorphic antigen genes or satellite markers or a combination of both (Hemmink *et al*., 2016; Patel *et al*., 2011).

iii) Precautions (hazards)

Care should be taken during the preparation of sporozoite stabilates to avoid aerosol infection of personnel with extraneous pathogens when ticks are being ground. Those grinding ticks should be educated in the potential hazards involved; access to areas where ticks are homogenised should be restricted to specified and informed personnel; personnel should wear protective clothing, including gloves and masks; and tick grinding should be carried out in a microbiological safety cabinet (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

2.3.3. Efficacy requirements

i) Capacity to protect against naturally transmitted theileriosis

Although successful final product batch testing (see Section C.2.2.4.iii) indicates that a vaccine batch is immunogenic, it is important to note that protection against homologous challenge does not necessarily indicate that the vaccine will protect against all *T. parva* strains encountered in the field, and that the 'needle' challenge used in pen trials does not necessarily reflect a challenge delivered by ticks. This can only be assessed by controlled field trials, which are expensive, complicated and lengthy. The need for and extent of field trials for each vaccine batch is the subject of debate and there is no agreed protocol. The least intensive approach, if veterinary authorities allow, is to closely monitor the initial 'roll-out' of the vaccine after successful final product batch testing as described above, with particular attention being given to the incidence of 'reactors' soon after vaccination and the occurrence of any 'breakthrough' cases following field exposure.

ii) Use in the field

The frozen vaccine is viably preserved in large liquid nitrogen repositories at the production facility and transported to farms in smaller liquid nitrogen containers. Field centres for storage and supply of vaccine can be set up in theileriosis-enzootic areas. The basic equipment required for field application of frozen vaccine includes a wide-mouthed jar for preparing a 38°C water bath, a thermometer for measuring the temperature of water, long forceps, face shield and temperature-resistant gloves. Application of the frozen vaccine to field cattle begins by putting on a face shield and temperature-resistant gloves. The required numbers of vials are withdrawn with the forceps from the canister of the liquid nitrogen refrigerator. When withdrawing the vials, the canister should be kept as deep as possible in the neck of the refrigerator to avoid quick warming of the remaining vials. Each withdrawn vial should be checked to ascertain that liquid nitrogen has not leaked inside. The liquid nitrogen does not alter the vaccine, but may cause the vial to explode when introduced in the water bath. Such a vial should be held at ambient temperature for 1–2 minutes to allow the nitrogen to escape and then processed in the usual way. The reported leaking of liquid nitrogen into a vial containing frozen vaccine has raised questions about the sterility of the frozen vaccine. However the system has been used for decades with no significant problem observed.

2.3.4. Breadth of immunity

Unlike *T. annulata*, where considerable cross-protection is observed among different strains in the field, a more complex situation exists for *T. parva*. Two strategies are used to try to overcome this antigenic complexity. A combination of three stocks, which provides a broad spectrum of protection, has been tested in a number of countries. Two large batches of a trivalent stabilate have been prepared by the International Livestock Research Institute (ILRI, Nairobi), the first in 1996 for the FAO and another one in 2008. These stabilates were prepared to the latest proposed standards and have been used safely and effectively in Tanzania. Further batches are being prepared at the African Union Centre for Ticks and Tick-Borne Diseases, Lilongwe, Malawi with increasing demand for the infection and treatment method of immunisation in *T. parva*-endemic areas in sub-Saharan Africa. If an immunising stabilate fails to protect against a 'breakthrough stock', this should be isolated, characterised, tested and considered for use, either alone, or as an addition to the current immunising stabilate. Another strategy is to prepare stabilates of national or local stocks for use within defined areas. This latter strategy is more costly in time and resources, but it avoids, to some extent, the introduction of new stocks into an area. With movement of cattle, there is a risk of the introduction of different stocks into an area, which may breakthrough the immunity provided by the local stock. Therefore the use of local or introduced stocks for immunisation needs to be carefully evaluated (Geysen, 2008; McKeever, 2007).

The infection and treatment method of immunisation is effective provided the appropriate quality assurance measures are enforced. In the longer term, the attendant delivery problems and the risk of induction of carrier states and disease transmission, emphasise the need for the identification of protective antigens for development of subunit vaccines.

2.3.5. Duration of immunity

There have been few reports of controlled experiments to determine the duration of immunity induced by infection and treatment, in either the presence or absence of field challenge. However, Burridge *et al.* (1972) established that cattle that had survived an experimental infection (without treatment), and were subsequently maintained in an ECF-free environment, survived a lethal homologous challenge up to 43 months later.

2.3.6 Stability

If kept in liquid nitrogen, the frozen vaccine has a practically unlimited shelf life.

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NB: There is a WOAH Reference Laboratory for theileriosis (see the WOAH Web site for the most up-to-date list: http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the WOAH Reference Laboratory for any further information on diagnostic tests, reagents and vaccines for theileriosis

NB: FIRST ADOPTED IN 1991; MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.4.16.

TRICHOMONOSIS

SUMMARY

Description and importance of the disease: *Trichomonosis is a venereal disease of cattle caused by* Tritrichomonas foetus*, a flagellate protozoan parasite.*

Trichomonosis is asymptomatic in bulls, however in cows the disease is characterised by infertility, abortion, embryonic and early fetal death, fetal maceration, pyometra and vaginal discharge. The disease has a world-wide distribution and, at one time, was of major economic importance as a cause of abortion and infertility, especially in dairy cattle. The widespread use of artificial insemination in many areas of the world has contributed to reduced prevalence. Nevertheless, trichomonosis is still of importance in countries with extensive farming practices where artificial insemination is not used.

Transmission of the disease is primarily by coitus, but mechanical transmission by insemination instruments or by gynaecological examination can occur. The organism can survive in whole or diluted semen at 5°C. Bulls over 3–4 years old are the main reservoir of the parasite as they tend to be long-term carriers, whereas most cows and young bulls (less than 3 years old) may clear the infection spontaneously. For these reasons samples from bulls are usually preferred for diagnosing and controlling the disease in herds.

Identification of the agent by in-vitro ***culture and microscopy:*** Tritrichomonas foetus *is a flagellate, pyriform protozoan parasite, approximately 8–18 μm long and 4–9 μm wide, with three anterior and one posterior flagellae and an undulating membrane. The organisms move with a jerky, rolling motion and are seen in culture tests of preputial samples of infected bulls and vaginal washings or cervico-vaginal mucus of infected cows, or sometimes in aborted fetuses.* Tritrichomonas foetus *can be cultured* in vitro*, and may be viewed in a wet mount or stained slide. The standard diagnostic method for bulls involves the appropriate collection, examination and culture of smegma from the prepuce and penis, while in cows the preferred sample is vaginal mucus. Smegma can be collected by a variety of means including preputial lavage, brushing or scraping the preputial cavity and glans penis at the level of the fornix with a dry insemination pipette. A number of* in-vitro *culture media exist, including a commercially available field culture test kit, which supports trichomonad growth and allows direct microscopic examination.*

Identification of the agent by molecular methods: *Bovine trichomonosis may also be detected by polymerase chain reaction (PCR) amplification. Both conventional and quantitative real-time PCR have been used successfully in the identification of* T. foetus *and the diagnosis of trichomonosis. Both methods have been used either in combination with culture or alone. The conventional PCR has increased sensitivity when combined with culture, while the quantitative PCR has been successfully used on clinical samples. The quantitative real-time PCR has been validated both in the USA and Canada and it is now available as a commercial kit. This assay is also used routinely in Australia.*

Serological tests: *Attempts have been made at developing immunological tests, including an agglutination test using mucus collected from the cervix and an antigen made from cultured organisms, and these were used as herd tests. However, these tests lack sensitivity and are not used for individual diagnosis of trichomonosis.*

Requirements for vaccines: *A partially efficacious, killed whole-cell vaccine is commercially available as either a monovalent, or part of a polyvalent vaccine containing* Campylobacter *and* Leptospira*.*

A. INTRODUCTION

Description and importance of the disease: Trichomonosis is a bovine venereal disease caused by the flagellate protozoan parasite, *Tritrichomonas foetus*.

Causal pathogen: *Tritrichomonas foetus* belongs to the genus *Tritrichomonas* in the family Trichomonadidae. *Tritrichomonas foetus* is pyriform, 8–18 μm long and 4–9 μm wide, with three anterior and one posterior flagellae, and an undulating membrane. Live organisms move with a jerky, rolling motion, and can be detected by light microscopy. Phase-contrast dark-field microscopy or other methods must be used to observe the details needed for identification. Detailed morphological descriptions, including electron microscopy studies, have been published (Warton & Honigberg, 1979). *Tritrichomonas foetus* has only the trophozoite stage and multiplies by longitudinal binary fission; sexual reproduction is not known to occur. Three serotypes are recognised based on agglutination (Skirrow & BonDurrant, 1988): the *'belfast'* strain, reported predominantly in Europe, Africa and the USA (Gregory *et al*., 1990); the *'brisbane'* strain described in Australia (Elder, 1964) and the *'manley'* strain, which has been reported in only a few outbreaks (Skirrow & BonDurrant, 1988)

The habitual hosts of *T. foetus* are cattle (*Bos taurus, B. indicus*). The bovine gastrointestinal tract hosts a number of other, commensal trichomonads e.g. *Pentatrichomonas hominis*, *Tetratrichomonas buttreyi*, *Tetratrichomonas pavlova*, *Tritrichomonas enteris* and *Pseudotrichomonas* species, which often contaminate preputial samples (Taylor *et al*., 1994). The number of flagellae, observed under phase contrast illumination or after staining, is an important morphological characteristic that can help differentiate *T. foetus* from other bovine flagellated parasites. However, non-*T. foetus* trichomonads are often difficult to distinguish from *T. foetus* based on culture and morphology (Taylor *et al*., 1994).

Tritrichomonas suis, a commensal of pigs, and bovine *T. foetus* are indistinguishable morphologically, serologically and antigenically. The use of modern molecular techniques such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA analysis (RAPD), variable length repeats (VLR), and internal transcribed spacers 1 and 2 (ITS1 and 2) polymerase chain reaction methods (PCR), supports the view that these two species are identical (Tachezy *et al*., 2002). More recently, *T. foetus* and *T. suis* were found to be identical at 9/10 loci and the use of the *T. suis* senior synonym has been suppressed in favour of *T. foetus* (Šlapeta *et al*, 2012).

Tritrichomonas foetus has been reported in domestic cats, horses and roe deer. Other species, such as goats, pigs, dogs, rabbits and guinea-pigs, have been experimentally infected (Levine, 1973). *Tritrichomonas foetus* has also been isolated from cats with diarrhoea and is now commonly known as the 'cat genotype' *T. foetus* (Šlapeta *et al*., 2012). *Tritrichomonas foetus* has also been reported to cause infections in humans including meningoencephalitis and peritonitis in immunocompromised and immunosuppressed individuals (Yao, 2012).

Transmission of infection occurs by coitus, or by gynaecological examination of cows using contaminated instruments. It may also occur via artificial insemination (AI) as semen from infected bulls may be passively contaminated by *T. foetus* present in the preputial cavity. Therefore, all bulls must be routinely checked for absence of *T. foetus* infection. Where AI along with diagnostic monitoring and culling of infected bulls is used, Trichomonosis has been controlled; however, it is still prevalent in the Americas, Australia, South Africa and Eastern European countries where extensive farming is still practised and natural mating is allowed.

The site of infection in bulls is primarily the preputial cavity (BonDurant, 1997), and little or no clinical manifestation occurs. For bulls older than 3–4 years, spontaneous recovery rarely occurs, resulting in a permanent source of infection in herds. In bulls under 3–4 years of age, infection may be transient. One of the proposed measures for controlling *T. foetus* infection in a herd is the use of bulls under 3–4 years of age rather than bulls older than 3–4 years (Yao, 2013).

Tritrichomonas foetus is present in small numbers in the preputial cavity of infected bulls, with some concentration in the fornix and around the glans penis (BonDurant, 1997). Chronically infected bulls show no gross lesions. In the infected cow, the initial lesion is a vaginitis, which, in pregnant animals, results in invasion of the cervix and uterus. Various sequelae can result, including a placentitis leading to early abortion (1–16 weeks), uterine discharge, and pyometra. In some cases, despite infection, pregnancy is not terminated by abortion and a normal, full-term calf is born. On a herd basis, cows may, following infection, exhibit irregular oestrous cycles, uterine discharge, pyometra, or early abortion (BonDurant, 1997; Skirrow & BonDurrant, 1988). Cows usually clear their infection within 90 days and acquire a short-lived immune protection to *T. foetus* for a period of at least a year and in some cases up to three years (BonDurant, 1997).

Differential diagnosis: Diseases such as campylobacteriosis, leptospirosis, brucellosis, neosporosis, chlamydiosis, bovine viral diarrhoea, infectious bovine rhinotracheitis and anaplasmosis, that may cause clinical signs including infertility, vaginitis, pyometra, abortions and vaginal discharge, should be excluded.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of trichomonosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Microscopy for morphological identification	+++	++	+++	+++	++	–
Conventional PCR on clinical samples	+	++	–	++	–	–
Conventional PCR in combination with culture	++	+++	–	+++	+	–
Real-time PCR	+++	+++	+++	+++	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

A tentative diagnosis of trichomonosis as a cause of reproductive failure in a herd is based on the clinical history, signs of early abortion, repeated returns to service, or irregular oestrous cycles. Confirmation of infection depends on the demonstration of *T. foetus* in placental fluid, stomach contents of the aborted fetus, uterine washings, pyometra discharge, vaginal mucus or preputial smegma. In infected herds, the most reliable material for diagnosis is either preputial or vaginal washings or scrapings (Buller & Corney, 2013; Yao, 2013).

The number of organisms varies in different situations. They are numerous in the aborted fetus, in the uterus several days after abortion and, in recently infected cows, they are plentiful in the vaginal mucus 12–20 days after infection. In the infected bull, *T. foetus* organisms are present on the mucosa of the prepuce and penis, apparently not invading the submucosal tissues. It is recommended to allow at least 1 week after the last service before taking a preputial sample.

1.1. Sampling techniques and transport conditions

A number of techniques for collecting preputial samples from bulls or vaginal samples from cows have been described. It is important to avoid faecal contamination, as this may introduce intestinal protozoa that may be confused with *T. foetus* (Taylor *et al.*, 1994). Contamination of samples should be minimised by removal of extraneous material and soiled hair from around the preputial orifice or vulva; however, cleansing of the area, particularly with disinfectants, is to be avoided, as this may reduce diagnostic sensitivity.

Samples collected from bulls, cows and aborted fetuses are tested by conventional methods (direct examination and culture) or by molecular methods. The sampling techniques are the same in both cases; however, samples for culture are inoculated into transport medium or culture medium, whereas those for molecular biology can be collected either in medium or in phosphate buffered saline (PBS) or normal saline.

1.1.1. Collection of samples: bulls

Samples can be collected from bulls by three methods, namely scraping, brushing or washing (Buller & Corney, 2013; Yao, 2013):

i) Scraping the preputial and penile mucosa with an artificial insemination pipette connected to a syringe or a bulb via a silicone rubber tube, is a common technique.

ii) Special brushes made of metal or plastic, may be used to collect smegma from the penis and prepuce (Yao, 2013). Plastic disposable brushes, with a hole in the tip (hollow 'brush' or 'rasper') are commercialised for this purpose. They are easy to use and fast. The tool is gently scraped along the surface of the penis and internal prepuce near the fornix. The collected smegma is rinsed into ~5 ml PBS or normal saline, or medium.

iii) Preputial lavage is still a common technique. A strong plastic tubing attached to a rubber bulb is inserted into the full length of the preputial cavity and the latter is washed with 20–30 ml PBS pH 7.2 or normal saline Collecting washes from the artificial vagina after semen collection is not recommended due to low diagnostic sensitivity (Gregory *et al.*, 1990).

Sampling techniques for bulls have been compared by several laboratories (reviewed in Yao, 2013). The results indicate that all three methods, i.e. brushing, scraping and lavage, provide similar analytical sensitivity, irrespective of the diagnostic method implemented afterwards, i.e. culture or PCR. Thus the sampling techniques should be chosen based on the context and local conditions.

Several laboratories have examined the effect on diagnostic sensitivity of repeated sampling (reviewed in Yao [2013]). For optimal sensitivity (95% or more) the first sampling should be done after a 1-week sexual rest and bulls should be sampled and tested three times at weekly intervals.

1.1.2. Collection of samples: cows

Samples from cows are collected by washing the vagina, or by scraping the cervix with an artificial insemination pipette or a brush. The collected mucus is rinsed into ~ 5 ml PBS or normal saline (if PCR is intended) or in medium.

1.1.3. Samples from aborted fetus

Abortions due to *T. foetus* may occur at any time during gestation from 2 months onwards, but most frequently at 3–5 months (Buller & Corney, 2013). When abortions in that period occur and *T. foetus* infection is suspected, the placenta and fetal fluids should be sampled, together with the lungs of the aborted foetus (Buller & Corney, 2013). The abomasal content is also reported to contain high numbers of *T. foetus* (Rhyan *et al.*, 1988).

Where samples must be submitted to a laboratory and cannot be delivered within 24 hours, a transport medium containing antibiotics should be used (e.g. a thioglycolate broth media with antibiotics [Bryan *et al.*, 1999]) or a commercial plastic pouch. PBS or normal saline is not a good transport medium if the samples are to be cultured (Bryan *et al.,* 1999).

During transportation, the organisms should be protected from extremes of temperature, especially if cultures are to be undertaken in the laboratory. The temperature should remain above 5°C and below 37°C during transport (Bryan *et al.*, 1999). Transport at 25°C followed by culture at 37°C is considered optimal for the survival and growth of *T. foetus* (Buller & Corney, 2013).

In conclusion, the choice of sampling and diagnostic techniques should take into account several factors including the transport conditions and possibilities, and the expected duration of transport.

1.2. Identification of *T. foetus* by direct examination or in culture

1.2.1. Direct detection of parasites

Direct detection of parasites by microscopy immediately after sample collection, or on reception of the sample in transport medium, can be attempted (see below for examination and identification criteria). However, the organisms are often too few to allow for direct detection from the original samples. Thus cultures should be prepared to allow multiplication of the parasites above the detection limit (around 10^4/ml; Bryan *et al.,* 1999).

1.2.2. Culture

Samples should be inoculated into culture media as soon as possible after collection and thereafter incubated at 30°C–37°C, for 48–72 hours or longer, depending on the culture medium used, before being examined (see Section B.1.2.2.1). Inoculated media should not be chilled or refrigerated as this would affect survival of *T. foetus*.

1.2.2.1. Culture media

Several culture media can be used. Diamond's trichomonad medium has been widely used for decades with some modifications over time (Bryan *et al.*, 1999). However, other culture media can be used, such as the liver infusion broth medium (Lun *et al*. 2000) also named TFM medium, Clausen's and Oxoid's media. Commercial culture kits are also available.

a) Composition of three commonly used culture media

i) Modified Diamond's medium (Bryan *et al.,* 1999; Lun *et al.,* 2000)

The modified Diamond's medium consists of: 2 g trypticase peptone, 1 g yeast extract, 0.5 g maltose, 0.1 g L-cysteine hydrochloride, and 0.02 g L-ascorbic acid and is made up with 90 ml distilled water containing 0.08 g each of K_2HPO_4 and KH_2PO_4, and adjusted to pH 7.2–7.4 with sodium hydroxide or hydrochloric acid. Following the addition of 0.05 g agar, the medium is autoclaved for 10 minutes at 121°C, allowed to cool to 49°C, and then 10 ml inactivated bovine serum (inactivated by heating to 56°C for 30 minutes), 100,000 units crystalline penicillin C and 0.1 g streptomycin sulphate are added aseptically. The medium is aseptically dispensed in 10 ml aliquots into sterile 16 × 125 mm screw-top vials and refrigerated at 4°C until use.

The incorporation of agar into the medium confines contaminating organisms largely to the upper portion of the culture medium, while helping to maintain microaerophillic conditions at the bottom where the trichomonads occur in largest numbers.

ii) *Tritrichomonas foetus* medium (TFM) (based on modified Plastridge's medium)

The TFM consists of: 12.5 g neutralised liver digest, 5 g tryptose dissolved in 500 ml distilled water. The pH is adjusted to 7.4 with sodium hydroxide or hydrochloric acid. Following addition of 1.5 g Bacto agar, the medium is autoclaved for 15 minutes at 121°C. An antibiotic solution containing 0.75 g penicillin and 0.082 g streptomycin is prepared in 100 ml distilled water. To prepare 1 litre TFM, 500 ml basal medium is combined to 500 ml sterile inactivated bovine serum and 10 ml antibiotic solution. This medium can be stored at –20°C.

Where a combination of convenience and sensitivity is required, a combined specimen transport and culture kit may be used (BonDurant, 1997; Borchardt *et al.*, 1992). The kit consists of a clear flexible plastic pouch with two chambers. The upper chamber contains special medium into which the sample is introduced. Field samples for direct inoculation into the culture pouch would normally be collected by the preputial scraping technique (BonDurant, 1997). Samples collected by preputial washing require centrifugation before introduction of the sediment into the upper chamber. Following mixing, the medium is forced into the lower chamber, and the pouch is then sealed and incubated at 37°C. Microscopic examination for trichomonads can be done directly through the plastic pouch (Borchardt *et al.*, 1992).

The quality of the water used is important and an antifungal can be added to the media to control yeast growth.

Quality control checks including sterility checks should be carried out on all batches of media.

It is important to make sure that the culture media are used before their established expiry date, as many media are not stable. In-house made media should normally be kept for no longer than 1 month at 5°C ± 3°C.

b) Growth characteristics in the different media

Lun *et al*. (2000) tested the three media described above, i.e. Diamond's, liver infusion broth medium and a commercial culture kit. They found that the three media supported the growth of *T. foetus* at 37°C, sixteen isolates from various geographical origins were tested. The growth characteristics of these isolates were different in the three media. For isolates grown in Diamond's the growth kinetics appeared less variable among isolates, the peak concentration tended to be reached earlier (by day 2–4 post-inoculation) and was generally higher (above 10^7 organisms per ml) than with the other two media. However, the parasites died faster after reaching the peak concentration in Diamond's medium. This has implications for monitoring of cultures (see Section B.1.2.3).

1.2.2.2. Culture inoculation

It is desirable to process samples collected by preputial wash or vaginal wash by centrifugation. If the sampling conditions in the field do not permit the use of a centrifuge, the samples should be decanted for 15–20 minutes on the bench. A volume of approximately 1 ml or less of the sediment or the pellet from the centrifugation step is then inoculated into culture media.

When using commercial sampling devices with a disposable brush, the latter is aseptically cut off directly into culture medium after sampling.

1.2.2.3. Culture conditions

Cultures should be maintained at a temperature within the range 30°C–37°C. In no case should the incubation temperature rise above 37°C.

A known isolate of *T. foetus* should be cultured in parallel with the test samples, as a control.

It is advisable to start examining culture media microscopically at day 2–4 following inoculation, which in most media will correspond to the peak concentration. If Diamond's medium is being used, a single examination at day 2–4 may be performed as the parasites are rapidly dying out in this medium from day 5 onwards (Lun *et al*. 2000). With other media supporting slower growth, such as liver broth infusion and even more so with commercial medium, it is recommended to examine the cultures at intervals until day 7 post-inoculation (Bryan *et al*., 1999; Lun *et al*., 2000). A single examination at day 7 is an alternative option with those media that support slower growth of *T. foetus* than Diamond's (Section B.1.2.3).

In conclusion, it should be emphasised that the different culture media described above are equally successful provided the culture procedures follow the general requirements and are adapted to the type of medium. Laboratories should evaluate what is most suitable for them in their own context, given their facilities and expertise, and considering the climatic, logistical and economic considerations.

1.2.3. Summary of times for optimal growth and survival for different media

The optimal growth for modified Plastridge's medium, *Trichomonas* medium and commercial kit media is after 2–7 days, and the survival time in culture is 1–7 days; for modified Diamond's

medium optimal growth is after 2–4 days and survival time in culture is 1–4/5 days (Buller & Corney, 2013).

1.2.4. *Tritrichomonas foetus* detection and identification by microscopic examination

Initial detection of organisms can be done by light microscopy, on a wet mount slide prepared directly from a drop of the sample or culture, or through the wall of a plastic pouch. When a drop of culture medium is examined, it should be carefully taken from the bottom of the tube, where the trichomonads are likely to concentrate due to the microaerophilic conditions.

The organisms may be seen under a standard compound microscope using a magnification of ×40 or ×80 initially, then ×100 or ×400. *Tritrichomonas foetus* is motile and normally exhibits jerky movements in wet preparations. Sufficient time, i.e. 2–5 minutes, should be devoted to examination of each slide, to allow the detection of parasites in low numbers and observation of the morphological features.

The pear-shaped organisms have three anterior and one posterior flagellae and an undulating membrane that extends nearly to the posterior end of the cell. They also have an axostyle that usually extends beyond the posterior end of the cell (Table 2).

Phase-contrast microscopy is very valuable in revealing these features. A rapid, Giemsa-based staining procedure may also be used (Buller & Corney 2013; Lun & Gajadhar, 1999). Both these techniques work best when relatively high numbers of organisms are present, especially the staining technique.

The staining of trichomonads is best performed using Lugol's iodine, which enhances the morphological features of the flagellae and the membrane (Lun & Gajadhar, 1999). Briefly, 1 ml of the culture containing the parasites is concentrated at high speed e.g. 16,000 ***g*** for 10 seconds. The supernatant is removed and the pellet is resuspended homogenously in the culture medium containing 10% bovine serum. A thin smear is prepared on a microscope slide using 10 µl of the suspension. The slide is air-dried and fixed for 1 minute in methyl alcohol and then stained in Lugol's iodine for 1 minute. The slide is subsequently stained with the rapid Giemsa method following the manufacturer's instructions. The slide is then washed thoroughly to remove any remaining stain and air dried before being examined at × 100 under oil-immersion (Buller & Corney, 2013). The staining allows the differentiation of *T. foetus* from other trichomonads as shown in Table 2.

Table 2 Morphological features of trichomonads (Buller & Corney, 2013)

Organism	Anterior flagella	Posterior flagella	Undulating membrane	Host
Tritrichomonas				
T. foetus	3	1	2–5	Cattle
T. enteris	3	1	3	Cattle
T. vaginalis	4	0	4	Human
Tetratrichomonas				
T. buttreyi	3 or 4 variable length	3–5	1	Pigs, cattle
T. pavlovi	4	1	2–4	Calves
Pentatrichomonas				
P. hominis	4	1	3 waves	Human, primates, cats, dogs, cattle

1.2.5. Overall sensitivity and specificity of culture and identification

Any estimate of the diagnostic sensitivity and specificity of the culture and identification test will be dependent on the efficacy of sample collection, handling and processing, as well as the composition and quality of the culture medium.

In bulls, a commercial pouch kit has a sensitivity of 92% (95% confidence interval, 84–96%), while the same device used in experimentally infected young cows had an apparent sensitivity of 88% through a 10-week period after infection (Kittel *et al.*, 1998).

Estimates for Diamond's and related media have been variable, possibly due to variation in composition and preparation, but range from 78% to 99% (Skirrow & BonDurrant, 1988). Until recently, it has been assumed that the specificity of the culture test was 100%, but this is likely to be an overestimation.

Diagnostic results with samples from bulls using either Diamond's medium or a field kit have shown that the two methods give comparable results (Borchardt *et al.*, 1992; Bryan *et al.*, 1999; Kittel *et al.*, 1998). It should be emphasised that not every sample taken from a particular bull known to be infected, will necessarily give a positive culture result. Even when conditions of sampling, transport, culture and identification are optimal, more than one negative sample should be obtained before there is reasonable assurance that the animal is not infected. This is the basis for most breeding regulations to specify that bulls above 6 months of age should be tested three times at weekly intervals before concluding on absence of *T. foetus* infection. For bulls below 6 months, or that have been kept with bulls only, one negative test is considered sufficient.

In females, as the infection is usually cleared within 90–95 days, it may be difficult to isolate organisms from individuals in the late stages of their infection.

The diagnosis of abortion induced by *T. foetus* may be relatively easy where an aborted foetus is recovered, because of the large number of organisms demonstrable in the fetal abomasal contents or placental fluids (Rhyan *et al.*, 1988). In addition, immunohistochemical techniques and molecular methods can be used to demonstrate *Tritrichomonas foetus* organisms in tissues of aborted foetuses.

1.2.6. Immunohistochemical techniques

Immunohistochemical techniques using monoclonal antibodies have been described for revealing *T. foetus* organisms in formalin-fixed tissues (Rhyan *et al.*, 1995). These techniques can be used to identify *T. foetus* in tissues from aborted fetuses (e.g. placenta and lungs).

1.3. Molecular methods – detection of nucleic acids

Several molecular methods have been described for the detection of *T. foetus* DNA prepared from cultures or directly from clinical samples. These include conventional and real-time PCR methods targeting conserved regions of the 5.8S ribosomal RNA gene and the flanking internal transcribed spacer regions (ITS). A PCR diagnostic test offers a number of potential advantages, including increased analytical sensitivity, faster diagnostic turnaround time, and the fact that the organisms in the collected sample are not required to be intact and viable.

1.3.1. DNA Extraction

The preferred samples for DNA extraction are smegma and vaginal mucus in PBS or normal saline, transport medium and cultures.

1.3.1.1. Heat lysis

One ml of sample (vaginal mucus or smegma) is centrifuged for 5 minutes at 12,000 ***g***. The supernatant is removed and the pellet is resuspended in 500 µl RNase/DNase-free water. The suspension is heated at 95°C for 10 minutes. The lysate is centrifuged at 2000 ***g*** for 3 minutes and 5 µl of the supernatant is tested.

1.3.1.2. Magnetic beads-based extraction

There is a variety of magnetic beads kits commercially available on the market. This methodology uses a robotic system; it is sensitive and suitable for high throughput set up, where numerous samples are submitted for *T. foetus* testing.

1.3.1.3. Spin column method

This method uses spin columns and is more suitable for manual extraction of a small number of samples. There is a number of spin column kits available on the market and they all perform similarly.

The sensitivity of conventional PCRs is enhanced when using purified DNA extracts obtained by the use of commercial kits, e.g. magnetic beads or spin column methods. The real-time PCR can be used for all three extraction methods. Increased sensitivity has been observed by McMillen & Lew (2006) when using the heat lysis method, which was not suitable for conventional PCR.

1.3.2. Conventional PCR

Felleisen *et al.* (1998) described a conventional PCR that uses primers TFR3 and TFR4 to differentiate *T. foetus* from other commensal trichomonads, which are faecal contaminants of clinical samples submitted for the diagnosis of bovine trichomonosis.

The primers used are TFR3 and TFR4, which target a 347bp region and have the following sequences:

TFR3 5'-CGG-GTC-TTC-CTA-TAT-GAG-ACA-GAA-CC-3'

TFR4 5'-CCT-GCC-GTT-GGA-TCA-GTT-TCG-TTA-A-3'

The PCR is usually conducted for 40 cycles with a 30 second denaturation at 94°C, 30 seconds annealing at 67°C and 90 seconds extension at 72°C for 90 seconds. The final extension is performed at 72°C for 15 minutes (Felleisen *et al.*, 1998).

The use of two sets of primers together, one set amplifying DNA from the trichomonad group (TFR1 and TFR2) and the *T. foetus*-specific set of primers TFR3 and TFR4 (Felleisen *et al.*, 1998) allowed the differentiation of *T. foetus* from other commensal trichomonads of the gastrointestinal tract that are often faecal contaminants of the bovine reproductive system (Campero *et al.*, 2003). *Tritrichomonas foetus* DNA is amplified by both sets of primers, while the DNA of commensal trichomonads is only amplified by the TFR1 and TFR2 primers.

The sequences of the generic primers TFR1 and TFR2, which amplify a 372 bp region of the ITS1 and ITS2 region of the trichomonad group, are as follows:

TFR1 5'-GTA-GGT-GAA-CCT-GCC-GTT-G-3'

TFR2 5'-ATG-CAA-CGT-TCT-TCA-TCG-TG-3'

The PCR is conducted for 30 cycles with a 30 second denaturation at 94°C, followed by 20 seconds annealing at 58°C and a 30 second extension at 72°C. The final extension was performed at 72°C for 20 minutes.

The TFR3 and TFR4 primers have also been used in conjunction with DNA chelating fluorescent dyes under real-time PCR conditions for research purposes rather than diagnostic screening for trichomonosis (Casteriano *et al.*, 2016).

Grahn *et al.* (2005) have also used the TFR1 and TFR2 primers in combination with a fluorophore, whereas the forward primer (TFR1) is labelled with a 6FAM fluorophore, resulting in increased sensitivity of the conventional PCR.

Hayes *et al.* (2003) have also used RFLP of the amplicon generated by primers TFR1 and TFR2 to differentiate various trichomonads including *T. foetus*.

A loop mediated isothermal amplification assay (LAMP) targeting *T. foetus* 5.8S rDNA has been reported with slightly higher sensitivity than the TFR3/TFR4 PCR and demonstrated increased specificity (Oyhenart *et al*., 2013). Due to the simplicity of the assay over PCR or real time PCR, the LAMP assay may be a cheaper alternative for low skill operators, however the assay has not been field tested at this stage.

1.3.3. Real-time PCR or quantitative PCR

A Minor Groove Binder (MGB) probe real-time PCR method described by McMillen & Lew (2006), based on the ITS-1 region within the same rDNA regions as the TFR3-TFR4 PCR primers, was shown to be highly specific and sensitive compared with culture/microscopy and the previously described PCR methods (McMillen & Lew, 2006). Assay sensitivity was evaluated with 10-fold dilutions of known numbers of *T. foetus* cells, and compared with that for microscopy following culture and the TFR3-TFR4 PCR assay. The probe-based real-time PCR assay detected a single cell per assay directly from non-cultured smegma or vaginal mucus and was 2500-fold and 250-fold more sensitive than microscopy following selective culture respectively and 500-fold more sensitive than culture followed by the TFR3-TFR4 PCR assay. When compared with TFR3-TFR4 amplification of *T. foetus* DNA from cultures, the real-time PCR was consistently 10-fold more sensitive for smegma samples at 0, 2 and 5 days post-culture. For vaginal mucus, both PCRs demonstrated equivalent sensitivities at days 0 and 2 with 10-fold increase in sensitivity for real-time PCR at day 5 post-culture. The sensitivity of the TFR3-TFR4 PCR assay was 10-fold lower compared with the real-time PCR assay when testing purified DNA extracted from clinical specimens. Furthermore, the sensitivity of the real-time PCR assay improved 500-fold when using crude cell lysates, which were not suitable as template for the conventional PCR assay (McMillen & Lew, 2006). Initial evaluations of this real-time PCR method showed that from 159 Australian diagnostic specimens, 14 bulls were positive by real-time PCR (directly from clinical specimens: smegma and vaginal mucus) with only three confirmed by selective culture/microscopy detection (Fisher's exact test $p<0.001$) (McMillen & Lew 2006). The real-time PCR was designed with a MGB probe. The substitution of the MGB probe with other probes e.g. black hole quenchers or TAMRA will result in false positive results. The real-time PCR uses the following primers and probe:

TFF2 (20μM) 5'-GCG-GCT-GGA-TTA-GCT-TTC-TTT-3'

TFR2 (20μM) 5'-GGC-GCG-CAA-TGT-GCA-T-3'

TrichP2 (5μM) 5'-6FAM-ACA-AGT-TCG-ATC-TTT-G-MGB-3'

The real-time PCR is performed using a 25 μl reaction containing commercially available mastermix, 900 nM of TFF2 and TFR2 primers and 80 nM of TichP2 probe. When using a uracyl DNA glycosilase (UDG) containing mastermix, the real-time PCR is conducted at 50°C for 2 minutes to activate the UDG and prevent any carryover contamination, followed by incubation at 95°C for 2 minutes, and then 40 cycles of denaturation at 95°C for 20 seconds and annealing/extension at 60°C for 45 seconds. The fluorescence is acquired in the green channel at the end of each annealing/extension step.

Robust evaluation of the commercial version of this real-time PCR assay has been undertaken as described by Effinger *et al.* (2014) using real-time PCR post-culture. This evaluation used 833 cultured smegma samples, which were tested by five laboratories in the USA using the commercial real-time PCR kit different DNA processing methods, PCR equipment and PCR cycling conditions. Pools consisting of one *T. foetus* positive and four negative smegma samples were also processed and 96% of the positive samples were detected in these pools. For individual sample testing an overall agreement of 95.89% was attained between the five participating laboratories. A recent study undertaken in Canada showed that real-time PCR detection of *T. foetus* in pooled preputial samples in PBS was more sensitive than culture methods. The study also demonstrated that there was no difference in the detection of *T. foetus* from known infected bulls between direct real-time PCR (from PBS), culture/microscopy or culture/real-time PCR.

Another real-time PCR method, which targets the gene encoding beta-tubulin 1 from *T. foetus,* has been developed. It is available on the market as commercial kits. Despite the high degree of conservation of beta tubulin genes in trichomonads, the test is claimed to be specific and it has broad range detection of *T. foetus* isolates. However, there is only limited and unpublished data on the evaluation of these kits.

PCR diagnostic frameworks are best determined for a particular region and country taking into account transport time to the diagnostic laboratory, temperature of the sample, and optimised DNA extraction and PCR methods used at the diagnostic laboratory. Currently, the best option is to use published protocols (adapted to the particular laboratory) or commercial *T. foetus* real-time PCR kits applied to transport medium or PBS, which guarantees fast preliminary results especially when using boiled lysates.

1.4. Combination of culture and PCR on 5-day cultures

Combining PCR methods and culture has been reported to yield higher sensitivity or improved specificity, and has been suggested as the most cost-effective and practical approach to assess bulls before breeding (Michi *et al*., 2016). However, it has also been reported that testing clinical samples from bulls directly by PCR is more sensitive than culture followed by PCR (McMillen & Lew, 2006), even though most laboratories process cultures as a priority.

The culture and PCR combination should be implemented in some specific situations where specificity is a problem, due to occurrence of other trichomonads that may result in false positives in culture. In view of the difficulty of distinguishing *T. foetus* from other trichomonads based on morphology, it is recommended that, whenever the facilities exist for DNA-based methods, cultures with trichomonads be systematically tested by PCR to confirm the presence of *T. foetus*.

2. Serological tests

Bulls do not develop prominent immune responses to *T. foetus*. Some immunological tests have been developed for the diagnosis of bovine trichomonosis, such as mucus agglutination test and intradermal test (Rhyan *et al.*, 1999) and an antigen-capture enzyme-linked immunosorbent was described more recently (BonDurant, 1997). However, these tests appear very limited in use due to low sensitivity or specificity, and thus they are not recommended for the detection of *T foetus* in individual animals.

Infected cows develop specific IgG1 and IgG2 antibodies that are present in the vaginal mucus and in serum, but these are not exploited for diagnostic purposes.

C. REQUIREMENTS FOR VACCINES

Whole cell vaccines for cows have been shown to offer protection and are available commercially (Corbeil, 1994) as either a monovalent vaccine or part of a polyvalent vaccine also containing *Campylobacter* and *Leptospira* spp. (BonDurant, 1997). These products have shown efficacy in the female but not in the bull.

One example of a method of whole cell vaccine production is by growing *T. foetus* (culture VMC-84) in modified Diamond's medium (Corbeil, 1994) and freezing the culture at –20°C for 60 minutes. After thawing, a suspension of 5×10^7 organisms/ml in PBS is added to the CL-vaccine.

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NB: At the time of publication (2018) there were no WOAH Reference Laboratories for trichomonosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991 AS TRICHOMONIASIS. MOST RECENT UPDATES ADOPTED IN 2018.

SECTION 3.5.

CAMELIDAE

CHAPTER 3.5.1.

CAMELPOX

SUMMARY

Camelpox is a wide-spread infectious viral disease of Old World camelids. New World camelids are also susceptible. It occurs throughout the camel-breeding areas of Africa, north of the equator, the Middle East and Asia, and has an important economic impact through loss of production and sometimes death. Camelpox does not occur in the feral camel population of Australia. Camelpox virus *belongs to the family* Poxviridae*, subfamily* Chordopoxvirinae*, genus* Orthopoxvirus*. The disease is characterised by fever, local or generalised pox lesions on the skin and in the mucous membranes of the mouth and respiratory tract. The clinical manifestations range from inapparent infection to mild, moderate and, less commonly, severe systemic infection and death. The disease occurs more frequently and more severely in young animals and pregnant females. Transmission is by either direct contact between infected and susceptible animals or indirect infection via a contaminated environment. The role of insects in transmission has been suspected because the disease is often observed after rainfall. Camelpox virus is very host specific and does not infect other animals. Zoonotic camelpox virus infection in humans associated with outbreaks in dromedary camels* (Camelus dromedarius) *was described in the north-eastern region of India during 2009. This was a single incident illustrating that camelpox is of limited public health importance.*

Detection of the agent: *The presumptive diagnosis of camelpox infection is based on clinical signs. However, infections of camels with contagious ecthyma* (*orf*)*, papilloma virus and reaction to insect bites are considered differential diagnoses in the early clinical stages and in mild cases of camelpox. Several diagnostic methods are available and, where possible, more than one should be used to make a confirmatory diagnosis of disease.*

The preferred method of laboratory confirmation of camelpox is by the use of polymerase chain reaction (*PCR*) *methods. Alternatively the characteristic, brick-shaped orthopoxvirions can be demonstrated in skin lesions, scabs or tissue samples using transmission electron microscopy* (*TEM*)*. Camelpox virus is distinct from the ovoid-shaped parapox virus, the aetiological agent of the principle differential diagnosis: camel orf. However, both viruses may be seen simultaneously by TEM as dual infections.*

Camelpox can be confirmed by demonstration of the camelpox antigen in scabs and pock lesions in tissues by immunohistochemistry. It is a relatively simple method that can be performed in laboratories where TEM is not available. In addition, the paraffin-embedded samples can be stored for a long period of time, enabling future epidemiological, retrospective studies.

Camelpox virus may be propagated on the chorioallantoic membrane (*CAM*) *of embryonated chicken eggs. After 5 days, characteristic lesions can be observed on the CAM. Camelpox virus shows typical cytopathic effect on a wide variety of cell cultures. Intracytoplasmic eosinophilic inclusion bodies, characteristic of poxvirus infection, may be demonstrated in infected cells using haematoxylin and eosin staining. The presence of viral nucleic acid may be confirmed by PCR, and different strains of camelpox virus may be identified using DNA restriction enzyme analysis. An antigen-capture enzyme-linked immunosorbent assay* (*ELISA*) *for the detection of camelpox virus has been described.*

Serological tests: *A wide range of serological tests is available to identify camelpox, including virus neutralisation and ELISA.*

Requirements for vaccines: *Both attenuated and inactivated vaccines are commercially available. Vaccination with live attenuated vaccine provides protection for at least 6 years and with inactivated vaccine for 12 months.*

A. INTRODUCTION

Camelpox occurs in almost every country in which camel husbandry is practised apart from the introduced dromedary camel in Australia and tylopods (llama and related species) in South America. Outbreaks have been reported in the Middle East, Asia and Africa (Mayer & Czerny, 1990; Wernery *et al*., 1997b). The disease is endemic in many countries and a pattern of sporadic outbreaks occurs with a rise in the seasonal incidence usually during the rainy season. For up-to-date information, consult WOAH WAHIS interface[1]).

Camelpox is caused by *Camelpox virus*, which belongs to the genus *Orthopoxvirus* within the family *Poxviridae.* Based on sequence analysis, it has been determined that the camelpox virus is the most closely related to variola virus, the aetiological agent for smallpox. Camels have been successfully vaccinated against camelpox with vaccinia virus strains. The average size of the virion is 265–295 nm. Orthopoxviruses are enveloped, brick-shaped and the outer membrane is covered with irregularly arranged tubular proteins. A virion consists of an envelope, outer membrane, two lateral bodies and a core. The double-stranded DNA genome is approximately 206 kbp. Virus replicates in the cytoplasm of the host cell, in so-called inclusion bodies. Camelpox virus haemagglutinates chicken erythrocytes, but the haemagglutination may be poor (Davies *et al*., 1975). Camelpox virus is ether resistant and chloroform sensitive (Davies *et al*., 1975; Tantawi *et al*., 1974). The virus is sensitive to pH 3–5 and pH 8.5–10 (Davies *et al*., 1975). Poxviruses are susceptible to various disinfectants including 1% sodium hypochlorite, 1% sodium hydroxide, 1% peracetic acid, 0.5–1% formalin and 0.5% quaternary ammonium compounds. The virus can be destroyed by autoclaving or boiling for 10 minutes and is killed by ultraviolet rays (245 nm wave length) in a few minutes (Coetzer, 2004).

The incubation period is usually 9–13 days (varying between 3 and 15 days). Clinical manifestations of camelpox range from inapparent and mild local infections, confined to the skin, to moderate and severe systemic infections, possibly reflecting differences between the strains of camelpox or differences in the immune status of the animals (Wernery *et al.,* 2014). The disease is characterised by fever, enlarged lymph nodes and skin lesions. Skin lesions appear 1–3 days after the onset of fever, starting as erythematous macules, developing into papules and vesicles, and later turning into pustules. Crusts develop on the ruptured pustules. These lesions first appear on the head, eyelids, nostrils and the margins of the ears. In severe cases the whole head may be swollen. Later, skin lesions may extend to the neck, limbs, genitalia, mammary glands and perineum. In the generalised form, pox lesions may cover the entire body. Skin lesions may take up to 4–6 weeks to heal. In the systemic form of the disease, pox lesions can be found in the mucous membranes of the mouth and respiratory tract (Kritz, 1982; Wernery *et al.,* 2014).

The animals may show salivation, lacrimation and a mucopurulent nasal discharge. Diarrhoea and anorexia may occur in the systemic form of the disease. Pregnant females may abort. Death is usually caused by secondary infections and septicaemia (Wernery *et al.,* 2014).

Histopathological examination of the early skin nodules reveals characteristic cytoplasmic swelling, vacuolation and ballooning of the keratinocytes of the outer stratum spinosum. The rupture of these cells produces vesicles and localised oedema. Perivascular infiltration of mononuclear cells and variable infiltration of neutrophils and eosinophils occurs. Marked epithelial hyperplasia may occur in the borders of the skin lesions (Yager *et al*., 1991).

There are only a few detailed pathological descriptions of internal camelpox lesions. The lesions observed on post-mortem examination of camels that die following severe infection with camelpox are multiple pox-like lesions on the mucous membranes of the mouth and respiratory tract. The size of the lesions in the lungs may vary in diameter between 0.5 and 1.3 cm, occasionally up to 4–5 cm. Smaller lesions may have a haemorrhagic centre. The lung lesions are characterised by hydropic degeneration, proliferation of bronchial epithelial cells, and infiltration of the

1 http://www.woah.org/en/animal-health-in-the-world/the-world-animal-health-information-system/the-world-animal-health-information-system/

affected areas by macrophages, necrosis and fibrosis (Kinne *et al*., 1998; Pfeffer *et al*., 1998a; Wernery *et al*., 1997a). Pox lesions are also observed in the mucosa of the trachea and retina of the eye causing blindness.

The morbidity rate of camelpox is variable and depends on whether the virus is circulating in the herd. Serological surveys taken in several countries reveal a high prevalence of antibodies to camelpox (Wernery *et al.,* 2014). The incidence of disease is higher in males than females, and the mortality rate is greater in young animals than in adults (Kritz, 1982). The mortality rate in adult animals is between 5% and 28% and in young animals between 25% and 100% (Mayer & Czerny, 1990).

Transmission is by either direct contact between infected and susceptible animals or indirect infection via a contaminated environment. The infection usually occurs by inhalation or through skin abrasions. Virus is secreted in milk, saliva, and ocular and nasal discharges. Dried scabs shed from the pox lesions may contain live virus for at least 4 months and contaminate the environment. The role of an arthropod vector in the transmission of the disease has been suspected. Camelpox virus has been detected by transmission electron microscopy (TEM) and virus isolation from the camel tick, *Hyalomma dromedarii*, collected from animals infected with camelpox virus. The increased density of the tick population during the rainy season may be responsible for the spread of the disease (Wernery *et al*., 1997a). However, other potential vectors may be involved, such as biting flies and mosquitoes.

Different strains of camelpox virus may show some variation in their virulence (Wernery *et al.,* 2014). Restriction enzyme analysis of viral DNA allows isolates to be compared. However, no major differences from the vaccine strain have so far been demonstrated (Wernery *et al*., 1997a).

Immunity against camelpox is both humoral and cell mediated. The relative importance of these two mechanisms is not fully understood, but it is believed that circulating antibodies do not reflect the immune status of the animal (Wernery *et al.,* 2014). Life-long immunity follows after natural infection. Live, attenuated vaccine provides protection against the disease for at least 6 years, probably longer (Wernery & Zachariah, 1999). Inactivated vaccine provides protection for 1 year only.

The camelpox virus is very host specific and does not infect other animal species, including cattle, sheep and goats. Several cases in humans have been described (Kritz, 1982); the most recent from India (Bera *et al*., 2011) with clinical manifestations such as papules, vesicles, ulceration and finally scabs on fingers and hands. However, such cases and even milder human infections seem to be rare illustrating that camelpox is of limited public health importance. Camelpox virus should be handled with appropriate biosafety and biocontainment measures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities).*

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of camelpox and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
TEM	–	–	–	+++	–	–
Virus isolation in cell culture	–	–	–	+++	–	–
Virus isolation on CAM	–	–	–	+++	–	–
Immuno-histochemistry	–	–	–	+++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
PCR	–	–	–	+++	–	–
Real-time PCR	–	–	–	+++	–	–
Detection of immune response						
ELISA	+++	+++	–	+	+++	+++
Virus neutralisation	+++	+++	–	+	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
TEM: = Transmission electron microscopy; CAM = chorioallantoic membrane of embryonated chicken eggs;
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

During the viraemic stage of the disease (within the first week of the occurrence of clinical signs) camelpox virus can be isolated in cell culture from heparinised blood samples, or viral DNA can be detected by the polymerase chain reaction (PCR) from blood in EDTA (ethylene diamine tetra-acetic acid). The blood samples should be collected in a sterile manner by venepuncture. Blood samples, with anticoagulant for virus isolation from the buffy coat, should be placed immediately on ice and processed as soon as possible. In practice, the samples can be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures.

Blood obtained for serum samples should be collected in plain tubes with no anticoagulant. The blood tubes should be left to stand at room temperature for 4–8 hours until the clot begins to contract, after which the blood is centrifuged at 1000 ***g*** for 10–15 minutes. Separated serum can be collected with a pipette and held at 4°C for a short period of time or stored at –20°C.

A minimum of 2 g of tissue from skin biopsies and organs should be collected for virus isolation and histopathology. Scabs from different locations of the body are also suitable for virus isolation and histopathology. They should be transported without additives in plain tubes. For the PCR, approximately 30–50 mg of tissue sample should be placed in a cryotube or similar container, kept at 4°C for transportation and stored at –20°C until processed. Tissue samples collected for virus isolation should be placed in a virus transport medium, such as Tris-buffered tryptose broth or minimal essential medium (MEM) without fetal calf serum (FCS), kept at 4°C for transportation and stored at –80°C until processed. Material for histology should be placed immediately after collection into ten times the sample volume of 10% formalin. The size of the samples should not exceed 0.5 cm × 1–2 cm. Samples in formalin can be transported at room temperature.

1. Detection of the agent

1.1. Transmission electron microscopy

TEM is a rapid method to demonstrate camelpox virus in scabs or tissue samples. However, a relatively high concentration of virus in the sample is required for positive diagnosis and camelpox virus cannot be differentiated from other *Orthopoxvirus* species. However, currently, TEM is the fastest method for distinguishing clinical cases of camelpox and orf respectively, although the poxvirus infections can be differentiated by serological techniques and by PCR (Mayer & Czerny, 1990).

1.1.1. Sample preparation

The size of a sample should be at least 30–50 mg. Mince the scabs or tissue sample with a disposable blade or sterile scissors and forceps. Grind the sample in a five-fold volume of phosphate-buffered saline (PBS) with antibiotics (such as 10^5 International Units [IU] penicillin and 10 mg streptomycin per ml) using a mortar and pestle with sterile sand. Transfer the sample into a centrifuge tube and freeze and thaw two to three times to release the virus from the cells. Vortex

the samples while thawing. Place the tubes on ice and sonicate once for 30 seconds at 80 Hz. Centrifuge at 1000 ***g*** for 10 minutes to remove the gross particles and collect the supernatant (Pfeffer *et al*., 1996; 1998b).

1.1.2. Test procedure

Place 10 µl of above-mentioned supernatant on poly-L-lysine-covered grids and incubate at room temperature for 5 minutes. Remove the fluid with a chromatography filter paper. Add one drop of 2% phosphotungstic acid (diluted in sterile water and pH adjusted to 7.2 with NaOH) to the grid, incubate at room temperature for 5 minutes and air dry. Examine the grid by TEM (Pfeffer *et al*., 1996; 1998b).

Camelpox virus has a typical brick-shaped appearance with irregularly arranged, tubular surface proteins. Parapoxviruses are slightly smaller, ovoid-shaped and the surface proteins are regularly arranged.

1.2. Virus isolation in cell cultures

Camelpox virus can be propagated in a large variety of cell lines including Vero, MA-104 and MS monkey kidney, baby hamster kidney (BHK), and Dubai camel skin (Dubca) as well as in primary cell cultures (lamb testis, lamb kidney, camel embryonic kidney, calf kidney, and chicken embryo fibroblast) (Davies *et al*., 1975; Tantawi *et al*., 1974).

The samples are prepared for virus isolation as described above in Section B.1.1.1.

1.2.1. Test procedure

Incubate 400 µl of the supernatant for 1 hour at room temperature and then overnight at 4°C. Filter the supernatant through a 0.45 µm filter and inoculate into a 25 cm^2 flask of confluent cells. Flush the filter with 0.5 ml of the maintenance medium used in the cell culture and incubate the flasks at 37°C for 1 hour. Add 6–7 ml of fresh medium into the flask and continue the incubation for about 5–7 days. If there is any reason to suspect fungal contamination, the contaminated medium must be discarded and 5 µg/ml of amphotericin B added to a new medium. The flasks must be monitored daily for 5–7 days.

Characteristic, plaque-type cytopathic effect (CPE) showing foci of rounded cells, cell detachment, giant cell formation and syncytia may appear as soon as 24 hours post-inoculation. Syncytia may contain up to 20–25 nuclei (Tantawi *et al*., 1974). The growth of camelpox virus in a cell culture can be confirmed by TEM, PCR or antigen-capture enzyme-linked immunosorbent assay (ELISA) (Johann & Czerny, 1993).

1.3. Virus isolation on chorioallantoic membrane of embryonated chicken eggs

Camelpox virus can be isolated on the chorioallantoic membrane (CAM) of 11- to 13-day-old embryonating chicken eggs. The eggs should be incubated at 37°C and after 5 days the eggs containing living embryos are opened and the CAM examined for the presence of characteristic pock lesions: dense, greyish-white pocks. Camelpox virus does not cause death in inoculated embryonated chicken eggs. The maximum temperature for the formation of pock lesions is 38.5°C. If the eggs are incubated at 34.5°C, the pocks are flatter and a haemorrhagic centre may develop (Tantawi *et al*., 1974).

1.4. Immunohistochemistry

Immunohistochemistry for the detection of camelpox viral antigen is a relatively fast method and can be used instead of electron microscopy to establish a tentative diagnosis (Nothelfer *et al*., 1995). Almost any polyclonal antibody against vaccinia virus is likely to produce reasonable results in this test because of the antigenic relatedness between vaccinia and camelpox viruses (Nothelfer *et al*., 1995).

1.4.1. Test procedure

The following procedure for immunohistochemistry is described by Kinne *et al.* (1998) and Pfeffer *et al.* (1998b). The entire skin pustule should be collected for the immunohistochemical

examination. Fix the tissue in 10% formalin, dehydrate through graded alcohols and embed in paraffin wax according to standard histopathological procedures. Cut approximately 3 µm sections and place on glass slides. Treat the deparaffinised and dehydrated sections with 3% H_2O_2, prepared in distilled water, for 5 minutes and wash with PBS. Incubate the slides for 60 minutes at 37°C with anti-vaccinia virus monoclonal antibody 5B4[2], diluted 1/500. Remove the monoclonal antibody by washing twice with cold PBS. Incubate the slides for 30 minutes with anti-mouse antibodies labelled with biotin. Wash with PBS for 5 minutes and incubate with streptavidin-peroxidase for 30 minutes. Wash again with PBS for 5 minutes and add diaminobenzidine as chromogen for 10 minutes. Examine the slides under the microscope using ×200–400 magnification for any brown-labelled pox antigen.

1.5. Polymerase chain reaction

The PCR is a fast and sensitive method for the detection of orthopoxviral DNA. Several gel-based PCR methods have been described for the detection of camelpox viral DNA (Balamurugan *et al*., 2009; Meyer *et al*., 1994; 1997; Ropp *et al*., 1995). A generic PCR assay, described by Meyer *et al.* (1994), allows the detection and differentiation of species of the genus *Orthopoxvirus* because of the size differences of the amplicons. Using the primer pair: 5'-AAT-ACA-AGG-AGG-ATC-T-3' and 5'-CTT-AAC-TTT-TTC-TTT-CTC-3', the gene sequence encoding the A-type inclusion protein (ATIP) will be amplified. The size of the PCR product, specific for the camelpox virus, is 881 bp.

1.5.1. Test procedure for gel-based PCR

Suspend a small aliquot of crusted scabs in 90 µl of lysis solution (50 mM Tris/HCl, pH 8.0, 100 mM Na_2 EDTA, 100 mM NaCl, 1% sodium dodecyl sulphate) and add 10 µl of proteinase K (20 mg/ml). Digest the sample for 10 minutes at 37°C prior to the disruption of the scab or tissue with a microfuge tube pestle. Add another 350 µl lysis solution and 50 µl of proteinase K, mix gently and incubate for 3 hours at 37°C. Extract the lysed suspension with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) and centrifuge at 8000 ***g*** at 4°C for 1 minute. Collect the upper aqueous phase and mix it again with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1). Centrifuge at 8000 ***g*** at 4°C for 1 minute and transfer the upper, aqueous phase to a new tube. Precipitate the DNA by adding 1/10 volume of 3 M sodium acetate and two volumes of ice-cold absolute ethanol. Place the mixture at –70°C for 30 minutes or –20°C overnight. Centrifuge at 15,000 ***g*** for 5 minutes at 4°C. Discard the supernatant and wash the pellet with 0.5 ml of 70% ethanol. Centrifuge at 15,000 ***g*** for 5 minutes. Discard the supernatant and air-dry the pellets. Resuspend the pellets in 10 µl of nuclease-free water. Alternatively, DNA can be extracted using commercially available DNA extraction kits.

DNA amplification is carried out in a final volume of 50 µl containing 2 µl of each dNTP (10 mM), 5 µl of 10 × PCR buffer, 1.5 µl of $MgCl_2$ (50 mM), 1 µl of each primer, 2.5 U *Taq* DNA polymerase, 1 µl DNA template and an appropriate volume of nuclease-free water.

Run the samples in a thermal cycler as follows: 5 minutes at 94°C; 1 minute at 94°C, 1 minute at 45°C, 2.5 minutes at 72°C. (29 cycles); 10 minutes at 72°C then hold at 4°C until analysis.

Mix 10 µl of a sample with loading dye solution and load in 1% agarose gel in TBE (Tris/Borate/EDTA) buffer containing ethidium bromide or cyanine nucleic acid stain. Load a parallel lane with a 100 bp DNA-marker ladder. Separate the products at 100 V for 30–40 minutes and visualise using an UV transilluminator. Confirm the positive reactions according to the amplicon size.

A commercial PCR kit has been developed that allows detection of *Orthopoxvirus* DNA and contains a second 'conventional' amplification system, consisting of primers to the haemagglutinin (HA) gene of the orthopoxvirus. The amplicon can be sequenced and identified by comparison with already existing orthopoxvirus sequences.

2 Monoclonal antibody 5B4 is available commercially. For further information, please contact the WOAH Reference Laboratory: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3.

1.6. Real-time PCR

A small quantity of test material (blood, skin lesion, tissue) is suspended in 200 µl of lysis buffer and 20 µl of proteinase K and incubated at 65°C for 1 hour. The lysed sample is extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) and centrifuged at 8000 ***g*** for 10 minutes. The upper aqueous phase is transferred to a clean tube and the DNA is precipitated by adding 1/10 volume 3 M sodium acetate and 2.5 volumes of ice-cold absolute ethanol. The mixture is placed at –20°C for 1 hour followed by centrifugation at 13,000 ***g*** for 10 minutes. The supernatant is discarded and the pellet is washed with 0.5 ml 70% ethanol. The dried DNA pellet is resuspended in 30 µl of nuclease-free water. Alternatively, DNA can be extracted using commercially available DNA extraction kits.

For standardisation of the PCR, extracted DNA from the purified virus is used to amplify a 166-bp product from the HA gene of the camelpox virus genome (Pfeffer *et al.,* 1998b). PCR is performed in a 20-µl reaction containing 2 µl of 10× PCR reaction buffer, 1.6 µl of $MgCl_2$, (25 mM), 0.4 µl of dNTP (10 mM), (0.4 µl) of *Taq* polymerase, 5 units/µl, 2 µl DNA (approximately 50–100 ng), 6 pmol of each primer, and 2.5 pmol of each probe.

The cycling conditions are as follows:

Step	Temp.	Time	Acquisition mode	No. of cycles
Initial denaturation	95°C	10 minutes		1
Amplification	95°C	10 seconds		
	60°C	20 seconds single	Single	40
Melt	95°C	00[3]	Continuous	1
	60°C	30 seconds		
	95°C	00[3]		

A positive result is indicated by an amplification that gives a cycle threshold (Ct) value of 37 or less.

2. Serological tests

All the viruses in the genus *Orthopoxvirus* cross-react serologically. However, within the genus only camelpox virus can cause pox-like lesions in camels. Parapox and camelpox viruses do not cross-react and so infections of camelpox and camel orf can be distinguished serologically. Most of the conventional serological tests are very time- and labour-consuming, which makes them unsuitable for primary diagnosis. However, serological tests are a valuable tool for secondary confirmatory testing and retrospective epidemiological studies in those areas where vaccination against camelpox is not practised.

2.1. Virus neutralisation test

In this method the test sera are titrated against a constant titre of camelpox virus (100 $TCID_{50}$ [50% tissue culture infectious dose]) on Vero cells.

2.1.1. Test procedure

i) Sera are inactivated at 56°C for 30 minutes in a water bath.

ii) Sera are diluted in a twofold dilution series across the 96-well, flat-bottomed, cell-culture grade microtitre plate in serum-free cell culture medium (25 µl volumes). Individual serum controls, together with negative and known positive control sera, must also be included in each test.

3 This is a continuous step, i.e. the temperature is increased to 95°C and then lowered to 60°C for 30 seconds and then again increased to 95°C before finishing the cycle.

iii) A dilution of stock virus made up to contain 100 $TCID_{50}$ per 25 µl is prepared using serum-free cell culture medium containing antibiotics.

iv) 25 µl of the appropriate stock virus dilution is added to every well containing 25 µl of each serum dilution, except the test serum control wells and cell control wells on each plate.

v) Plates are covered and incubated 1 hour at 37°C in 5% CO_2 atmosphere.

vi) A cell suspension is made from 3–4 days old Vero cells using a concentration that will ensure confluent monolayers in the microtitre plate wells within 18–24 hours after seeding.

vii) A volume of 100 µl of cell suspension is added to every well, the plates are sealed with tape and incubated at 37°C in 5% CO_2 atmosphere for 3–4 days.

viii) Plates are examined microscopically for CPE and results are recorded on a work sheet. Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer is intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in half of the test wells is the 50% end-point titre of that serum. If necessary, the titre can be determined by the Spearman–Kärber method. A titre of 1/8 or greater is considered to be positive.

2.2. Enzyme-linked immunosorbent assay for the detection of antibodies against camelpox virus

The following procedure for the antibody ELISA for *Camelpox virus* is described by Azwai *et al*. (1996) and Pfeffer *et al*. (1998b). The following description gives general guidelines for the test procedure.

2.2.1. Preparation of the antigen

i) Harvest the cell culture when 100% infected with camelpox virus. Freeze and thaw two to three times. Sonicate for 30 seconds at 80 Hz on ice to release the virus from the cells.

ii) Centrifuge at 1000 ***g*** for 10 minutes and collect the supernatant.

iii) Centrifuge the supernatant at 45,000 ***g*** at 4°C for 1 hour. Re-suspend the pellet in PBS.

iv) Add NaCl to a final concentration of 330 mM and polyethylene glycol (PEG 6000) to a final concentration of 7%.

v) Stir overnight at 4°C, centrifuge at 3000 ***g*** at 4°C for 10 minutes and wash the pellet twice with 15 mM NaCl.

vi) Freeze and thaw, and treat with 1% non-ionic detergent (Nonidet P40, Sigma) at 37°C for 3 hours.

vii) Freeze and thaw and centrifuge at 3000 ***g*** for 10 minutes at 4°C.

viii) Collect the supernatant and dialyse at least three times against PBS.

ix) Measure the protein concentration as described by Lowry *et al*. (1951).

x) Store the aliquots at –20°C.

2.2.2. Preparation of rabbit anti-camel IgG horseradish-peroxidase conjugate

Rabbit anti-llama and anti-camel IgG horseradish-peroxidase conjugates are commercially available. The method for producing monoclonal antibodies for camel IgM and IgG has been described by Azwai *et al*. (1995). However, rabbit anti-camel IgG horseradish-peroxidase can be replaced with a commercially available protein A-peroxidase, *Staphylococcus aureus*/horseradish conjugate.

i) Precipitate camel sera twice adding saturated ammonium sulphate to a final concentration of 40% (v/v) (29.6% ammonium sulphate [w/v]) at room temperature. Centrifuge at 12,000 ***g*** for 15 minutes and dissolve in PBS, pH 7.2. Dialyse against several changes of PBS overnight.

ii) Separate the immunoglobulins using gel filtration chromatography: a column (2.6 × 100 cm) can be used to separate the salt precipitated immunoglobulins (IgM and IgG) by size. Elution can be effected with PBS at 20 ml/hour and 6 ml fractions can be collected. Determine the protein concentrations by absorbance at 280 nm.

2.2.3. Antiserum production

Immunise rabbits with a subcutaneous injection of camel IgG emulsified in appropriate adjuvant. The animals should be immunised three times to booster antibody production. Collect the serum and store at –20°C until used.

2.2.4. Test procedure

i) Coat 96-well microtitre ELISA plates with prepared antigen at 1 µg/ml in carbonate/bicarbonate buffer, 0.05 M, pH 9.6 (100 µl per well).

ii) Incubate the ELISA plates in a humid chamber (100% humidity) at 37°C for 1 hour and then overnight at 4°C.

iii) Wash off the unbound antigen with PBS containing 0.05% Tween 20 (PBS/Tween) three times.

iv) Add 100 µl of test and control serum at a predetermined optimal dilution in blocking buffer (PBS containing 0.05% Tween 20, and 1% fat-free milk powder) in duplicate wells.

v) Incubate the plates for 30 minutes at 37°C.

vi) Wash the plates three times with PBS/Tween.

vii) Dilute rabbit anti-camel IgG horseradish-peroxidase conjugate or protein A-peroxidase, *Staphylococcus aureus*/horseradish conjugate at a predetermined working dilution in blocking buffer and add 100 µl into the wells.

viii) Incubate the plates at 37°C for 30 minutes.

ix) Wash the plates three times with PBS/Tween.

x) Make the reaction visible using 100 µl per well of chromogen 3.3' 5.5'-tetramethylbenzidine (TMB) with hydrogen peroxide and incubate 15 minutes at 37°C with shaking.

xi) Stop the reaction 10 minutes later by 2 M H_2SO_4 at a volume of 50 µl/well.

xii) Measure the values with a photometer at a wave length of 450 nm. Seropositivity can be calculated as values above the mean +2 standard deviations from negative control sera.

C. REQUIREMENTS FOR VACCINES

C1. Live attenuated vaccine

1. Background

1.1. Rationale and intended use of the product

Currently live attenuated and inactivated vaccines are commercially available. The live attenuated vaccine was prepared from a strain isolated from a dromedary camel calf that had generalised camelpox (Wernery, 2000). The live attenuated vaccine gives long-term protection against camelpox (Wernery & Zachariah, 1999). However, a booster vaccination is recommended for young animals at the age of 8–12 months, 2–3 months after the initial vaccination, to avoid interference by maternal antibodies. When inactivated vaccine is used, the animals must be vaccinated annually.

Guidelines for the production of the veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Samples (such as crusty material from the nose, skin lesions or scabs) are collected from a camel calf showing generalised camelpox lesions. The samples are crushed in MEM with antibiotics, centrifuged, sterile-filtered and inoculated onto confluent Vero cells and on fetal camel skin cell line (Dubca). CPE is observed after 4 days of incubation at 37°C. When 80% of the cells are infected, cell culture is harvested, frozen and thawed. This procedure is done three times followed by sonication at 80 Hz on ice to release the virus. The suspension is clarified by centrifugation at 1000 ***g*** for 10 minutes. The supernatant is collected and the identity of camelpox virus is confirmed using different test methods. After the 10^{th} passage on Vero cells, a plaque test is carried out and the bigger plaque is chosen for the vaccine preparation. The plaque-purified virus is then passaged 110 times on Vero cells to attenuate the virus and designated as the master seed virus (MSV). The MSV is kept freeze dried and frozen at –80°C.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The purity and identity of the seed virus and the cells used for the vaccine production is proved. The seed virus is demonstrated free from contamination with adventitious viruses, bacteria, fungi or mycoplasma.

2.2. Method of manufacture

2.2.1. Procedure

The cells used for the production of vaccine preparation are prepared using a seed-lot system. The seed virus is cultured on Vero cells. When the cell monolayer is confluent, the cells are infected with the vaccine virus. Cell culture is harvested when the cells are 100% infected with camelpox virus. The supernatant is clarified, mixed with a stabiliser, bottled and freeze-dried.

2.2.2. Requirements for ingredients

Cell cultures and all animal origin products used in the production and maintenance of cells are proved free of adventitious viruses, bacteria, fungi and mycoplasma.

2.2.3. In-process controls

CPE is checked during cultivation of working virus seed. Uninoculated control cells should retain their morphology until the time of harvesting. Virus multiplication is demonstrated by titration with the harvested supernatant.

2.2.4. Final product batch tests

i) Sterility/purity

The procedure for testing for sterility and freedom from contamination of biological materials intended for veterinary use is described in chapter 1.1.9.

ii) Safety

Using the recommended route of administration, each batch of vaccine is tested in ten naive camels, using ten times the recommended dose per animal. The animals are observed for 7–14 days for any adverse reactions.

iii) Batch potency

The amount of virus present in the live attenuated vaccine is titrated on cell culture and the end titre is calculated.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Live attenuated camelpox vaccine causes no clinical signs in camelpox-susceptible Old Word camels and New World camels. Less than 1% of animals show a rise in temperature by a maximum of 1°C.

ii) Reversion-to-virulence

There are no reports of reversion to virulence by the live attenuated vaccine.

iii) Precautions

There is a low risk of human infection as camelpox is host specific. However, self-inoculation may lead to zoonotic camelpox virus infection with a vaccine virus.

2.3.2. Efficacy

Efficacy in susceptible animals is demonstrated in naive dromedaries. The experimental animals should be vaccinated twice with the live attenuated vaccine and 3 weeks after the last vaccination the camels should be challenged with a virulent camelpox field strain. The virulence of the challenge virus should be demonstrated by inoculation of the virus into unvaccinated control animals. The vaccinated animals should not show any clinical signs whereas the unvaccinated group should develop characteristic clinical signs of camelpox. The long-term immunity provided by the live attenuated vaccine can also be confirmed by challenging the vaccinated animals 6 years later. The efficacy of the live attenuated vaccine should be further evaluated by measuring the antibody levels against camelpox virus 21–30 days after vaccination using ELISA and virus neutralisation test.

The live attenuated vaccine can protect dromedaries from an infection for at least 6 years, but may be lifelong.

2.3.3. Stability

Vaccines should be stored at 4–8°C, with minimal exposure to light. The shelf life is determined by virus titration.

C2. Inactivated vaccine

1. Background

1.1. Rationale and intended use of the product

An inactivated vaccine has been available since 1992 and used mainly in North Africa to prevent the disease (El Harrak & Loutfi, 1999). The inactivated vaccine is prepared from a strain isolated during the 1984 outbreak in Morocco from a dromedary camel presenting with generalised camelpox (El Harrak *et al.,* 1991). The inactivated vaccine gives good protection against camelpox after a double injection administered at a 3- to 6-month interval followed by the annual booster. The vaccine is recommended from the age of 8–12 months to avoid interference by maternal antibodies.

Guidelines for the production of the veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The production of commercially available inactivated camelpox vaccine is described below.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Pox lesions from an adult dromedary camel presenting generalised camelpox infection are removed, crushed in PBS with antibiotics, centrifuged, sterile-filtered and inoculated onto the chorioallantoic membrane of specific pathogen free eggs for the first isolation (El Harrak *et al.,* 1991). The pox lesions obtained are passed on confluent Vero cells serially seven times. A CPE is routinely observed after 3–4 days of incubation at 35°C. When 80% of the cells are infected, viral suspension is harvested, frozen and thawed. This procedure is done twice to release the virus. The suspension is collected and the identity of the camelpox virus is proven using different test methods. The virus at its eighths passage level was designated the master seed virus (MSV) and named Laayoune T8 strain. The MSV is kept frozen at –80°C.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The purity, identity and titre of the seed virus as well as the purity of the Vero cells used for the vaccine production is proved. The seed virus is demonstrated free from contamination with adventitious viruses, bacteria, fungi or mycoplasma.

2.1.3. Validation as a vaccine strain)

Laayoune T8 strain was validated as a vaccine strain after three successive passages on Vero cells using the limiting dilution method to purify the virus. CPE characteristics and the virus titre are reproducible and freedom from extraneous agents proven.

2.2. Method of manufacture

2.2.1. Procedure

The seed virus is grown on Vero cells. Cells used for vaccine production are prepared using a seed-lot system. The infection with the seed virus takes place on confluent Vero cells in roller bottles or in bio-generators with micro-carriers. Viral suspension is harvested when the cells are 80% infected with camelpox virus. The virus is inactivated using betapropiolactone then mixed with aluminium hydroxide as an adjuvant and bottled.

2.2.2. Requirements for ingredients

The seed virus, cell cultures and all animal origin ingredients used in the production are proved free of adventitious viruses, bacteria, fungi and mycoplasma.

2.2.3. In-process controls

Cell sterility and purity are tested during the production process. The harvested virus is tested for sterility and infectious titre, the inactivated antigen is also checked for sterility and complete inactivation.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use is found in chapter 1.1.9.

ii) Identity

Identity of the vaccine virus is confirmed by PCR on the inactivated final product.

iii) Safety

Using the recommended route of administration, each batch of vaccine is tested in two naive camels, using two times the recommended field dose per animal. The animals are observed for 14 days for any adverse reactions.

iv) Batch potency

The amount of virus present in the inactivated vaccine is measured by titration on cell culture before inactivation and by real time PCR carried out on antigen before addition of the adjuvant.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

The inactivated camelpox vaccine is produced according to good manufacturing practices (GMP) standards and procedures recommended for inactivated veterinary vaccines.

2.3.2. Safety requirements

i) Target and non-target animal safety

Inactivated camelpox vaccine causes no clinical signs or rise in body temperature when administered to camelpox-susceptible animals. An inflammatory reaction due to the presence of the adjuvant may appear at the injection site with no influence on the animal's health.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Not applicable for inactivated vaccines.

iii) Precautions (hazards)

There are no precautions necessary as camelpox is host specific.

2.3.3. Efficacy requirements

Efficacy is demonstrated in six naive calf dromedaries challenged with a virulent field strain after two injections with the inactivated vaccine at a 3-month interval. The virulent strain is titrated on the animal skin comparatively on four vaccinated and two unvaccinated control animals. The obtained infectious titre on vaccinated animals is at least 1.5 log ID_{50} less than the titre obtained on control animals. In addition, vaccinated animals do not show any signs of generalised disease. The efficacy of the inactivated vaccine was also proven by the development of antibodies after vaccination that were evaluated by ELISA and the virus neutralisation test (El Harrak & Loutfi, 1999).

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

There is no vaccine available permitting a DIVA strategy for camelpox control.

2.3.5. Duration of immunity

The long-term immunity caused by the inactivated vaccine was also confirmed by challenging the vaccinated animals 1 year after the primary vaccination. Duration of the conferred immunity with inactivated vaccine lasts for a minimum of 1 year after double injection of the vaccine, as tested on young naïve dromedary camels. After multiple vaccinations, immunity may be longer in adult dromedaries; no vaccination failures reported in the field with animals not regularly vaccinated.

2.3.6. Stability

Vaccines should be stored at 4–8°C, with minimal exposure to light. The shelf life is 24 months.

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NB: There is a WOAH Reference Laboratory for camelpox (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for camelpox

NB: FIRST ADOPTED IN 2008. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.5.2.

MIDDLE EAST RESPIRATORY SYNDROME (INFECTION OF DROMEDARY CAMELS WITH MIDDLE EAST RESPIRATORY SYNDROME CORONAVIRUS)

SUMMARY

Description of the disease: *Middle East respiratory syndrome* (*MERS*) *is a viral respiratory infection of humans and dromedary camels that is caused by a coronavirus called Middle East respiratory syndrome coronavirus* (*MERS-CoV*). *MERS-CoV is an enveloped, positive-sense, single-stranded RNA virus of the genus* Betacoronavirus*, first identified in 2012. Since July 2015, infection with MERS-CoV has been reported in over 21 countries and so MERS-CoV has become a great concern for public health.*

Dromedary camels (Camelus dromedarius) *have been confirmed by several studies to be the natural host and zoonotic source of MERS-CoV infection in humans. Other species may be susceptible to infection with MERS-CoV, however, their epidemiological significance has not been proven. MERS-CoV has been reported to cause little to no disease in camels, and infections have sometimes been associated with mild respiratory signs consisting of nasal discharge, lacrimation and mild fever in young camels. While the impact of MERS-CoV on animal health is very low, human infections have a significant public health impact.*

Detection of the agent: *Nasal swabs are the preferred samples for laboratory detection of MERS-CoV infection in camels. They can be screened for MERS-CoV RNA using a real-time RT-PCR targeting the upstream region of the envelope gene. The presence of viral nucleic acid can be confirmed by either a positive RT-PCR result on both the two specific genomic targets, such as by testing with a real-time RT-PCR targeting the open reading frame 1a gene or a single positive target with sequencing of a second target.*

Serological tests: *Serum samples can be screened for the presence of MERS-CoV-specific antibodies by using a MERS-CoV enzyme-linked immunosorbent assay, a MERS-CoV neutralisation assay or a MERS-CoV pseudo-particle neutralisation assay.*

Requirements for vaccines: *Research on MERS-CoV camel vaccine development is ongoing.*

A. INTRODUCTION

Middle East respiratory syndrome coronavirus (MERS-CoV) is a zoonotic virus from dromedary camels (*Camelus dromedarius*) causing significant mortality and morbidity in humans in the Arabian Peninsula, and was first reported from Saudi Arabia in 2012 (Zaki *et al.,* 2012). Sporadic human cases of MERS have occurred and continue to occur over a wide geographical range with most cases reported from the Arabian Peninsula.

MERS-CoV belongs to lineage C of the genus *Betacoronavirus* in the family *Coronaviridae* under the order *Nidovirales*. MERS-CoV is an enveloped positive-sense single-stranded RNA virus and its single-stranded RNA genome has a size of approximately 30 kb (Chan *et al.,* 2015).

Dromedary camels have been shown to be the natural reservoir from where spill-over to humans can occur (Haagmans *et al.,* 2014). Human-to-human infection is also reported, especially in healthcare settings (Hui *et al.,* 2018). Although Africa has the largest number of dromedary camels, and MERS-CoV is endemic in these camels,

locally acquired zoonotic human MERS is confined to the Arabian Peninsula and has not been reported from Africa to date. There are viral genetic and phenotypic differences in viruses from different parts of Africa that may be relevant to differences in zoonotic potential, highlighting the need for studies of MERS-CoV at the animal–human interface (Chu *et al*., 2018). The lack of routine surveillance information about MERS-CoV circulation in dromedary camels restricts the understanding of the transmission dynamics and epidemiology in dromedary camel populations (Aguanno *et al.,* 2018).

Published studies have indicated that MERS-CoV or viral RNA from MERS-CoV have been identified in dromedary camels in countries in the Middle East and North Africa; antibodies to MERS-CoV have been identified in samples taken from camels in the Middle East and Africa. Antibodies to MERS-CoV have been detected with a prevalence range of from 0 to 100% in populations of camels in the Middle East and African countries. MERS-CoV is mainly acquired in dromedaries when they are less than 1 year of age, and the proportion of seropositivity increases with age to a seroprevalence of 100% in adult dromedaries (Wernery *et al.,* 2017). In general, only minor clinical signs of disease have been observed in infected dromedary camels and most MERS-CoV infections do not appear to cause any clinical signs (Chu *et al.,* 2014). MERS-CoV infections have also been detected in camels with MERS-CoV antibodies, both in calves with maternal antibodies as well as older camels that had already acquired antibodies from a previous infection. However, virus replication and the virus load are generally lower in infected seropositive animals compared with seronegative camels (Meyer *et al*., 2016).

Clinical signs of disease in camels that have been described after experimental and field infections are nasal discharge, fever and loss of appetite (Adney *et al.,* 2014; Hemida *et al.,* 2014; Khalafalla *et al.,* 2015). A systemic review of the global status of MERS-CoV in dromedary camels is provided by Sikkema *et al.* (2019).

Under experimental conditions, the disease observed in young adult dromedary camels was clinically benign with the absence of overt illness and with a large quantity of MERS-CoV and viral RNA detected in nasal swab specimens from camels. Histopathological examination revealed that the infectious virus was detected in the upper respiratory tract including nasal turbinates, olfactory epithelium, pharynx, and larynx. Specifically, the respiratory epithelium in the nasal turbinates is the predominant site of MERS-CoV replication in camels. In the lower respiratory tract, infectious virus was detected in the trachea. No viral antigen or lesions were detected in the alveoli. The large quantities of MERS-CoV shed in nasal secretions suggest that camel-to-camel and camel-to-human transmission may occur readily through direct contact and large droplet, or possibly fomite transmission (Adney *et al.,* 2014).

MERS-CoV antibodies have been detected in llamas and alpacas (David *et al.,* 2018; Reusken *et al*., 2016). Naturally, other species of animals including sheep, goats, cattle, water buffalo and wild birds have tested negative for the presence of antibodies to MERS-CoV (Hemida *et al.,* 2013; Reusken *et al*., 2016). However, recently, a single report from Africa that followed surveillance of other domestic mammalian species, such as sheep, goat, cow and donkeys, that were in contact with infected camels found the animals to be seropositive for MERS-CoV; domestic livestock in contact with MERS-CoV-infected camels may therefore be at risk of infection (Kandeil *et al*., 2019). It has also been shown that Bactrian camels and Bactrian X dromedary hybrids can get naturally infected with MERS-CoV, when brought to countries where dromedaries are reared (Lau *et al.,* 2020).

B. DIAGNOSTIC TECHNIQUES

Diagnosis should always use a combination of techniques based on history, the purpose of the testing and the stage of the suspected infection. For a definitive interpretation, combined epidemiological, clinical and laboratory information should be evaluated carefully.

Nasal swabs are preferred specimens for laboratory detection of MERS-CoV infection in camels. Viral RNA can be extracted using any of the commercial kits and then screened for MERS-CoV RNA using a conventional or a real-time reverse-transcription polymerase chain reaction (RT-PCR) targeting the upstream regions of the envelope (UpE) gene. Positive samples should be confirmed by testing with a real-time RT-PCR targeting the open reading frame 1a (ORF1a) gene or 1b (ORF1b) gene. The presence of viral nucleic acid can be confirmed by either a positive RT-PCR result on both the two specific genomic targets, such as by testing with a real-time RT-PCR targeting the open reading frame 1a (ORF1a) gene or a single positive target with sequencing of a second target. Serum samples can be screened for the presence of MERS-CoV-specific antibodies by using a MERS-CoV enzyme-linked immunosorbent assay (ELISA), a MERS-CoV neutralisation assay or a MERS-CoV pseudo-particle neutralisation test.

The collection of specimens and their transport to the laboratory should comply with the standards of Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*.

All the test methods described below should be validated in each laboratory using them (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Table 1. Test methods available for diagnosis of MERS and their purposes

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Real-time RT-PCR	–	+++	+	+++	+++	–
Antigen detection	–	+	+	++	++	–
Virus isolation and identification	–	+	–	+++	–	–
Detection of immune response						
Indirect IgG ELISAs	++	–	++	–	++	+
Pseudo-particle neutralisation assay	+	–	+	–	+	+++
PRNT	+	–	+	–	+	+++
VNT	+	–	+	–	+	+++

Key: +++ = recommended for this purpose; ++ = recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; IgG ELISA = immunoglobulin G enzyme-linked immunosorbent assay; PRNT = plaque reduction neutralisation test; VN = virus neutralisation.

1. Detection of the agent

1.1. Specimen collection and storage

Nasal swabs are the samples of choice for virus isolation. Sterile plain cotton swabs are taken from both nostrils by inserting the swabs deep into the nasal cavity and turning them 10 to 20 times in the nose. These swabs are immediately submerged into viral transport medium supplemented with antibiotics (e.g. tissue culture medium 199 with 5% bovine serum albumin (BSA), benzylpenicillin (2×10^6 IU/litre), streptomycin (200 mg/litre), polymyxin B (2×10^6 IU/litre), gentamicin (250 mg/litre), nystatin (0.5×10^6 IU/ litre), ofloxacin hydrochloride (60 mg/litre) and sulfamethoxazole (0.2 g/litre). The samples can be stored in a cool box with ice packs if a –80°C freezer is reachable in 48 hours or they can be frozen in a liquid nitrogen tank immediately after the sampling. Swabs should be processed immediately upon arrival at the laboratory or kept in a –80°C freezer until use.

1.2. Isolation in cell culture

i) Test procedure (Chu *et al.*, 2014; Woo *et al.*, 2016)

a) The day before inoculation of the samples, prepare wells of African green monkey kidney (Vero) cells (24-well format) that can reach 80% confluence the next day. Apart from Vero cells, other common cell lines, such as rhesus monkey epithelial kidney cells (LLC-MK2) and human liver cancer cells (Huh 7), can also be used for virus isolation.

b) Thawed samples are vortexed briefly, followed by a brief centrifugation at 1000 ***g*** for 5 minutes.

c) Samples can be filtered through a 0.45 μm filter (optional).

d) Retrieve the prepared Vero cell culture and wash the cells gently with sterile phosphate-buffered saline (PBS) for three times.

e) Add 300 μl of a sample to a designed well of Vero cells.

f) Incubate the mixture at 37°C in a CO_2 incubator for 1 hour.

g) Retrieve the plate and add 700 μl of virus culture medium (DMEM [Dulbecco's modified Eagle's medium] with 1% Pen/Strep, 1% sodium pyruvate and 2% fetal calf serum) into each well. Treated cells are then cultured in a CO_2 incubator for 24 hours.

h) Inoculum is removed after the incubation and 1 ml of fresh virus culture medium is added into each well. Treated cells are then cultured for another 3–5 days.

i) Check for cytopathic effect (CPE) under microscope every day. Infected cells show CPE effects with rounded, aggregated and granulated giant cells detaching from the monolayer at about day 3 post-infection.

j) Harvest the supernatant from cultures where 60% of cells have CPE.

k) Aliquot the supernatant and keep them at a –80°C freezer until use.

l) Confirm the identity of virus isolate using RT-PCR specific for MERS-CoV.

1.3. Real-time reverse-transcription polymerase chain reaction (RT-PCR) assay

Currently described tests are an assay targeting the upstream regions of the E protein gene (UpE) and an assay targeting the open reading frame 1b (ORF 1b) and/or an assay targeting ORF 1a. The assay for the UpE target is recommended for screening, whereas the ORF 1b or ORF 1a assay is recommended for confirmation. There are many commercially available kits for the UpE target MERS-CoV real-time RT-PCR assays, but these kits lack proper validation for use with camel samples (Mohamed *et. al.,* 2017). Therefore, any kit to be used must be validated according to WOAH standards (Chapter 1.1.6) in each individual laboratory before it can be used for routine diagnosis.

1.3.1. Real-time RT-PCR targeting the upstream of the E protein gene (UpE)

The protocol recommended is based on the method described by Corman *et al.* (2012a).

i) Test procedure

a) Nasal swabs are preferred samples for laboratory detection of MERS-CoV infection in camels. Different methods for RNA isolation have been described and a large variety of commercial kits are available; the RNA extraction step should be appropriate to the sample to be tested and must also be validated in the laboratory.

b) Reverse transcription is performed according to the manufacturer's instructions. Many one- and two-step RT-PCR kits formulated for application with probes are commercially available and should all provide satisfactory results.

c) Prepare a master mix for the number of samples under test plus one extra sample. For example, 12.5 μl of 2 × reaction buffer provided with the one-step RT-PCR system with Taq polymerase containing 0.4 mM of each dNTP and 3.2 mM magnesium sulphate, 1 μl of reverse transcriptase/Taq mixture, 0.4 μl of a 50 mM magnesium sulphate solution, 1 μg of non-acetylated BSA, 400 nM concentrations of primer upE-Fwd (GCA-ACG-CGC-GAT-TCA-GTT)

and primer upE-Rev (GCC-TCT-ACA-CGG-GAC-CCA-TA), as well as 200 nM of probe upE-Prb (6-carboxyfluorescein [FAM])-CTC-TTC-ACA-TAA-TCG-CCC-CGA-GCT-CG-6-carboxy-N,N,N,N'- tetramethylrhodamine [TAMRA]).

d) Add 20 µl PCR reaction mix to each PCR tube or well of a real-time PCR plate followed by 5 µl of the prepared RNA to give a final reaction volume of 25 µl. Spin for 1 minute in a suitable centrifuge.

e) Place the tubes or the plate in a real-time thermal cycler for PCR amplification and run the following programme: 55°C for 20 minutes, followed by 95°C for 3 minutes and then 45 cycles of 95°C for 15 seconds, 58°C for 30 seconds.

f) BSA can be omitted if using a PCR instrument with plastic tubes as this component only serves to enable glass capillary-based PCR cycling.

g) The procedure given may require modification to accommodate individual laboratory or different RT-PCR kit requirements.

h) Real-time RT-PCR-based diagnostics should be interpreted with caution because a positive RT-PCR result does not necessarily indicate the presence of infectious virus (MacLachlan *et al.,* 1994). Furthermore, results of this procedure are difficult to correlate quantitatively to virus titre due to the imbalance between viral genomic and sub-genomic transcripts (Corman *et al.,* 2012a).

i) For the results to be valid, the positive control should give the amplification curve and no curve should be observed in the negative control.

j) The threshold cycle (Ct value) used in the interpretation of the results should be defined by individual laboratories using the appropriate reference material.

1.3.2. Real-time RT-PCR targeting the open reading frame 1b (ORF 1b)

The assay for ORF 1b has the same conditions as for the UpE real-time RT-PCR, except primer and probe sequences are: ORF1b-Fwd (TTC-GAT-GTT-GAG-GGT-GCT-CAT), primer ORF1b-Rev (TCA-CAC-CAG-TTG-AAA-ATC-CTA-ATT-G), and probe ORF1b-Prb (6-carboxyfluorescein [FAM])-CCC-GTA-ATG-CAT-GTG-GCA-CCA-ATG-T-6-carboxy N,N,N,N'-tetramethylrhodamine [TAMRA]) (Corman *et al.,* 2012a).

1.3.3. Real-time RT-PCR targeting the open reading frame 1a (ORF 1a)

The procedure to perform this test is described by Corman *et al.* (2012b). The conditions are also the same as for the UpE real-time RT-PCR, except primer and probe sequences are: Orf1a-Fwd (CCA-CTA-CTC-CCA-TTT-CGT-CAG) and Orf1a-Rev (CAG-TAT-GTG-TAG-TGC-GCA-TAT-AAG-CA), as well as 200 nM of probe Orf1a-Prb (6-carboxyfluorescein [FAM]-TTG-CAA-ATT-GGC-TTG-CCC-CCA-CT-6-carboxy-N,N,N,N'-tetramethylrhodamine [TAMRA]) (Corman *et al.,* 2012b).

1.4. Antigen detection

1.4.1. MERS-CoV immunochromatographic test (ICT)

This procedure is used to detect MERS-CoV directly from samples of suspected camels and can be used in the field or laboratories for rapid diagnosis. The assay is based on the immune-chromatographic detection of nucleocapsid protein to MERS-CoV using a rapid strip test (Song *et al.,* 2015). Commercial kits that detect recombinant nucleocapsid antigen of MERS CoV are available. The kits should be validated for the specific species of interest and for the specific purpose(s) for which they are to be used. Please see the WOAH Register for kits certified by the WOAH[1].

The procedure is fit for the qualitative detection of MERS-CoV antigens from nasal, nasopharyngeal swabs or nasal aspirates in dromedary camels for the following purposes:

1 http://www.woah.org/en/scientific-expertise/registration-of-diagnostic-kits/background-information/

i) Detection of MERS-CoV infected herds (herd test) with acutely infected animals with high virus loads;

ii) A supplemental test to estimate prevalence of infection to facilitate risk analysis, e.g. surveys, herd health schemes and disease control programmes.

For sample preparation, use individual and new sterile swab for each camel. Collect the nasal swab specimens using sterile swab. Insert the swab through the nostril which presents more secretion. Rotate the swab a few times on the respiratory epithelium of the nose. The swab specimen should be placed immediately into sterile tubes containing 2–3 ml of viral transport media or the assay diluent tube.

i) Test procedure

a) Insert each swab sample without transport medium into the assay diluent tube, swirl the swab head against the inside of the assay diluent tube, squeeze remaining buffer from swab and take out the swab. Treat swabs collected in a transport medium in the same manner.

b) Dispense 100 µl from the assay diluent tube into a test tube.

c) Directly pipette 100 µl of the swab sample into the same test tube and mix well by vortex or by any other means.

d) Remove the test strip from the foil pouch and place immediately into the test tube.

e) Read the test result after 10~15 minutes, samples should not be interpreted after 20 minutes.

f) Each strip used in this assay contains a control line that indicates that the assay is working.

g) With every batch of sample always run a confirmed RT-PCR negative sample and a confirmed RT-PCR positive sample as negative and positive controls, respectively.

The presence of the purple line on both the control (C) and test (T) position is the threshold determination. The test sample is positive when two lines (C line and T line) both appear and negative when only the C line appears. Lines consist of the immune reaction of the gold conjugate and target analytes. Gold conjugate consists of colloidal gold and MERS-CoV antibody.

ii) Result interpretation

a) Negative result: Only one control (“C”) band appears.

b) Positive result: Test (“T”) band and control(“C”) band appear.

c) Invalid: Control (“C”) fails to appear. If the control band is not visible within the result window after performing the test, the result is considered invalid. It is recommended that the sample be re-tested using a new test kit.

iii) Quality control

Each strip used in this assay contains a control line that indicates that the assay is working. With every batch of sample always run a confirmed RT-PCR negative sample and a confirmed RT-PCR positive sample as negative and positive controls, respectively. ICT can be negative when not enough virus is in the sample

2. Serological tests

Several assays are available for detection of MERS-CoV antibodies in dromedary camels. Currently the most widely used technique is the enzyme-linked immunosorbent assay (ELISA) for the detection of IgG. Virus neutralisation tests have also been used to detect antibodies against MERS-CoV in the serum of dromedary camels (Hemida *et al.,* 2013; Meyer *et al.,* 2014; Reusken *et al.,* 2013a; 2013b). Samples collected from animals for antibody testing may contain live virus and appropriate inactivation steps should be put in place.

2.1. Enzyme-linked immunosorbent assay

Several ELISAs have been developed and one test is commercially available. The ELISA is a reliable and sensitive test to detect antibodies against MERS-CoV. The use of the antigenically divergent S1 subunit

of the MERS-CoV spike protein allows for the detection of antibodies specific for MERS-CoV. IgM ELISAs that allow diagnosis of recent infections have not been described.

2.1.1. Indirect IgG ELISA

i) Test procedure

a) Coat each well of the 96-well ELISA plate with 100 µl of recombinant S1 protein at 1 µg/ml in PBS, seal the plate and leave overnight at 4°C.

b) Wash the plates three times with approximately 300 µl PBS per well.

c) Block the plates with 200 µl blocking buffer containing 1% BSA/0.5% Tween20 in PBS for 1 hour at 37°C.

d) Dilute both control (positive and negative) and test sera 1/100 in blocking buffer.

e) Add 100 µl of the diluted sera in designated wells in duplicate.

f) Incubate the plates for 1 hour at 37°C. Avoid drying by putting plates in a humid chamber.

g) Following the incubation step, wash the ELISA plates with wash buffer (0.05% Tween20 in PBS) three times with 300 µl of wash buffer per well.

h) Next, wells are incubated with 100 µl of a goat anti-llama biotin conjugate (diluted 1:1000 in blocking buffer).

j) Incubate for 1 hour at 37°C.

j) The plates are washed four times with PBS.

k) Add 100 µl of ready to use TMB (tetramethyl benzidine) chromogenic substrate to each well and allow the plates to stand at room temperature for a few minutes, while avoiding exposure to direct light.

l) Stop the reaction with 100 µl stop solution, and read the plates using ELISA plate reader at 450 nm.

2.2. MERS-CoV neutralisation assays

Neutralisation tests are the most specific diagnostic serological tests, but these tests can only be performed with live virus and are not recommended for use outside laboratories without appropriate biosecurity facilities. Alternative neutralisation assays not requiring handling of highly virulent MERS-CoV using pseudotyped viruses are available.

2.2.1. Pseudo-particle neutralisation assay

i) Production of human coronavirus (HcoV)-MERS spike pseudo-particles

The pseudo-particle used in this test expresses full-length spike protein of MERS-CoV. It is intended to be used for screening serum samples that can neutralise MERS-CoV virus. Unlike the standard virus neutralisation test for MERS-CoV, no infectious MERS-CoV is involved in this assay and the entire work can be performed safely in standard biosafety level 2 settings. Plasmids (pNL Luc-E-R- and pcDNA-S) required for making pseudo-particles are available upon request (Perera *et al.,* 2013).

ii) Preparation of pseudo-particles

a) Trypsinise a flask of human 293T cells (70–80% confluence, in 75 cm^2 flask).

b) Resuspend the trypsinised cells with 10 ml of DMEM complete medium (DMEM + pyruvate with 10% FBS (fetal bovine serum), 10 mM HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid), 5% Pen/Strep).

c) Harvest the suspended cells, followed by a centrifugation (450 ***g*** for 5 minutes).

d) Discard the supernatant and resuspend the cells with fresh DMEM complete medium.

e) Count cells using a haemocytometer.

f) Adjust the cell density to 5×10^5 per ml with DMEM complete medium.

g) Transfer 5 × 10^6 cells onto a 10 cm^2 dish and incubate the freshly prepared cell culture in a CO_2 incubator for overnight. Make sure cells are evenly distributed on a dish before incubation.

h) On the day of transfection, prepare a solution mixture by adding 15 μg of pNL Luc-E^-R^- and 15 μg of pcDNA-S to 350 μl of sterile distilled water. Add 56 μl of 2 M $CaCl_2$ to the mixture and top up the mixture to 450 μl using sterile distilled water. Slowly add dropwise 450 μl of 2× HEPES buffered saline to the diluted DNA mixture. Briefly vortex the solution mixture and incubate it at room temperature for 2 minutes.

i) Evenly dispense the diluted DNA solution dropwise to the 10 cm^2 dish and mix the solution by moving the dish back and forth and sideway several times.

j) Incubate the transfected cells in a CO_2 incubator at 37°C for 16–18 hours.

k) Replace the medium with DMEM complete medium without disturbing the monolayer. Incubate the treated cells for another 2 days.

l) Harvest the supernatant at day 3 post-transfection. Centrifuge the supernatant at 450 ***g*** for 5 minutes. Filter the centrifuged supernatant with a sterile 0.45 μm filter.

m) Make aliquots of the filtrate and store them at –80°C until use.

iii) Pseudo-particle titration

a) Seed 1 × 10^4 Vero cells in a well (96-well plate format) similar to the protocol as described above. Culture the cells in a CO_2 incubator at 37°C overnight.

b) Prepare two-fold diluted pseudo-particle solution (range of dilution factors: 1 to 2048) by mixing the pseudo-particle with virus culture medium (DMEM + pyruvate with 2% FBS, 10 mM HEPES, 5% Pen/Strep) immediately before the infection. 200 μl for each studied concentration is sufficient for performing the test in triplicate.

c) Retrieve the cultured cells, discard the culture medium and add 50 μl of virus culture medium to each well.

d) Transfer 50 μl of prepared pseudo-particle solution to the corresponding wells in the plate. Incubate the treated cells in a CO_2 incubator at 37°C overnight.

e) Add 100 μl of virus culture medium in to each well at 24 hours post-infection and incubate the cells for another 48 hours.

f) At 72 hours post-infection, check the luciferase activity of infected cells using a commercially available luciferase assay kit. The final working concentration used for pseudo-particle neutralisation assays is the most diluted concentration that has a maximum luciferase activity in this titration assay.

iv) Pseudo-particle neutralisation assay

a) Prepare Vero cells in a 96-well plate format on the day before infection as described above.

b) Add 16 μl of heat-treated serum (56°C, 30 minutes) into 144 μl of virus culture medium (DMEM + pyruvate, 2% FBS, 10 mM HEPES, 5% Pen/Strep), then mix the diluted serum with 160 μl of diluted pseudo-particle at the predetermined concentration (see above). Keep the mixture on ice for 60 minutes. Include appropriate positive and negative controls for each run.

c) Retrieve the cultured Vero cells, discard the culture medium and transfer 100 μl of incubated pseudo-particle to the corresponding wells in triplicate. Incubate the treated cell in a CO_2 incubator at 37°C for 24 hours.

d) Add 100 μl of virus culture medium to each well at 24 hours post-infection and incubate the culture at 37°C for another 48 hours.

e) At 72 hours post-infection, check the luciferase activity of infected cells using a commercially available luciferase assay kit. Use data from negative controls for background subtraction. Data from positive controls representative 100% luciferase activity. Assay cut-off value is 90% of inhibition of the average luciferase activity from positive controls.

f) This assay screens for the presence of MERS-CoV-specific neutralising antibodies using 20× diluted serum samples. Neutralising antibody titres of positive samples can be

determined in a similar fashion by using two-fold serially diluted sera in the test (starting dilution: 20×).

2.2.2. Plaque reduction neutralisation test

The plaque reduction neutralisation test (PRNT) may be used to determine the presence of antibodies in naturally infected animals and in vaccinated animals. The test is highly specific and can be used to test serum of any species. The PRNT80 (i.e. 80% reduction) or PRNT90 conducted in a cell culture system is generally accepted as the standard assay system for the quantitative determination for neutralisation antibody activity in serum samples. The following technique uses a 96 well format.

i) Test procedure

a) Samples are first inactivated at 56°C for 30 minutes.

b) Prepare 50 µl of two-fold serial dilutions of heat-inactivated serum in RPMI1640 medium supplemented with clemizole penicillin (penicillin G), streptomycin, and 1% FBS (1% culture medium) using 96-well round-bottom plates. Starting dilution should be 1/10 and include known positive and negative control sera.

c) Dilute MERS-CoV in 1% culture medium to a dilution of 10000 tissue culture infective dose per ml ($TCID_{50}$/ml) of MERS-CoV. Add 50 µl of this suspension to the wells.

d) Incubate for 60 minutes at 37°C in a humidified atmosphere with 5% CO_2.

e) Inoculate onto Huh-7 cells that were grown in flat-bottom 96-well plates.

f) Wash with phosphate buffered saline and add 100 µl of 1% culture medium. Incubate for 8 hours at 37°C in a humidified atmosphere with 5% CO_2.

g) The cells are then fixed with 3.7% formalin for 15 minutes at 20°C. After removal of the formalin plates are soaked in 70% ethanol and kept overnight at 4°C.

h) Cells are stained using an anti-MERS-CoV N protein mouse monoclonal antibody or other specific antisera against MERS-CoV. A secondary peroxidase-labelled goat anti-mouse IgG1 or other appropriate antibody is subsequently applied. The signal can be developed using a precipitate forming TMB substrate. The number of infected cells per well are counted using an inverted microscope or image analyser.

i) The neutralisation titre of each serum sample is determined as the reciprocal of the highest dilution resulting in an at least 80 or 90% reduction in the number of infected cells. A titre of ≥ 20 is considered to be positive.

2.2.3. Virus neutralisation test

i) Test procedure

a) Samples are first inactivated at 56°C for 30 minutes.

b) Prepare 50 µl of two-fold serial dilutions of heat-inactivated serum in Iscove's modified Dulbecco's medium (IMDM) supplemented with clemizole penicillin (penicillin G), streptomycin, and 1% FBS (1% culture medium) using 96-well round-bottom plates. Starting dilution should be 1/10 and include known positive and negative control sera.

c) Dilute MERS-CoV in 1% culture medium to a dilution of 2000 tissue culture infective dose per ml ($TCID_{50}$/ml) of MERS-CoV. Add 50 µl of this suspension to the wells.

d) Incubate for 60 minutes at 37°C in a humidified atmosphere with 5% CO_2.

e) Inoculate the virus suspension onto Vero cells that were grown in flat bottom 96 well plates in 10% culture medium.

f) Incubate for 60 minutes at 37°C in a humidified atmosphere with 5% CO_2.

g) Wash with phosphate buffered saline and add 200 µl 1% culture medium. Incubate for 5 days at 37°C.

h) Determine the endpoint titres.

C. REQUIREMENTS FOR VACCINES

So far, among the available vaccine candidates, only three have been tested in dromedary camels: pVaxA, a DNA-based vaccine, adjuvanted MERS-rCoV, spike protein subunit vaccine and MVA vaccine, a viral-vector-based vaccine (Adney *et al.,* 2019; Haagmans *et al.,* 2016; Muthumani *et al.* 2015).

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NB: There is a WOAH Reference Laboratory for MERS (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for MERS

NB: FIRST ADOPTED IN 2021.

SECTION 3.6.

EQUIDAE

CHAPTER 3.6.1.

AFRICAN HORSE SICKNESS (INFECTION WITH AFRICAN HORSE SICKNESS VIRUS)

SUMMARY

Description of the disease: *African horse sickness (AHS) is an infectious but noncontagious viral disease affecting all species of equidae caused by an* orbivirus *of the family* Reoviridae *and characterised by alterations in the respiratory and circulatory functions. AHS is transmitted by at least two species of* Culicoides*. Nine different serotypes have been described.*

All serotypes of AHS occur in eastern and southern Africa. AHS serotypes 9, 4 and 2 have been found in North and West Africa, from where they occasionally spread into countries surrounding the Mediterranean. Examples of outbreaks that have occurred outside Africa are: in the Middle East (1959–1963), in Spain (serotype 9, 1966, serotype 4, 1987–1990), and in Portugal (serotype 4, 1989).

Laboratory diagnosis of AHS is essential. Although the clinical signs and lesions are characteristic, they can be confused with those of other equine diseases.

As a viral disease, the laboratory diagnosis of AHS can be based on the identification of infectious virus, virus nucleic acid, viral antigens or specific antibodies. A wide variety of laboratory tests have been adapted for the detection of both AHS virus (AHSV) and specific antibodies.

Identification of the agent: *It is particularly important to perform virus isolation and serotyping whenever AHS outbreaks occur outside the enzootic regions in order to choose a homologous serotype for the vaccine.*

AHSV can be isolated from blood collected during the early febrile stage. For virus isolation, the other tissues of choice for diagnosis are spleen, lung, and lymph nodes, collected at necropsy. Sample preparations can be inoculated in cell cultures, such as baby hamster kidney-21 (BHK-21), monkey stable (MS), African green monkey kidney (Vero) or insect cells (KC), intravenously in embryonated eggs. Several enzyme-linked immunosorbent assays (ELISAs) for the rapid detection of AHSV antigen in blood, spleen tissues and supernatant from infected cells have been developed. Identification of AHSV RNA has also been achieved using a reverse-transcription polymerase chain reaction (PCR) method. Virus isolates can be serotyped by a type-specific serological test such as virus neutralisation (VN), by type-specific reverse-transcription PCR or by sequencing.

Serological tests: *Horses that survive natural infection develop antibodies against the infecting serotype of AHSV within 8–12 days post-infection. This may be demonstrated by several serological methods, such as complement fixation test, ELISA, immunoblotting and VN. The latter test is used for serotyping.*

Requirements for vaccines: *Attenuated (monovalent and polyvalent) live vaccines for use in horses, mules and donkeys, are currently commercially available. Subunit vaccines have been evaluated experimentally.*

A. INTRODUCTION

African horse sickness (AHS) (Peste equina africana, Peste equine) is an infectious, non-contagious arthropod-borne disease of equidae, caused by a double-stranded RNA orbivirus belonging to the family *Reoviridae*. The genus *Orbivirus* also includes bluetongue virus and epizootic haemorrhagic disease virus, which have similar morphological and biochemical properties with distinctive pathological and antigenic properties as well as host ranges. Nine antigenically distinct serotypes of AHSV have been identified by virus neutralisation; some cross-reaction has been observed between 1 and 2, 3 and 7, 5 and 8, and 6 and 9, but no cross-reactions with other known orbiviruses occur. The virus can be inactivated at 72°C for 120 minutes (confirmed by three blind passages in the Vero cell line).

The virion is an unenveloped particle of a size around 70 nm. The genome of AHS virus (AHSV) is composed of ten double-stranded RNA segments, encoding seven structural proteins (VP1-7), most of which have been completely sequenced for AHSV serotypes 4, 6 and 9 (Roy *et al*., 1991; Venter *et al*., 2000; Williams *et al*., 1998), and four nonstructural proteins (NS1, NS2, NS3, NS3A) (Grubman & Lewis, 1992; Laviada *et al*., 1993). Proteins VP2 and VP5 form the outer capsid of the virion, and proteins VP3 and VP7 are the major inner capsid proteins. Proteins VP1, VP4 and VP6 constitute minor inner capsid proteins. The NS3 proteins are the second most variable AHSV proteins (Van Niekerk *et al*., 2001), the most variable being the major outer capsid protein, VP2. This protein, VP2, is the determinant of AHSV serotypes and, together with VP5, the target for virus neutralisation activity (Martinez-Torrecuadrada *et al*., 2001). At least two field vectors are involved in the transmission of the virus: *Culicoides imicola* and *C. bolitinos*.

AHS is enzootic in sub-Saharan Africa, although occasional outbreaks have occurred in northern Africa (1965, 1989–1990, 2007–2010), the Middle East (1959–1961), and in Europe (Spain: 1966, 1987–1990, and Portugal: 1989) (Sanchez-Vizcaíno, 2004).

There are four classical clinical forms of AHS: pulmonary, cardiac, mixed, and horse sickness fever. The peracute, pulmonary form occurs in fully susceptible animals and has a short course, often only a few hours, and a high mortality rate. The animal exhibits respiratory distress, an extended head and neck, and profuse sweating. Terminally, froth exudes from the nostrils. The cardiac, oedematous form has a more subacute course with mortality reaching 50%. The head and neck may show severe swelling that can extend down to the chest. Swelling of the supraorbital fossae is characteristic and may include conjunctival swelling with petechiae. Paralysis of the oesophagus may result in aspiration pneumonia and sublingual haemorrhages are always a poor prognostic sign. The mixed, acute form is most commonly seen and has features of both the cardiac and pulmonary forms. Mortality can reach 70%. Horse sickness fever is an often overlooked, mild form of the disease and is seen in resistant equidae such as zebra and donkeys (Coetzer & Guthrie, 2005).

Clinical cases have also been described in dogs, with acute respiratory distress syndrome or sudden death. The mortality in dogs is high, and they may play a role in spread of the disease (Oura, 2018). Historically, infection was attributable to the consumption of infected horse meat, however more recent evidence includes the suspicion of vector-transmission (O'Dell *et al.,* 2018; van Sittert *et al* 2013).

The disease has both a seasonal (late summer/autumn) and a cyclical incidence with major epizootics in southern Africa during warm-phase events, such as occurrences of El Niño (Baylis *et al*., 1999). Mortality due to AHS is related to the species of equidae affected and to the strain or serotype of the virus. Among equidae, horses are the most susceptible to AHS with a mortality rate of 50–95%, followed by mules with mortality around 50%. In enzootic regions of Africa, donkeys are very resistant to AHS and experience only subclinical infections. In European and Asian countries, however, donkeys are moderately susceptible and have a mortality rate of 10%. Zebras are also markedly resistant with no clinical signs, except fever, and may have extended viraemia (up to 40 days).

A laboratory diagnosis is essential to establish a correct and confirmatory diagnosis. Although some clinical signs and lesions are characteristic, AHS can be confused with other diseases. For example, the supraorbital swelling, which is often present in horses with subacute AHS, is, in combination with an appropriate history, sufficient for a tentative diagnosis. Other signs and lesions are less specific for AHS, and other diseases such as equine encephalosis, equine infectious anaemia, Hendra virus, equine viral arteritis, piroplasmosis and purpura haemorrhagica should be excluded (WOAH, 2010).

Attenuated (monovalent and polyvalent) live vaccines for use in horses, mules and donkeys, are currently commercially available.

There is no evidence that humans can become infected with any field strain of AHSV, either through contact with naturally or experimentally infected animals or by virus manipulation in laboratories. Laboratory manipulations should be performed with appropriate containment determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of African horse sickness and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Real-time RT-PCR	+	+++	+	+++	++	–
Agarose gel-based RT-PCR	–	+	+	++	+	–
Virus isolation	–	++	–	+++	–	–
Detection of immune response						
ELISA (serogroup specific based on VP7)	+++	++	++	++	+++	++
CFT	+	+	+	+	+	+
VN	+	+	–	+	+	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
VN = virus neutralisation; CFT = complement fixation test.
[(a)] A combination of agent identification methods applied on the same clinical sample is recommended.

Several techniques are already available for AHS viral identification ranging from the rapid capture (indirect sandwich) enzyme-linked immunosorbent assay (ELISA) (Rubio *et al.,* 1998), using either polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs), to the polymerase chain reaction (PCR) test, including reverse-transcription (RT) PCR for discrimination of the nine AHSV serotypes or virus isolation in cell culture. If possible more than one test should be performed to diagnose an outbreak of AHS, especially the index case. The initial test can be a quick test such as ELISA or PCR, followed by virus isolation in tissue culture. Virus neutralisation (VN) for serotype identification, type-specific RT-PCR or sequencing should be performed as early in the outbreak as possible so that the serotype can be identified and the correct vaccine selected.

At present, there are no international standards for viruses or diagnostic reagents, and there is no standard methodology for the identification of AHSV. However, a viral and antibody panel has been evaluated, and comparative studies between different ELISAs for AHSV antigen and antibody determination have been carried out in different laboratories, including in the European Union (EU) Reference Laboratory for AHS. The results have demonstrated a high level of correlation for both antigen and antibody determination with an in-house test and commercial kits. Similar studies have been conducted with several RT-PCR assays also providing a high level of correlation. Further information on comparative studies of different test methods and kits is available from the

WOAH Reference Laboratories for AHS. A very important aspect of the diagnosis is the selection of samples and their safe transportation to the laboratory.

1. Identification of the agent

1.1. Virus isolation

Unclotted whole blood collected during the early febrile stage of the disease from sick animals, as well as small pieces (2–4 g) of spleen, lung and lymph nodes from animals that have died, are the samples of choice for diagnosis. Samples should be kept at 4°C during transportation and short-term storage prior to processing.

1.1.1. Cell culture

Successful direct isolation of AHSV has been performed on baby hamster kidney (BHK-21), monkey stable (MS) and African green monkey kidney (Vero) mammalian cell lines and on *Culicoides* and mosquito insect cell lines. Blood samples collected in an appropriate anticoagulant can be used undiluted as the inoculum. After 15–60 minutes of adsorption at ambient temperature or at 37°C, the cell cultures are washed and maintenance medium is added. Alternatively and more commonly, the blood is washed, lysed and diluted 1/10. This procedure removes unwanted antibody, which could neutralise free virus, and promotes release of virus associated with the red blood cell membranes. When tissue samples, such as spleen, lung, etc., are used, a 10% tissue suspension is prepared in phosphate buffered saline (PBS) or cell culture medium, containing antibiotics.

A cytopathic effect (CPE) may appear between 2 and 10 days post-infection with mammalian cells. Three blind passages should be performed before considering the samples to be negative. No CPE is observed in insect cells but the presence of the virus can be detected in the supernatant after 5–7 days by real-time RT-PCR. Supernatant from infected insect cells can then be passed onto mammalian cells, which will show CPE after one or two passages.

1.2. Nucleic acid methods

1.2.1. Reverse-transcription polymerase chain reaction

RT-PCR is a highly sensitive technique that provides a rapid identification of AHS viral nucleic acid in blood and other tissues of infected animals. This technique has greatly improved the laboratory diagnosis of AHS by increasing the sensitivity of detection and shortening the time required for the diagnosis. The RT-PCR procedure will detect virus-specific nucleic acid after the virus is no longer viable and capable of establishing a new infection in either insects or mammalian cells. Therefore, positive results do not necessarily indicate the presence of infectious virus.

Several agarose gel-based RT-PCR assays for the specific detection of AHSV RNA have been described targeted at viral segments 3, 7 or 8 (Aradaib, 2009; Bremer *et al*., 1998; Laviada *et al*., 1997; Sakamoto *et al*., 1994; Stone-Marschat *et al*., 1994; Zientara *et al*., 1994). The most widely used method employs primers corresponding to the 5' end (nucleotides 1–21) and 3' end (nucleotides 1160–1179) of RNA segment 7 (coding for VP7) amplifying the complete viral segment (Zientara *et al.,* 1994).

Real-time RT-PCR methods for the highly sensitive and specific detection of AHSV RNA have been developed based on the use of a pair of primers and a labelled probe from conserved sequences of viral segments 3, 5 or 7 (Agüero *et al*., 2008; Bachanek-Bankowska *et al.,* 2014; Fernández-Pinero *et al*., 2009; Rodriguez-Sanchez *et al*., 2008). A duplex real-time RT-PCR has also been described that targets segments 7 and 8 of the genome (coding for NS1 and NS2 respectively) (Quan *et al*., 2010).

Although both gel-based and real-time RT-PCR procedures can detect reference strains from the nine virus serotypes, real-time RT-PCR provides advantages over agarose gel-based RT-PCR methods, with its faster analysis time, higher sensitivity, and suitability for high-throughput automation. Nevertheless, gel-based RT-PCR methods, particularly those amplifying long RNA

fragments (Laviada *et al*., 1997; Zientara *et al*., 1994), can be very useful in the further genetic characterisation of the virus by sequencing of the amplicons. In addition, it may be beneficial in laboratories without the capacity to perform real-time RT-PCR.

In 2015 the WOAH Reference Laboratories for AHS carried out an international ring trial to gather information on the performance of the different methods used in the main AHSV diagnostic laboratories. Ten different RT-PCR protocols were evaluated. Although in this trial some methods could only be tested in one or two laboratories, they produced very good results and therefore are suitable for further evaluation and validation. The study identified that the real-time RT-PCR methods of Agüero *et al* (2008) and Guthrie *et al.* (2013) correctly detected all the representative strains included in the international ring trial with a high sensitivity in the analysis of field samples. These methods are validated for certification of individual animals prior to movement, and are described below.

Details of AHSV gel-based RT-PCR and real-time RT-PCR methods are given below.

To assure a good reaction it is necessary to extract from the sample an AHSV RNA of high quality. The extraction of nucleic acids from clinical samples can be performed by a variety of in-house and commercially available methods.

1.2.2. Agarose gel-based RT-PCR procedure (Zientara *et al.,* 1994)

Denaturation of extracted RNA has to be performed prior to the RT-PCR procedure as the AHSV genome consists of double-stranded RNA. The sequences of the PCR primers used are 5'-GTT-AAA-ATT-CGG-TTA-GGA-TG-3', which corresponds to the messenger RNA polarity, and 5'-GTA-AGT-GTA-TTC-GGT-ATT-GA-3', which is complementary to the messenger RNA polarity.

All the components required for the reverse transcription and PCR are included in the reaction tube containing the denatured RNA. A one-step RT-PCR is carried out by incubating in a thermocycler as follows: 45 minutes to 1 hour at 37–55°C, 5–10 minutes at 95°C, then 40 cycles of: 94–95°C for 1 minute, 55°C for 1–1.5 minutes, 70–72°C for 2–2.5 minutes, followed by a final extension step of 7–8 minutes at 70–72°C. Analysis of the PCR products is carried out by agarose gel electrophoresis. AHS-positive samples will resolve in a 1179 base-pair band that can be used as template in the sequencing reaction, using the PCR primers independently to obtain the nucleotide sequence of viral segment 7.

1.2.3. Real-time RT-PCR procedure (Agüero *et al.,* 2008)

This group-specific real-time RT-PCR has been employed with very good results by the participating national reference laboratories of the EU Member States in annual proficiency tests for the period 2009–2015. Moreover, in an international ring trial organised in 2015 under the auspices of the WOAH Reference Laboratory network, it was found to be one among other top-ranking protocols.

a) Protocol

The assay targets AHSV segment 7 (VP7) and is described in Agüero *et al.* (2008). It is able to detect all known AHSV types and strains currently circulating.

i) RNA extraction from blood and tissue samples

Commercial kits are widely available; the RNA extraction step can be performed according to the procedures specified in each kit.

ii) Several one-step real-time RT-PCR kits are commercially available that can be used depending on local or case-specific requirements, kits used and equipment available. Some basic steps as described by Agüero *et al.* (2008) are given below. (For primers and probe sequences see Table 2).

iii) Primer stock concentration is diluted to a working concentration of 8 µM whereas probe is diluted to a working concentration 50 µM.

iv) 2.5 µl of each primer working stock 8 µM (final concentration 1 µM) is added to each well of the PCR plate (or tube or strip) that will contain RNA samples, positive or negative controls. The plate is held on ice.

v) 2 µl of RNA samples, including test and positive and negative controls, is added to each well.

vi) Samples are subjected to heat denaturation at 95°C for 5 minutes, followed by rapid cooling on ice for further 5 minutes.

vii) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared following the manufacturer's instructions. Probe should be included in a final concentration of 0.25 µM (0.1 µl of probe working stock, 50 µM per sample).

viii) 13 µl of master mix is distributed in each well on the PCR plate containing the denatured primers and RNA.

ix) The plate is placed in a real-time thermal cycler programmed with the following profile:
48°C × 25 minutes
95°C × 10 minutes
40 cycles: 95°C × 15 seconds, 55°C × 35 seconds, 72°C × 30 seconds

If reagents and thermal cycler allowing fast reactions are employed then the following program can be used:
48°C × 25 minutes
95°C × 10 minutes
40 cycles: 97°C × 2 seconds, 55°C × 30 seconds
Fluorescence data are acquired at the end of the 55°C step.

Note: times and temperatures may vary and should be optimised for the reagents or kit used.

b) Interpretation of the results

The assay is considered not valid if atypical amplification curves are obtained. If this is the case, the assay must be repeated.

The assay is considered positive when a typical amplification curve is obtained and the Ct value (the number of polymerase chain reaction (PCR) cycles required for fluorescent signal to exceed the background) is lower or equal to the defined Ct threshold (35) within 40 PCR cycles (Ct≤35).

The assay is considered inconclusive when a typical amplification curve is obtained and the Ct value is higher to the defined Ct threshold (35) within 40 PCR cycles (Ct≥35).

The assay is considered negative when a horizontal amplification curve is obtained and does not cross the threshold line within 40 PCR cycles.

c) Diagnostic characteristics

i) Cut-off determination

The positive cut-off for the test method is less than 35 PCR cycles (Ct≤35).

The negative cut-off for the test method is 40 PCR cycles.

Test results between the positive and negative cut-offs (35 and 40 PCR cycles) are considered inconclusive (35 ≤ Ct ≤ 40).

ii) Diagnostic sensitivity and specificity

The diagnostic specificity (DSp) and diagnostic sensitivity (DSe) were calculated according to the procedure detailed in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*.

In total 186 known negatives and 132 known positive samples were analysed to estimate DSe and DSp of the Agüero AHS real-time RT-PCR method, which is higher than the minimum

number required (73) for an estimated DSe and DSp of 95% allowing a 5% error. DSe and DSp were 97% and 100%, respectively. It can thus be concluded that the number of known status samples used to calculate these diagnostic parameters was sufficient to comply with WOAH requirements.

d) Reproducibility

Reproducibility of the Agüero real-time RT-PCR method in the international ring trial cited above was at least 93.55%, correctly identifying all positive and negative samples included in the panel. All laboratories detected dilutions of positive samples to at least 10^{-5}. Similar results have been reported in other proficiency test programmes.

Inactivated virus of serotypes 1–9 reference strains can be obtained from the WOAH Reference Laboratory in Spain to set up the RT-PCR detection method.

1.2.4. Real-time RT-PCR procedure (Guthrie *et al.,* 2013)

a) Protocol

The test method presented here is adapted from Guthrie *et al.* (2013) and is capable of detecting all known AHSV types and strains currently circulating. The assay targets AHSV segment 7 (VP7). The procedure given may require modification to accommodate individual laboratory or different RT-PCR kit requirements.

i) RNA extraction from blood and tissue samples

Commercial kits are widely available; the RNA extraction step can be performed according to the procedures specified in each kit.

ii) Kits for the one-step real-time RT-PCR are available commercially. Below are some basic steps as described by Guthrie *et al.* (2013), which can be modified depending on local or case-specific requirements, kits used and equipment available.

iii) Primer and probe mix stock solutions are made up in a 25× concentration at 5 μM for the forward and reverse primers and 3 μM for the probe.

iv) 5 μl of RNA samples, including test and positive and negative controls, are added to appropriate wells of the PCR plate (or tube or strip).

v) Samples are subjected to heat denaturation at 95°C for 2 minutes, and held on ice for at least 3 minutes.

vi) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared, following the manufacturer's instructions. 1 μl of 25× primer probe mix stock solution (from step iii above) is included in the master mix to give a final concentration in each well of 200 nM for each primer and 120 nM of the probe.

vii) 20 μl of master mix is distributed in each well on the PCR plate containing the denatured RNA.

viii) The plate is placed in a real-time thermal cycler programmed for reverse transcription and cDNA amplification or fluorescence detection as suggested by the manufacturers.

The following thermal profile is an example:
48°C × 10 minutes
95°C × 10 minutes
40 cycles: 95°C × 15 seconds, 60°C × 45 seconds

Note: times and temperatures may vary and should be optimised for the reagents or kit used.

b) Interpretation of the results

Note: the positive/inconclusive/negative cut-off values shown should be validated or adjusted in individual laboratories according to the reagents and equipment in use.

Samples are classified as "AHSV positive" if the normalised fluorescence for the AHSV real-time RT-PCR assay exceeds a 0.1 threshold within 36 PCR cycles in all replicates of a sample.

Samples are classified as "AHSV Inconclusive" if the normalised fluorescence for the AHSV real-time RT-PCR assay exceeds a 0.1 threshold between 36 and 40 PCR cycles in any replicate of a sample.

Samples are classified as "AHSV negative" if the normalised fluorescence for the AHSV assay did not exceed a 0.1 threshold within 40 PCR cycles in all replicates of a sample and if the normalised fluorescence for the internal positive control assay exceeded a 0.1 threshold within 33 PCR cycles.

c) Diagnostic characteristics

i) Cut-off determination

The positive cut-off for the test method is less than 36 PCR cycles.

The negative cut-off for the test method is 40 PCR cycles.

Test results between the positive and negative cut-offs (36 and 40 PCR cycles) are considered inconclusive.

ii) Diagnostic sensitivity and specificity

The DSe and DSp of the AHSV real-time RT-PCR for detection of AHSV nucleic acid in whole blood samples were estimated by comparison with virus isolation using a two-test two-population Bayesian latent class model that allowed for conditional dependence (correlation) among test results. A total of 503 equine blood samples collected from individual horses with pyrexia and one or more clinical signs typical of AHS were used to represent AHS suspect cases. Blood samples were also collected from two separate healthy populations of horses (503 and 98 horses each, respectively) that were not vaccinated against AHS and that were highly unlikely to have been exposed to natural infection with AHSV; these samples were used to represent AHS negative cases.

The median diagnostic specificity of the test method exceeded 99.9%.

The median diagnostic sensitivity of the test method exceeded 97.8%.

d) Reproducibility

In the international ring trial cited above the Guthrie FRET probe real-time RT-PCR method demonstrated sensitivity in excess of 88.1%, a specificity of 100%, correctly identifying all positive and negative samples included in the panel. All laboratories detected dilutions of positive samples to at least 10^{-5}.

***Table 2. Comparison of the real-time RT-PCR methods of Agüero* et al. (*2008*) *and Guthrie* et al. (*2013*)**

	Agüero *et al.,* 2008	**Guthrie *et al.,* 2013**
Target	Group specific (VP7)	Group specific (VP7)
Primers (5'-3')	CCA-GTA-GGC-CAG-ATC-AAC-AG	AGA-GCT-CTT-GTG-CTA-GCA-GCC-T
	CTA-ATG-AAA-GCG-GTG-ACC-GT	GAA-CCG-ACG-CGA-CAC-TAA-TGA
Probe (5'-3')	FAM-GCT-AGC-AGC-CTA-CCA-CTA-MGB	FAM-TGC-ACG-GTC-ACC-GCT-MGB
Annealing temperature	55°C	60°C
Number of amplification cycles	40	40

	Agüero *et al.,* 2008	Guthrie *et al.,* 2013
Analytical sensitivity (LOD)	Dilution 10^{-5} of a viral suspension of AHSV-4 reference strain with a titre of $10^{6.3}$ $TCID_{50}$/ml, which corresponded to a Ct of 34.3±0.5 ($10^{1.3}$ $TCID_{50}$/ml)	Dilution of 3.02×10^{-6} of a AHSV positive blood samples, with corresponding Ct of 35.71
Diagnostic specificity	100%	99.9%.
Diagnostic sensitivity	97%	97.8%.

1.3. AHSV typing

Until recently, the VN test has been the method of choice for typing as well as the 'gold' standard test for identifying AHSV isolated from the field using type-specific antisera (Verwoerd, 1979). This technique takes 5 or more days before results are obtained. The development of type-specific gel-based RT-PCR (Maan *et al.,* 2011; Sailleau *et al.,* 2000), and real-time RT-PCR using hybridisation probes (Koekemoer, 2008) targeting AHSV Seg-2 for identification and differentiation of AHSV genotypes, provides a rapid typing method for AHSV in tissue samples and blood. These methods can be used to very significantly increase the speed and reliability of detection and identification (compared with VN tests) of the nine serotypes of AHSV. Type-specific real-time RT-PCR assays based on the use of labelled DNA probes–MGB probes have been developed recently by Bachanek-Bankowska *et al*., 2014, Weyer *et al.,* 2015).

However, the genetic variation that may appear over time in the AHSV genome, in particular in the VP2 coding region, where specific primers/probes for typing assays have to be designed, makes the detection of all genetic variants within each serotype by this type of technique difficult. Therefore, although molecular methods are able to rapidly type AHSV in many positive field samples, VN should be kept as the gold standard for serotyping AHSV isolates.

2. Serological tests

Indirect and competitive blocking ELISAs using either soluble AHSV antigen or a recombinant protein VP7 (Hamblin *et al*., 1990; Laviada *et al*., 1992; Maree & Paweska, 2005; Wade Evans *et al.,* 1993) have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especially for large-scale investigations (Rubio *et al*., 1998). Both of these tests have been recognised by the European Commission (2002). The competitive blocking ELISA can also be used for testing wildlife as species-specific anti-globulin is not required with this method. An immunoblotting test has also been adapted for anti-AHS antibody determination (Laviada *et al*., 1992), which is especially suitable for small numbers of sera. The complement fixation (CF) test has been widely used, but some sera are anti-complementary, particularly donkey and zebra sera.

2.1. Blocking enzyme-linked immunosorbent assay

The competitive blocking ELISA technique detects specific antibodies against AHSV, present in any equine species. VP7 is the main antigenic protein within the molecular structure of AHSV and it is conserved across the nine AHSV serotypes. An MAb directed against VP7 is used in this test, allowing high sensitivity and specificity. Moreover, other species of equidae (e.g. donkeys, zebra, etc.) can be tested thus preventing the problem of specificity experienced occasionally using the indirect ELISAs. VP7 recombinant antigen is non-infectious, which provides a high level of security (European Commission, 2002).

The principle of this test is to block the specific reaction between the recombinant VP7 protein absorbed on an ELISA plate and a conjugated MAb against VP7. AHSV antibodies present in a suspect serum sample will block this reaction. A decrease in the amount of colour is evidence of the presence of AHSV antibodies in the serum sample.

The competitive blocking ELISA is commercially available. The reproducibility of the test was assessed in an international ring trial (Durán-Ferrer *et al.,* 2018).

2.1.1. Test procedure

i) *Solid phase:* coat 96 well ELISA plates with 50–100 ng of recombinant AHSV-4 VP7 diluted in carbonate/bicarbonate buffer, pH 9.6. Incubate overnight at 4°C.

ii) Wash the plates three times with PBS 0.1× containing 0.135 M NaCl and 0.05% (v/v) Tween 20 (washing solution). Gently tap the plates onto absorbent material to remove any residual wash.

iii) *Test samples:* serum samples to be tested, and positive and negative control sera (if not ready to use by kit manufacturer), are diluted 1/5 in diluent containing 0.35 M NaCl, 0.05% Tween 20; and 0.1% Kathon, 100 µl per well. Incubate for 1 hour at 37°C.

iv) Wash the plates five times with PBS 0.1× containing 0.135 M NaCl and 0.05% (v/v) Tween 20 (washing solution). Gently tap the plates onto absorbent material to remove any residual wash.

v) *Conjugate:* dispense 100 µl/well of horseradish peroxidise-conjugated MAb anti-VP7 at optimal dilution in a suitable diluent. The MAb and diluent may be included in commercial kits. Incubate for 30 minutes at 37°C.

vi) Wash the plates as described in step iv.

vii) *Substrate/chromogen:* add 100 µl/well substrate/chromogen solution, e.g. ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline]-6-sulphonic acid) 5 mg/ml diluted 1/10 in 0.1 M phosphate/citrate buffer, pH 4, containing 0.03% H_2O_2, and incubate for 10 minutes at room temperature.

Colour development is stopped by adding 100 µl/well of 2% (w/v) of SDS. Alternative chromogen systems may be used (e.g. tetramethyl benzidine).

viii) Read the plates at 405 nm.

ix) *Validation of the assay:* positive control lower than 0.2 and negative control higher than 1.0.

x) *Interpretation of results:* determine the blocking percentage (BP) of each sample by applying the following formula:

$$BP = \frac{\text{Abs (Control Neg)} - \text{Abs (sample)}}{\text{Abs (Control Neg)} - \text{Abs (Control Pos)}} \times 100$$

Samples showing BP value lower than 45% are considered negative for antibodies to AHSV. Samples showing BP value higher than 50% are considered positive for antibodies to AHSV. Samples with BP value between 45% and 50% are considered doubtful and must be retested. If the result is the same, resample and test 2 weeks later.

2.2. Indirect enzyme-linked immunosorbent assay

The recombinant VP7 protein has been used as antigen[1] for AHSV antibody determination with a high degree of sensitivity and specificity (Laviada *et al.,* 1992; Maree & Paweska, 2005). Other advantages of this antigen are its stability and its lack of infectivity. The conjugate used in this method is a horseradish peroxidase anti-horse gamma-globulin reacting with horse, mules and donkeys. The method described by Maree & Paweska (2005) uses protein G as conjugate that also reacts with zebra serum.

2.2.1. Test procedure

i) *Solid phase:* Coat 96 well ELISA plates with recombinant AHSV-4 VP7 diluted in carbonate/bicarbonate buffer, pH 9.6. Incubate overnight at 4°C.

ii) Wash the plates five times with distilled water containing 0.01% (v/v) Tween 20 (washing solution). Gently tap the plates on to absorbent material to remove any residual wash.

1 The antigen can be provided on request by the Centro de Investigación en Sanidad Animal (CISA), Spain. The delivery time is 4–6 months.

iii) Block the plates with PBS, pH 7.2 + 5% (w/v) skimmed milk, 200 µl/well, for 1 hour at 37°C.

iv) Remove the blocking solution and gently tap the plates on to absorbent material.

v) *Test samples:* Serum samples to be tested, and positive and negative control sera, are diluted 1/25 in PBS + 5% (w/v) skimmed milk + 0.05% (v/v) Tween 20, 100 µl per well. Incubate for 1 hour at 37°C. For titration, add twofold dilution series from 1/25 (100 µl/well), one serum per plate column, in duplicate columns, and do the same with positive and negative controls. Incubate for 1 hour at 37°C.

vi) Wash the plates as described in step ii.

vii) *Conjugate:* Dispense 100 µl/well of horseradish peroxidase conjugated anti-horse gamma-globulin diluted in PBS + 5% milk + 0.05% Tween 20, pH 7.2. Incubate for 1 hour at 37°C.

viii) Wash the plates as described in step ii.

ix) *Chromogen/Substrate:* Add 200 µl/well of chromogen/substrate solution (10 ml 80.6 mM DMAB [3-(dimethylamino) benzoic acid] + 10 ml 1.56 mM MBTH [3-methyl-2-benzothiazolinone hydrazone] + 5 µl H_2O_2). Colour development is stopped by adding 50 µl of 3 N H_2SO_4 after approximately 5–10 minutes (before the negative control begins to be coloured). Other chromogens such as ABTS, tetramethyl benzidine or orthophenyldiamine can also be used.

x) Read the plates at 600 nm (or 620 nm).

xi) *Interpretation of results:* Calculate the cut-off value by adding 0.06 to the value of the negative control. (0.06 is the standard deviation derived with a group of 30 negative sera) Test samples giving absorbance values lower than the cut-off are regarded as negative. Test samples giving absorbance values greater than the cut-off + 0.15 are regarded as positive. Test samples giving intermediate absorbance values are doubtful and a second technique must be employed to confirm the result.

2.3. Complement fixation

The CF test has been used extensively in the past, but currently its use is decreasing and has been replaced in many laboratories by ELISA as a screening technique. This progressive replacement is because of the higher sensitivity and degree of standardisation of ELISA as well as a significant number of sera with anti-complementary activity. Nevertheless, the CF test is a useful tool in endemic areas for the demonstration and titration of group-specific IgM antibodies against AHSV notably following a recent infection or vaccination.

2.3.1. Reagents

i) Veronal buffered saline containing 1% gelatin (VBSG).

ii) Serum samples, free from erythrocytes, must be heat inactivated: horse serum at 56°C, zebra serum at 60°C and donkey serum at 62°C, for 30 minutes.

iii) The antigen is a sucrose/acetone extract of AHSV-infected mouse brain. The control antigen is uninfected mouse brain, extracted in the same way. In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. In the test, four to eight units are used. The antigen may also be obtained by inoculation of the virus in suitable cell culture (see Section B.1 above).

iv) The complement is a normal guinea-pig serum.

v) The haemolysin is a hyperimmune rabbit serum against sheep red blood cells (SRBCs).

vi) The SRBCs are obtained by aseptic puncture of the jugular vein and preserved in Alsever's solution[2] or sodium citrate.

vii) The haemolytic system (HS) is prepared by diluting the haemolysin to contain two haemolytic doses and using this to sensitise washed SRBCs. The SRBCs are standardised to a 3% concentration.

viii) *Control sera:* A positive control serum is obtained locally and validated. Serum from a healthy antibody-negative horse is used as the negative control serum.

2.3.2. Test procedure

i) The reaction is performed in 96-well round-bottom microtitre plates in a final volume of 100 µl/well or in tubes if the macro-technique is used, at 4°C for 18 hours.

ii) All the sera, samples and controls are diluted 1/5 in VBSG and 25 µl of each serum is added in duplicate. A twofold dilution series of each serum is done from 1/5 to 1/180.

iii) Add 25 µl of the antigen diluted according to the previous titration.

iv) Add 25 µl of the complement diluted according to a previous titration.

v) Incubate at 4°C for 18 hours.

vi) 25 µl of HS is added to all wells on the microtitre plate.

vii) The plate is incubated for 30 minutes at 37°C.

viii) Plates are then centrifuged at 200 ***g***, and the wells are scored for the presence of haemolysis. Control of sera, complement, antigen and HS are used

ix) Results are read using 50% haemolysis as the end point. The inverse of the highest dilution of serum specifically fixing complement with the CF antigen is called the titre.

x) A titre of 1/10 or more is positive, under 1/10 is negative.

2.4. Virus neutralisation (VN)

Serotype-specific antibody can be detected using the VN test (House *et al*., 1990). The VN test may have additional value in epidemiological surveillance and transmission studies, mainly in endemic areas where multiple serotypes are likely to be present.

2.4.1. VN test procedure

i) From a starting dilution of 1/5, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample two wells are used at each dilution. Control positive and negative sera should also be included in each batch of tests. An equal volume (e.g. 25 µl) of a stock of AHSV containing 100 $TCID_{50}$ (50% tissue culture infective dose) is added to each well.

ii) Serum/virus mixtures are incubated for 60 minutes at 37°C 5% CO_2 and 95% humidity prior to the addition of 0.1 ml of Vero cell suspension (200,000 cells/ml) to each test well.

iii) A back titration of virus stock is prepared for each test using four wells per tenfold dilution, 25 µl per well. Test plates are incubated at 37°C, 5% CO_2, 95% humidity for 4–5 days, until the back titration indicates that the stock virus contains 30–300 $TCID_{50}$.

iv) After incubation for 4–5 days, the test is read using an inverted microscope. Wells are scored for the presence or absence of CPE. The presence of CPE in the wells containing the serum sample indicates that the tested serum does not contain specific neutralising antibodies against the virus in the assay that cannot neutralise the virus, therefore producing cell lysis with the consequent destruction of the cell layer.

2 20.5 g dextrose (114 mM), 7.9 g sodium citrate $2H_2O$ (27 mM), 4.2 g NaCl (71 mM), H_2O to 1 litre. Adjust to pH with 1 M citric acid.

By contrast, the absence of CPE in the wells containing the serum sample indicates that the tested serum does contain specific neutralising antibodies against the virus in the assay that can neutralise the virus, therefore maintaining intact the cell layer.

v) Alternatively, the plates are then fixed and stained in a solution of 0.15% (w/v) crystal violet in 2% (v/v) glutaraldehyde and rinsed or they may be fixed with 70% ethanol and stained with 1% basic fuchsin.

vi) The 50% end-point titre of the serum is calculated by the Spearman–Kärber method and expressed as the negative $\log_{10}$.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Polyvalent or monovalent live attenuated AHS vaccines, based on the selection in Vero cell culture of genetically stable macroplaques, have been used for the control of AHSV in and out of Africa (Erasmus, 1976; Sanchez-Vizcaíno, 2004). Polyvalent vaccines are commercially available.

An inactivated monovalent (serotype 4) AHSV vaccine based on virus purification and inactivation with formalin was produced commercially in the early 1990s (House *et al*., 1992), but is not available at the present time. Subunit AHSV vaccines based on serotype 4 outer capsid protein VP2 and VP5 plus inner capsid protein VP7, derived from single and dual recombinant baculovirus expression vectors have been used experimentally in different combinations to immunise horses (Martinez *et al*., 1996). The protective efficacy of VP2 in a subunit vaccine was also evaluated (Scanlen *et al*., 2002). However, these vaccines are not commercially available.

2. Outline of production and minimum requirements for conventional vaccines

At present only the live attenuated AHS vaccines (polyvalent or monovalent) are commercially available. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

3. Live attenuated African horse sickness vaccine

3.1. Characteristics of the seed

3.1.1. Biological characteristics

The seed virus is prepared by selection in Vero cells of genetically stable large plaques from low passage levels of AHSV. The plaque mutants are then further multiplied by three passages in Vero cells. A large quantity of this antigen is lyophilised and stored at –20°C as seed stock antigen.

3.1.2. Quality criteria

The seed virus must be shown to be free of contaminating viruses, bacteria and mycoplasmas by the appropriate techniques. The serotype identity of the seed virus is confirmed.

3.2. Method of manufacture

3.2.1. Procedure

At the onset of a production run, working antigens are produced from the seed stock antigen in either BHK-21 or Vero cell cultures. The working antigens are tested for sterility, purity and identity and should contain at least 1×10^6 plaque-forming units (PFU)/ml of infectious virus.

3.2.2. Requirements for substrates and media

Roller bottle cultures of Vero or BHK-21 cells are grown using gamma-irradiated bovine serum in the growth medium. Once the cultures are confluent, the medium is poured off and the cells are seeded with the working antigens. After 1 hour, maintenance medium is added to the cultures. Incubation is continued at 37°C for 2–3 days. When the CPE is advanced, both cells and supernatant medium are harvested. The products from the same serotype are pooled and stored at 4°C.

3.2.3. In-process control

The pooled harvests of the individual serotypes are tested for sterility and assayed for infectivity by plaque titration on Vero cell cultures. The minimum acceptable titre is 1×10^6 PFU/ml.

Finally, two multivalent vaccines are constituted by mixing equal volumes of serotypes 1, 3, 4 and 2, 6, 7, 8 respectively. Serotypes 5 and 9 are not included in vaccine formulations. A monovalent type can also be prepared. After addition of suitable stabiliser, the vaccine is distributed in 1.0 ml volumes into glass vials and freeze-dried.

3.2.4. Final product batch test

i) Sterility

Following lyophilisation, five bottles of vaccine are selected at random and tested for sterility by internationally accepted methods. Tests for sterility and freedom from contamination of biological materials intended for veterinary use are given in chapter 1.1.9.

ii) Safety

Innocuity of a vaccine is determined by the inoculation of reconstituted vaccine into mice (0.25 ml intraperitoneally), guinea-pig (1.0 ml intraperitoneally), and a horse (5.0 ml subcutaneously). All the animals are observed daily for 14 days. The rectal temperature of the horse is taken twice daily for 14 days and should never exceed 39°C.

iii) Batch potency

Potency is largely based on virus concentration in the vaccine.

The minimum immunising dose for each serotype is about 1×10^3 PFU/dose. The infectivity titre of the final product is assayed by plaque titration in Vero cell cultures and should contain at least 1×10^5 PFU/dose. The horse used for safety testing is also used for determining the immunogenicity of a vaccine.

Serum samples are collected on the day of vaccination and 21 days later, and are tested for neutralising antibodies against each serotype by the plaque-reduction test using twofold serum dilutions and about 100 PFU of virus. The horse should develop a neutralising antibody titre of at least 20 against at least three of the four serotypes in the quadrivalent vaccine.

3.3. Requirements for authorisation

No specific guideline is described for AHS vaccine. However a guideline is described in the EU for Bluetongue virus under exceptional circumstances that could probably be used for AHS virus. This guideline includes the minimum date requirements for the authorisation under exceptional circumstances for vaccine production for emergency use against bluetongue virus (Regulation EC Nº726/2004, in particular Articles 38, 39 and 43 thereof and Article 26 of Direction 2001/82/EC), including guidance measures to facilitate the rapid inclusion of new or different virus serotypes.

4. Vaccines based on biotechnology

4.1. Vaccines available and their advantages

None is available commercially. Experimental subunit vaccines have been described (Section C.1.1 *Rationale and intended use of the product*).

4.2. Special requirements for biotechnological vaccines, if any

None.

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NB: There are WOAH Reference Laboratories for African horse sickness (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for African horse sickness

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2019.

CHAPTER 3.6.2.

CONTAGIOUS EQUINE METRITIS

SUMMARY

Description and importance of disease: *Contagious equine metritis is an inflammatory disease of the proximal and distal reproductive tract of the mare caused by* Taylorella equigenitalis*, which usually results in temporary infertility. It is a non-systemic infection, the effects of which are restricted to the reproductive tract of the mare.*

When present, clinical signs include endometritis, cervicitis and vaginitis of variable severity and a slight to copious mucopurulent vaginal discharge. Recovery is uneventful, but prolonged asymptomatic or symptomatic carriage is established in a proportion of infected mares. Direct venereal contact during natural mating presents the highest risk for the transmission of T. equigenitalis *from a contaminated stallion or an infected mare. Direct venereal transmission can also take place by artificial insemination using infective raw, chilled and possibly frozen semen. Indirectly, infection may be acquired through fomite transmission, manual contamination, inadequate observance of appropriate biosecurity measures at the time of breeding and at semen-collection centres. Stallions can become asymptomatic carriers of* T. equigenitalis*. The principal sites of colonisation by the bacterium are the urogenital membranes (urethral fossa, urethral sinus, terminal urethra and penile sheath). The sites of persistence of* T. equigenitalis *in the majority of carrier mares are the clitoral sinuses and fossa and infrequently the uterus. Foals born of carrier mares may also become carriers. The organism can infect equid species other than horses, e.g. donkeys.*

Detection and Identification of the agent: *Swabs should be taken from designated genital sites. Culture or real-time polymerase chain reaction (PCR) should be used for agent identification. To avoid loss of viability for culture, individual swabs should be fully submerged in Amies charcoal medium and transported to the testing laboratory under temperature-controlled conditions for plating out within 48 hours of collection. Growth of* T. equigenitalis *is likely to take 3–6 days at 37°C on specialised media in an atmosphere of 5–10% CO_2. An incubation time of at least 7 days is advisable before certifying cultures negative for* T. equigenitalis*. Identification should include biochemical characterisation, antigenic testing using specific antibodies and molecular genotyping. The fastidious nature of* T. equigenitalis *makes it difficult to isolate. Two real-time PCR tests are also available for agent identity testing and have some advantages over culture. Swabs for real-time PCR do not need to be inserted into bacterial transport medium. Other PCR assays are available and additional detection assays such as the immunofluorescent antibody test have been developed and, ultimately, test-breeding of stallions for detection of the carrier state has been used as an adjunct to agent identity testing.*

Serological tests: *Serology has been used for detecting recent, but not chronic, infection in the mare. Serum antibody to* T. equigenitalis *can be detected in mares for 3–7 weeks after infection. It may also be demonstrated in the occasional carrier mare, but never in the stallion. No individual serological test described to date has been shown reliably to detect infection. Serological tests can be used as an adjunct to culture for* T. equigenitalis *in screening mares recently bred to a carrier stallion, but must not be used as a substitute for culture.*

Requirements for vaccines: *Effective vaccines are not yet available.*

A. INTRODUCTION

1. Description and impact of the disease

Contagious equine metritis was first described in the United Kingdom (UK) in 1977, after which it was diagnosed in a number of countries world-wide. It first presented as disease outbreaks characterised by a mucopurulent vaginal discharge originating from inflammation of the endometrium and cervix, resulting in temporary infertility. Mares may experience more than one episode of the disease in a short period. Most mares recover uneventfully, but some may become carriers of the causal organism, *Taylorella equigenitalis*, for many months. Infection does not always adversely affect conception and abortion due to *T. equigenitalis* is a very rare occurrence. Many primary cases are subclinical, and a frequent indicator of infection is the mare returning in oestrus prematurely after being bred to a putative carrier stallion. Infection in a stallion is subclinical.

The carrier state plays an important role in the dissemination of the bacterium. The urogenital membranes of the stallion become contaminated at coitus or by contact with fomites typically employed in semen collection. The carrier state may persist for many months or years. Most carrier mares are clitoral carriers and poor hygienic measures when breeding may also spread the organism. Prior infection is not fully protective as the serum antibody only persists for a few weeks after infection so control of infection has relied solely on prevention of transmission. The organism can be eliminated by treatment with antibiotics combined with antiseptic washing and cleaning of the affected sites. *Taylorella equigenitalis* is not known to infect humans and it should be handled in the laboratory with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

2. Nature and classification of the pathogen

Taylorella equigenitalis is a Gram-negative, non-motile, bacillus or cocco-bacillus that is often pleomorphic (up to 6 μm long) and may exhibit bipolar staining. It is catalase positive, phosphatase positive, and strongly oxidase positive. It is otherwise inert in tests for biochemical activity.

3. Differential diagnosis

The fastidious slow growing organism can be isolated in the laboratory from swabs of colonisation sites in the reproductive tract of stallions and mares (urethral fossa, urethral sinus, terminal urethra and penile sheath; clitoral fossa, clitoral sinuses and endometrium) using the correct atmospheric conditions and is currently the preferred procedure for international trade or movement. Designated swabbing sites are usually specified for international movement, by the competent authorities.

Molecular testing methods such as polymerase chain reaction (PCR) and particularly real-time PCR are now commonly used to detect *Taylorella* both from swabs and culture plates. They have the advantage of speed of result and can usually differentiate between *T. equigenitalis* and *T. asinigenitalis*.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of contagious equine metritis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Bacterial isolation and identification	+++	+++	+++	+++	+++	–
IFAT	+	+	+	+	+	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Real-time PCR	+++	+++	+++	+++	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
IFAT = indirect fluorescent antibody test; PCR = polymerase chain reaction.

1. Detection and identification of the agent

1.1. Culture techniques

Sampling and transport prior to *Taylorella* isolation and identification needs special attention. Swabs must be placed in a transport medium with activated charcoal, such as Amies medium, to absorb inhibitory by-products of bacterial metabolism (Swerczek, 1978). The numbers of viable *T. equigenitalis* decline on swabs over time, and this effect is more pronounced at higher temperatures (Sahu *et al.,* 1979). Swabs must be kept cool during transportation and should arrive and be plated out at the laboratory no later than 48 hours after they were taken.

Various bacteria exist on the urogenital membranes of horses as harmless commensals that may interfere with the culture of *T. equigenitalis* by obscuring its presence. Washing and antibiotic treatment may control this problem but may sublethally damage *T. equigenitalis,* allowing it to persist on the urogenital membranes but rendering it impossible to grow on laboratory media. Swabbing for *T. equigenitalis*, therefore, should not recommence until at least 7 days (systemic treatment) or 21 days (local treatment) following treatment.

The fastidious nature of T. *equigenitalis* makes it difficult to isolate. Test breeding of stallions has been used to increase the sensitivity of detection of the carrier state and it has been a valuable adjunct to cultural examination. The numbers of *Taylorella* present on the external genitalia of stallions can be very low and may be missed by culturing alone, but can be detected after multiplication in the mare that has been test bred. The use of test breeding as an additional diagnostic tool can be especially important in countries that are considered free from contagious equine metritis.

Culture media is produced by heating reconstituted agar base containing 5% (v/v) lysed horse blood to 70–80°C for 12 minutes ('chocolate' blood agar), which is cooled to 45–50°C and trimethoprim (1 μg/ml), clindamycin (5 μg/ml), and amphotericin B (5–15 μg/ml) is added (Timoney & Powell, 1982). Lysed horse blood contains thymidine phosphorylase, which will inactivate thymidine, thus allowing the trimethoprim to exert its selective effect. This is the preferred medium for isolating *T. equigenitalis* therefore each swab must be inoculated onto this medium. It will successfully isolate both streptomycin resistant and sensitive biotypes of the pathogen; suppress the growth of many commensal bacteria and inhibit fungal growth. As inhibiters may prevent the isolation of some strains of *T. equigenitalis*, swabs should also be inoculated on to 5% 'chocolate' blood agar with a rich peptone agar base containing additional cysteine (0.83 mM), sodium sulphite (1.59 mM) and a fungicide (5–15 μg/ml amphotericin B). *Taylorella equigenitalis* can be cultivated on blood agar, but will grow better on 'chocolate' blood agar as described above. Some manufacturers produce a peptone agar base that supports the growth of *T. equigenitalis*. An important feature of all good *T. equigenitalis* media is the absence of fermentable carbohydrates. The fermentation of carbohydrates by other bacteria inhibits *T. equigenitalis* growth (Atherton, 1983; Fernie *et al.,* 1980). A third medium containing streptomycin sulphate (200 μg/ml) can be used to inhibit the growth of other bacteria that might obscure *T. equigenitalis* (Swerczek, 1978); however, the streptomycin-sensitive biotype will not be detected on this medium; and it should only be used in conjunction with medium without streptomycin. All culture media should be subjected to quality control and must support growth of a small inoculum of the suspect organism before their use on suspect samples. The reference strain of *T. equigenitalis* must also be cultured in parallel with the test samples to ensure that the culture conditions are optimal for isolation of this organism.

Plates must be incubated at 35–37°C in 5–10% (v/v) CO_2 in air or by use of a candle jar. At least 72 hours is normally required before colonies of *T. equigenitalis* become visible, after which time daily inspection is needed. Rarely, visual detection of colonies may take up to 14 days (Ward *et al.,* 1984). A standard incubation time of at least 7 days is advisable before certifying cultures negative for *T. equigenitalis*. Plates should be examined for contaminants after the first 24 hours' incubation. Colonies of *T. equigenitalis* may be up to 2–3 mm in diameter, smooth with an entire edge, glossy and yellowish grey. Laboratories should be aware that certain countries may require the prolonged incubation period as a standard procedure and should therefore ascertain the particular import requirements of those countries and/or indicate the incubation period on which their cultural findings are based. Growth of other bacteria, for example *Proteus mirabilis*, may be so extensive that the laboratory cannot issue a negative result. In this event, further swabs should be requested.

If a slow-growing organism is isolated that fits the description for cellular morphology and that is strongly oxidase and catalase positive, it can be tested for reactivity with *T.-equigenitalis*-specific antiserum.

1.2. Serotyping methods

A variety of serotyping tests has been developed to confirm that a culture is *T. equigenitalis,* ranging in complexity from slide agglutination to direct or indirect immunofluorescence. Each method has its advantages and disadvantages. The disadvantage of the slide agglutination test is that occasionally autoagglutination of isolates occurs. Culturing in bottled CO_2 in air, as opposed to in a candle jar, may reduce autoagglutination (Ter Laak & Wagenaars, 1990). Immunofluorescence may be of value in the identification of auto-agglutinating isolates; a validated indirect immunofluorescence test for the detection of *T. equigenitalis* in swabs from the reproductive tract of stallions and mares is commercially available.

Antiserum is produced by vaccinating rabbits with killed *T. equigenitalis*. A standard strain, such as NCTC 11184[1], should be used for immunisation. However, the most important consideration is the specificity of the antiserum produced. It should agglutinate *T. equigenitalis*, but fail to agglutinate other bacteria that might be cultured from horse urogenital membranes. In particular, it should not agglutinate any oxidase-positive and Gram-negative rods, such as *Mannheimia haemolytica*, *Actinobacillus equuli, Bordetella bronchiseptica* (to which *T. equigenitalis* is closely related, see Bleumink-Pluym *et al.* (1993), *Oligella urethralis* and *Pseudomonas aeruginosa*. *Taylorella asinigenitalis* has similar, though not identical, colonial appearance and cultural characteristics and gives identical biochemical test results to those used to confirm the identity of *T. equigenitalis*. There is even serological cross-reactivity between the two organisms. Differentiation of *T. asinigenitalis* from *T. equigenitalis* is possible using the PCR or 16S rDNA sequencing and biochemical reactivity (Baverud *et al.,* 2006; Breuil *et al.,* 2011; Duquesne *et al.,* 2007; Wakeley *et al.,* 2006). Monoclonal antibodies are available commercially that provide a highly specific means of identifying *T. equigenitalis*.

A latex agglutination kit is available commercially for the antigenic identification of *T. equigenitalis*. It is based on polyclonal antibodies produced using methods similar to those described above. This is widely used by routine testing laboratories for the confirmation of the identity of colonies growing on selective medium that give a biochemical reaction consistent with *T. equigenitalis*. As *T. equigenitalis* is antigenically relatively distinct, and small amounts of cross-reactive antibody are easily absorbed during production of the reagent, the test has proved to be highly specific and sensitive. It should be emphasised that it will not necessarily distinguish strains of *T. equigenitalis* from *T. asinigenitalis.*

1.3. Immunofluoresence methods (IFAT)

Antibody-based methods can also be used for the direct detection of *T. equigenitalis* in swabs taken from sampling sites. Both in-house and commercially available indirect immunofluorescence antibody tests (IFAT) have been described (Breuil *et al.,* 2010). Reported sensitivity and specificity are 93% and 100%, respectively (Breuil *et al.,* 2010). It is important that kits used have been fully validated in accordance with

1 Obtainable from the National Collection of Type Cultures, Colindale, London, UK: https://www.phe-culturecollections.org.uk/collections/nctc.aspx.

Chapter 1.1.6. *Validation of diagnostic assays for infectious diseases of terrestrial animals*. Kits should preferably be selected from those listed on the WOAH Register[2].

1.4. Matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry (MALDI-TOF MS)

Matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry (MALDI-TOF MS) has also been described as a reliable method for identification of *Taylorella* culture colonies and for the differentiation of *T equigenitalis* and *T. asinigenitalis* with appropriately expanded reference spectra, using direct spotting of 48-hour suspect colonies (Petry *et al.,* 2019).

1.5. Molecular methods

Molecular testing methods such as PCR and real-time PCR have been applied to the detection of *T. equigenitalis* both directly (using swabs taken from sampling sites) and indirectly (on cultures grown from swabs). To mitigate the possibility of false negative results, it is recommended that, whenever possible, several colonies suspected of being of the *Taylorella* genus are selected for confirmation by PCR. In Japan the field application of the PCR was evaluated for the eradication of contagious equine metritis. It was demonstrated that the PCR was more sensitive than culture for the detection of *T. equigenitalis* from genital swabs of horses (Anzai *et al.,* 1999; 2002). Laboratory ring trial data also indicate improved detection of *T. equigenitalis* by PCR compared with culture (Mawhinney, 2020; Petry *et al.*, 2018).

1.5.1. Real-time PCRs

A real-time PCR was developed for use directly on genital swabs and compared with culture (Wakeley *et al.,* 2006, Mawhinney *et al.*, 2019); it has subsequently been used for pre-breeding screening studies (Ousey *et al.,* 2009) and surveillance and eradication schemes in various countries (Belloy *et al.,* 2012; Jeoung *et al.,* 2018; May *et al.,* 2016). A second real-time PCR was developed and used successfully for the diagnosis of CEM (Nadin-Davies *et al.,* 2015). These two real-time PCRs have been shown to be highly specific and reproducible in different laboratories with slightly higher sensitivity than culture; they are the recommended PCR assays.

i) Real-time PCR 1 (Wakeley *et al.,* 2006)

This PCR has been used widely for detection of CEM. It has been shown to be robust to minor modifications of methodology and different DNA extraction techniques when validated in-house (May *et al.,* 2016; Petry *et al.,* 2018). This real-time PCR uses two separate PCRs, one that specifically amplifies a portion of the 16S rDNA of *T. equigenitalis* and *T. asinigenitalis,* and a second control PCR that amplifies a region of the 16S rDNA of many commensal bacteria found in the genital tract of horses and that acts to confirm inoculation of the swab and DNA extraction. Two probes, TEquiFAM and TAsiniHEX, corresponding to the two species of *Taylorella*, are labelled with different fluorophores to discriminate the amplicons and used in the *Taylorella*-specific PCR (Table 2a). In the control PCR, another probe labelled with a different fluorophore is used for each sample.

The preparation of DNA samples from isolated bacteria as well as genital swabs has been described by Wakeley *et al.* (2006). Isolated bacteria are suspended in 1.5 ml PBS (pH 7.4), transferred to Eppendorf tubes and heated at 95–100°C for 20 minutes in a heating block, after which the tubes are centrifuged at 18,000 ***g*** for 1 minute in a microcentrifuge. Each swab is immersed and manually agitated in 0.2 ml of 0.1 M PBS (pH 7.4) in a 1.5 ml Eppendorf tube for 5 seconds, a negative extraction control is also prepared using 0.2 ml of nuclease free water. The Eppendorf tube is then centrifuged at 18,000 ***g*** in a microcentrifuge for 30 seconds to pellet the bacteria. The supernatant is aspirated and disposed of and the pellet is re-suspended in 100 µl nuclease free water. The re-suspended pellet is heated at 95–100°C for 15 minutes in a heat block prior to centrifugation at 18,000 ***g*** for 1 minute in a

2 https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-5

microcentrifuge. This supernatant is the DNA extract or lysate. The lysate can be used immediately or stored at –20°C. Each reaction uses 2–5 µl of the lysate.

Alongside the samples, a *T. equigenitalis*, *T. asinigenitalis* and a no template control are tested on each plate. Cycling involves initial denaturation for 2 minutes at 94°C followed by 40 amplification cycles of denaturation for 5 seconds at 94°C, primer annealing for 10 seconds at 60°C and extension and data collection for 15 seconds at 72°C. A control real-time PCR for bacterial 16S rDNA (BactUniF, ACTA CGT-GCC-AGC-AGC-C; BactUniR, GGA-CTA-CCA-GGG-TAT-CTA-ATC-C) using the 16SrDNA ROX, TGT-TTG-CTC-CCC-ACG-CTT-TCG-CAC-BHQ2 probe, is run in parallel in a separate well to ensure the extraction and test process has worked. Minor variations from the published method, such as DNA extraction methods using proprietary kits, master mix compositions or internal controls, should be laboratory validated (in-house). The PCR is performed on a suitable real-time PCR machine. A positive test has a CT value ≤ 40 for the specific probe. A valid *Taylorella* PCR test requires the control 16S rDNA PCR to be positive for the sample in question.

ii) Real-time PCR 2 (Nadin-Davies *et al.*, 2015)

This real-time PCR has not been as widely used, but is also highly reproducible when used as described. The assay is composed of a single primer set with two probes specific for *T. equigenitalis* (EQUI-VIC; if available HEX can also be used) and *T. asinigenitalis* (ASINI-FAM) (Table 2a). A third probe, HA5-CY5, TCT-ACG-AGA-GAA-CCT-CTC-CGA-GCT-CAG-CT-BHQ2, is specific to a cloned sequence in a spiked-in plasmid and is used as an internal positive control (IPC). The spiked-in plasmid contains the TAYQ primer sequences flanking a 148 bp sequence from human adenovirus 5 corresponding to bases 28297 through 28407 of GenBank Accession number M73260.

To prepare the test sample, each swab is extracted and placed into 1.0 ml sterile nuclease free water in a microcentrifuge tube, vortexed for 5 seconds, and allowed to stand for 5 minutes. At the same time, a negative extraction control is also prepared using 1.0 ml of nuclease free water. After standing in the microcentrifuge tube, the swab is returned to its original vial. The sample is then centrifuged for 5 minutes at 17,949 ***g***, the supernatant aseptically removed and discarded, and the pellet re-suspended in 20 µl of 10 mM TE buffer pH 8.0. Suspensions are heated for 15 minutes at 100°C then cooled. Prior to testing, samples are centrifuged for 5–10 seconds to remove any liquid from the lid and tested immediately or stored at –20°C until tested. Stored samples are centrifuged for 30 seconds prior to testing to pellet debris. The supernatant is the DNA extract or lysate.

Each reaction is composed of 2 µl of supernatant added to 18 µl of mastermix containing 500 nM each of TayQF and TayQR, 250 nM of EQUI-VIC, and 125 nM of ASINI-FAM. Minor variations in the mastermix or concentrations must be laboratory validated (in-house). The described assay includes an IPC consisting of a manufactured sequence that is added to each well together with the sample DNA and negative extraction control and specific probe. The IPC is detected by a corresponding probe included in the master mix. Failure to amplify this control indicates inhibition of the PCR. Each run includes a standard curve (five dilution series) of DNA template controls for *T. equigenitalis* together with IPC to demonstrate PCR efficiency. Individual wells each containing *T. asinigenitalis* PCR control and IPC, *Oligella urethralis* and IPC, IPC alone, and a no template control are also included on every plate, to demonstrate PCR specificity and fitness for purpose. Samples are tested in duplicate. PCR cycle conditions are as follows: 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 55°C for 1 minute. A CT value ≤ 40 for the specific probe is considered positive.

Alternative laboratory validated variations of controls have been used, however every PCR plate should include a no template negative extraction control, *T. equigenitalis* and *T. asinigenitalis* positive controls and an IPC control, as a minimum. A control test to show the presence of bacteria or bacterial DNA to confirm that the swabs have been inoculated is also advisable.

Table 2. Primer sequences for use in recommended real-time PCR tests

Primer 1 (forward)		Primer 2 (reverse)		Probe (only for real-time PCRs)		Reference
Name	**Sequence (5' → 3')**	**Name**	**Sequence (5' → 3')**	**Name**	**Sequence (5' → 3')**	
Tay377 for	CCG-CGT-GTG-CGA-TTG-A	Tay488 rev	TTT-GCC-GGT-GCT-TAT-TCT-TCA	Tequi FAM-probe Tasini HEX-probe	6FAM-AAA-GGT-TTG-TGT-TAA-TAC-CAT-GGA-CTG-CTG-ACG-G-BHQ1 HEX-AAA-GTT-TTA-GGA-TAA-TAC-CCT-AGG-ATG-CTG-ACG-G-BHQ1	Wakeley *et al.*, 2006
TAYQF	CGC-GTG-TGC-GAT-TGA-A	TAYQR	GCC-GGT-GCT-TAT-TCT-TCA	EQUI-VIC (or HEX)	(HEX) VIC-AGG-TTT-GTG-TTA-ATA-CCA-TGG-ACT-GCT-GAC-QSY7 (BHQ1 if using HEX)	Nadin-Davies *et al*, 2015
				ASINI-FAM	FAM-AGT-TTT-AGG-ATA-ATA-CCC-TAG-GAT-GCT-GAC-GGT-BHQ1	

If using HEX in these two real-time PCRs, the *Taylorella* species that use the HEX and FAM labels are opposite (Table 2).

Maintaining the viability of the organism is not necessary for the recommended real-time PCRs, so swabs may be transported at ambient temperature. For PCR testing it is not necessary to use transport medium to convey swabs to the laboratory. Swabs in plain sleeves without transport medium can be used. It is recommended that swabs for PCR should be tested no more than 7 days after sampling.

The direct detection of *T. equigenitalis* by real-time PCR has several advantages over isolation of the bacteria by culture. First, PCR is less vulnerable to contaminating flora, which reduces the number of false-negative results. Secondly, the turnaround time of the PCR is much shorter than the minimum 7-day culture time with isolation. Thirdly, as only DNA is detected rather than viable organisms, the need for rapid transport of specimens to the laboratory is reduced. Fourthly these PCRs also differentiate *T. equigenitalis* from *T. asinigenitalis*. A strict PCR regime to avoid DNA cross contamination should be deployed in diagnostic laboratories.

1.5.2. Other PCRs

Other conventional and real-time PCRs have been developed and commercial PCR kits are available for the detection of *T. equigenitalis*. Before use in a diagnostic laboratory these must be fully validated to WOAH Standards (Chapter 1.1.6) as fit for use for defined purposes.

1.5.3. Sequencing-based methods

Sequencing-based methods, such as sequencing of the 16s rRNA gene, have been used to confirm the identification of *Taylorella* spp. (Erdman *et al*., 2011). Advances in whole genome sequencing have led to additional identification and typing tools as well as improved molecular characterisation of *Taylorella* spp. The multi-locus sequence typing (MLST) scheme has been used for typing of *Taylorella* spp. and defining clonal complexes that further describe genetic relationships between the sequence types (Duquesne *et al.,* 2013). Recent studies have also demonstrated the use of MLST, single nucleotide polymorphism analysis, and other genomic methods to characterise strains, evaluate detailed genetic relationships between isolates, and generate phylogenetic trees, which may prove useful in understanding transmission patterns and for epidemiological tracing (Duquesne *et al*., 2020; Hicks *et al.,* 2018,). New research has explored targeted amplification from semen and swabs for culture independent sequence typing using MLST (May *et al.,* 2019).

1.6. Use of agent identity tests

1.6.1. Testing post-treatment

After a horse has been treated to eliminate *T. equigenitalis*, either culture or PCR may be used to determine the continued presence of infection or carrier state. Detection by culture can be inhibited by carry-over of the antimicrobial agent in the swab onto the culture plate, sub-lethal damage to the organism preventing its growth *in vitro* or due to overgrowth by competing organisms. PCR detects the DNA of both viable and non-viable organism. If treatment does not involve adequate washing of debris from the anatomical sites used for swabbing, then DNA from the dead organism may persist. For these reasons it is common to test by either method at least 21 days after treatment. In most cases adequate treatment will result in negative tests using both methods. However if discordant results between culture and PCR are found, it is advisable to assume that infection may still be present and consider re-treatment to eliminate the organism.

1.6.2. Semen testing

Table 1 refers to the tests validated for use with genital swabs rather than semen. There are little available data on the relative diagnostic sensitivity of testing semen compared with genital swabs from the stallion (Al-Kass *et al.,* 2019; Erdman *et al.,* 2011). Semen testing is therefore usually used as an adjunct to rather than a substitute for swabbing the horse. There are limited data on the use of either culture or PCR in semen or the viability of the organism in transit. Published data indicate the organism can remain viable after freezing semen, and it can be readily recovered from fresh semen by culture. Its growth in culture is often completely inhibited by antibiotics in semen extender (Klein *et al.,* 2012; Olivieri *et al.,* 2011), but this is not always the case, and semen may remain infective in the presence of extender (Delerue *et al.,* 2019). Further data from a semen spiking study indicated that culture can be very sensitive if the semen is pure and fresh, but extended semen containing antibiotics showed a rapidly reduced detection level, and refrigerated storage of semen samples over many days resulted in poor detection of *T. equigenitalis* by culture for both pure and extended semen due to overgrowth with commensal organisms. A real-time PCR (Wakeley *et al.,* 2006) with a DNA extraction step used on the same samples was reasonably sensitive and not diminished by extender nor by storage. There is an inherent limit on the analytical sensitivity of PCR due to the quantity of semen that is used in the DNA extraction and reaction steps, but it is not known if this limits the diagnostic sensitivity in carrier horses as there are little data on expected viable cell counts of *T. equigenitalis* in semen from infected horses. A study using real-time PCR on frozen extended semen samples from a naturally infected horse indicated that testing multiple aliquots from each ejaculate may enhance the sensitivity in detecting whether the donor horse was infected (Schulman *et al.,* 2016). The sensitivity of culture from frozen extended semen has not been widely reported but the limitations of culture for fresh extended semen at least apply to frozen samples. Aliquots of semen and swabs dipped in semen have both been used as samples for culture and PCR, and the same conditions for transit are applied as for horse swab samples.

2. Serological tests

No serological test described to date will, by itself, reliably detect infection for diagnosis and control. However, the complement fixation test has been used successfully as an adjunct to culture for *T. equigenitalis* in screening mares between 21 and 45 days after being bred to a suspect carrier stallion.

C. REQUIREMENTS FOR VACCINES

Effective vaccines that protect against contagious equine metritis or prevent colonisation by *T. equigenitalis* are currently unavailable.

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NB: There are WOAH Reference Laboratories for contagious equine metritis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for contagious equine metritis

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.6.3.

DOURINE IN HORSES (*TRYPANOSOMA EQUIPERDUM* INFECTION)

SUMMARY

Dourine is a chronic or acute contagious disease of breeding equids that is transmitted directly from animal to animal during coitus. The causal organism is Trypanosoma (Trypanozoon) equiperdum. Trypanosoma equiperdum *is primarily a tissue parasite that is rarely detected in the blood. There is no known natural reservoir of the parasite other than infected equids. It is present in the genital secretions of both infected males and females. The incubation period, severity, and duration of the disease vary considerably; it is often fatal, however spontaneous recoveries do occur as do latent carriers and subclinical infections. Donkeys and mules are more resistant than horses and may remain inapparent carriers. Infection is not always transmitted by an infected animal during every copulation. Although adaptation to other hosts is not always possible, dogs, rabbits, rats and mice can be infected experimentally and be used to isolate and maintain strains of the parasite indefinitely.* Trypanosoma equiperdum *strains are best stored in liquid nitrogen.*

The clinical signs are marked by periodic exacerbation and relapse, ending in death, sometimes after paraplegia, or, possibly, recovery. Moderate fever, local oedema of the genitalia and mammary glands, cutaneous eruptions, incoordination, facial and lip paralysis, ocular lesions, anaemia, and emaciation may all be observed. Oedematous cutaneous plaques, 5–8 cm in diameter and 1 cm thick, are still considered as pathognomonic, although they are also occasionally found in equids infected with T. evansi.

Detection of the agent: *Definitive diagnosis depends on the recognition of clinical signs, and identification of the parasite and evidence of sexual transmission. As this is rarely possible, diagnosis is usually based on clinical signs, serological or molecular evidence and epizootiological context.*

Serological tests: *Humoral antibodies are present in infected animals with or without clinical signs. The complement fixation test (CFT) is used to confirm infection in clinical cases or in latent carriers. Non-infected animals, especially donkeys, often yield unclear results. The indirect fluorescent antibody test can be used to confirm infection or resolve inconclusive CFT results. Enzyme-linked immunosorbent and immunochromatographic assays are also used.*

Molecular tests: *Genetic markers that allow unequivocal differentiation of* T. equiperdum *from* T. evansi *within the subgenus* Trypanozoon *are missing.*

Requirements for vaccines: *There are no vaccines available for this parasite. The only effective control is through the slaughter of infected animals. Good hygiene is essential during assisted mating because infection may be transmitted through contaminated fomites.*

A. INTRODUCTION

Dourine is a chronic or acute contagious disease of equids that is primarily transmitted directly from animal to animal during coitus. Other routes of transmission, e.g. vertical or congenital, may be possible but have not been documented. The causal organism is *Trypanosoma equiperdum* (Doflein, 1901).

Trypanosoma equiperdum is a flagellated extracellular protozoan parasite belonging to the order Kinetoplastida, the family Trypanosomatidae and the subgenus *Trypanozoon*. Other species within this subgenus are *T. brucei* causing nagana (Chapter 3.4.14 *Nagana: infections with salivarian trypanosomoses* [*excluding* Trypanosoma evansi *and* T. equiperdum]) and *T. evansi* causing surra (Chapter 3.1.21 *Surra in all species* [Trypanosoma evansi *infection*]).

Both species are able to cause some similar clinical signs to dourine in chronic infections such as ventral oedema, emaciations, anaemia and neurological signs (Büscher *et al*., 2019). However, dourine initially often presents with genital lesions that may progress to neurological and chronic disease over a period of weeks to month. Morphologically and genetically these three species are very similar.

Dourine is also known under other names: mal de coït, syphilis du cheval, el dourin, morbo coitale maligno, Beschälseuche, slapsiekte, sluchnaya bolyezni, and covering disease (Barner, 1963; Hoare, 1972). So far, no human case has been reported.

Although the disease has been known since ancient times, its nature was established only in 1896 when Rouget discovered trypanosomes in an infected Algerian horse (Rouget, 1896). *Trypanosoma equiperdum* is primarily a tissue parasite that is rarely detected in the blood. There is no known natural reservoir of the parasite other than infected equids.

Dourine is transmitted during copulation, more commonly from stallion to mare, but also from mare to stallion, due to the presence of the parasite in the seminal fluid and mucous exudate of the penis and sheath of the infected male, and in the vaginal mucus of the infected female. Initially, parasites are found free on the surface of the mucosa or between the epithelial cells of a newly infected animal. Invasion of the tissues takes place, and oedematous patches appear in the genital tract. Parasites then may pass into the blood, where they are carried to other parts of the body such as the central nervous system. In typical cases, this metastatic invasion gives rise to characteristic cutaneous plaques.

The incubation period, severity and duration of the disease vary considerably. In South Africa, the disease is typically chronic, usually mild, and may persist from 6 months to 2 years (Henning, 1955). In Ethiopia and Mongolia, dourine appears to be endemic rather than epidemic (Davaasuren *et al*., 2017; Hagos *et al*., 2010). In other areas, such as Northern Africa and South America, the disease tends to be more acute, often lasting only 1–2 months or, exceptionally, 1 week. Although dourine is a fatal disease with an average mortality of 50% (especially in stallions), spontaneous recovery can occur. Subclinical infections are recognised. Donkeys and mules are more resistant than horses. In donkeys, the disease passes often unperceived whereas their semen and vaginal secretions contain infective trypanosomes.

As trypanosomes are not continually present in the genital tract throughout the course of the disease, transmission of the infection does not necessarily take place during every copulation involving an infected animal. Transmission of infection from mare to foal can occur via the mucosa, such as the conjunctiva (Hoare, 1972). Trypanosomes were found in the mammary gland of a non-lactating mare (Parkin, 1948) and in skin samples after examination by immunohistochemistry (Pascucci *et al*., 2013; Scacchia *et al*., 2011). Not all *T. equiperdum* strains can be readily adapted to laboratory animals such as rodents or rabbits. It has been reported that adaptation to rats is possible after isolation in rabbits by intratestis inoculation (Schneider & Buffard, 1900; Soldini, 1939). Direct *in-vitro* isolation from the urethral tract has also been achieved (Suganuma *et al*., 2016). Rodent- and *in-vitro*-adapted strains can be maintained indefinitely and cryopreserved. Antigens for serological tests are commonly produced from infected laboratory rodents or *in-vitro* propagated parasites.

Dourine is marked by stages of exacerbation, tolerance or relapse, which vary in duration and which may occur once or several times before death or recovery. The clinical signs most frequently noted are: pyrexia, tumefaction and local oedema of the genitalia and mammary glands, oedematous cutaneous eruptions, knuckling of the joints, incoordination, facial and lip paralysis, ocular lesions, anaemia, and emaciation. A frequent but not constant sign is the formation of oedematous plaques consisting of raised lesions in the skin, up to 5–8 cm in diameter and 1 cm thick. The plaques usually appear over the ribs, although they may occur anywhere on the body, and usually persist for between 3 and 7 days. Oedematous plaques have long been considered pathognomonic, but have also been described in *T. evansi* infections (Van den Bossche *et al*., 2009).

Generally, the oedema disappears and returns at irregular intervals. During each recess, an increasing extent of permanently thickened and indurated tissue can be seen. The vaginal mucosa may show raised and thickened semi-transparent patches. Folds of swollen membrane may protrude through the vulva. It is common to find oedema of the mammary glands and adjacent tissues. Depigmentation of the genital area, perineum, and udder may occur. In the stallion, the first clinical sign is a variable swelling involving the glans, penis and prepuce. The oedema extends posteriorly to the scrotum, inguinal lymph nodes, and perineum, and anteriorly along the inferior abdomen. In stallions of heavy breeds, the oedema may extend over the whole floor of the abdomen.

Pyrexia is intermittent; nervous signs include incoordination, mainly of the hind limbs, lips, nostrils, ears, and throat. Facial paralysis is usually unilateral. In fatal cases, the disease is usually slow and progressive, with increasing anaemia and emaciation, although the appetite remains good almost throughout.

At post-mortem examination, gelatinous exudates are present under the skin. In the stallion, the scrotum, sheath, and testicular tunica are thickened and infiltrated. In some cases, the testes are embedded in a tough mass of sclerotic tissue and may be unrecognisable. In the mare, the vulva, vaginal mucosa, uterus, bladder, and mammary glands may be thickened with gelatinous infiltration. The lymph nodes, particularly in the abdominal cavity, are hypertrophied, softened and, in some cases, haemorrhagic. The spinal cord of animals with paraplegia is often soft, pulpy and discoloured, particularly in the lumbar and sacral regions.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of dourine and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Microscopic observation	–	+	+	+++	–	–
PCR/ real-time PCR	–	+	+	+++	+	–
Detection of immune response						
CFT	++	+++	+++	+++	+++	–
IFAT	++	+	++	+	++	–
ELISA	+++	+	+++	+	+++	–
ICT	+	+	+	+	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; CFT = complement fixation test; IFAT = indirect fluorescent antibody test;
ELISA = enzyme-linked immunosorbent assay; ICT = immunochromatographic test.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended

1.1. Overview of parasitological methods

A confirmed diagnosis depends on the recognition of clinical signs, demonstration of the parasite and evidence of infection via coitus. This is rarely possible because: (a) although the clinical signs and gross lesions in the developed disease may be pathognomonic, they cannot always be identified with certainty, especially in the early stages or in latent cases; they can be confused with other conditions, such as coital exanthema, or infections with other trypanosomes such as *T. evansi* (Büscher *et al*., 2019); (b) the trypanosomes are usually sparsely present and are extremely difficult to find, even in oedematous areas; and (c) knowledge about the transmission route is often lacking.

Recently, new *T. equiperdum* strains have been isolated in Ethiopia (Dodola), Italy (ICT 2011), Venezuela (TeAp-N/D1) and Mongolia (IVM-t1), although these isolates still have to be further characterised (Hagos *et al*., 2010; Pascucci *et al.,* 2013; Sánchez *et al*., 2015a, 2015b; Suganuma *et al*., 2016). In practice, diagnosis is based on clinical evidence supported by the mode of transmission, serology and histopathology.

In infected animals, trypanosomes are present, in low numbers only, in lymph and oedematous fluids of the external genitalia, in the urethral and vaginal mucus (Parkin, 1948) and in exudates of plaques and mammary glands (Pascucci *et al*., 2013; Scacchia *et al*., 2011). They are usually undetectable in the blood, but may be found in the urethral or vaginal mucus collected from preputial or vaginal washings or scrapings 4–5 days after infection. The skin of the area over the plaque should be washed, shaved and dried, and the fluid contents aspirated by syringe. Blood vessels should be avoided. The fresh aspirate is examined microscopically for motile trypanosomes. These are present for a few days only, so lesions should be examined at regular intervals. As the parasite is rarely found in thick blood films, the use of concentration techniques is recommended, such as capillary tube centrifugation, or mini-anion exchange centrifugation technique (Lanham & Godfrey, 1970; Woo 1970).

Trypanosoma equiperdum is relatively easy to distinguish morphologically from *T. congolense* and *T. vivax,* both of which can infect horses. However, in countries where *T. evansi* or *T. brucei* occur, it is difficult to distinguish *T. equiperdum* microscopically (morphology, motility) from these other members of the subgenus *Trypanozoon*. In particular, *T. equiperdum* and *T. evansi* cannot be differentiated on the basis of morphological criteria. Both are monomorphic, slender trypomastigotes with a free flagellum, although stumpy forms have occasionally been reported. For typical strains the parasites range in length from 15.6 to 31.3 μm.

1.2. Detection of trypanosomal DNA and differential diagnosis

Kinetoplast DNA in the mitochondrion is the most remarkable characteristic in the order Kinetoplastida. In field situations, akinetoplastic strains of *T. evansi* (no kinetoplast visible when stained with Giemsa) were found in infected animals, but this situation was not observed in *T. equiperdum*. The suggestion by Li *et al.* (2007) that *T. equiperdum* can be distinguished from *T. evansi* by the apparent presence of intact maxi-circles in *T. equiperdum* remains controversial. On the other hand, the absence of the RoTat 1.2 variant surface glycoprotein (VSG) could be a molecular marker to differentiate *T. equiperdum* from *T. evansi* type A infections in horses (Claes *et al*., 2003). Although no *T. equiperdum*-specific polymerase chain reaction (PCR) method is available, subgenus *Trypanozoon*-specific PCR and real-time PCR can be used for detection of *T. equiperdum* DNA (Benfodil *et al.,* 2020; Masiga *et al*., 1992) (see also Chapter 3.1.21 *Surra in all species* [Trypanosoma evansi *infection*]). A highly sensitive real-time PCR for the *Trypanozoon* subgenus has been applied on tissues and fluid samples from a naturally dourine-infected horse, enabling the detection of low numbers of parasites (Becker *et al*., 2004; Pascucci *et al*., 2013).

Table 2. Primers and probes described for Trypanozoon-specific conventional and real-time PCR that can be used for detection of* T. equiperdum *DNA

Target	Primers/probe sequences (5' → 3')	Amplicon length	Reference
177 bp repeats	TBR1: GAA-TAT-TAA-ACA-ATG-CGC-AG	164 bp	Masiga *et al*,. 1992
	TBR2: CCA-TTT-ATT-AGC-TTT-GTT-GC		
177 bp repeats	Tb177F: AAC-AAT-GCG-CAG-TTA-ACG-CTA-T	134 bp	Becker *et al*., 2004; Pascucci *et al*., 2013
	Tb177B: ACA-TTA-AAC-ACT-AAA-GAA-CAG-CGT-TG		
18S rDNA	M18SF: CGT-AGT-TGA-ACT-GTG-GGC-CAC-GT	150 bp	Benfodil *et al*., 2020
	M18SR: ATG-CAT-GAC-ATG-CGT-GAA-AGT-GAG		
	M18SP: TCG-GAC-GTG-TTT-TGA-CCC-ACG-C-MGB-VIC		

2. Serological tests

Humoral antibodies are present in infected animals, whether they display clinical signs or not. The complement fixation test (CFT) is used to confirm clinical evidence and to detect latent infections. Uninfected equids, particularly donkeys and mules, often give inconsistent or nonspecific reactions because of the anticomplementary effects of their sera. In the case of anticomplementary sera, or for clinical diagnostic purposes, the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISAs) may provide additional information. There

are no internationally adopted protocols. Cross-reactions are possible due to the presence of other trypanosomes in some countries, for example, *T. cruzi, T. vivax* and *T. evansi*. *Trypanosoma equiperdum* is closely related to other *Trypanozoon* trypanosomes, including *T. brucei* and *T. evansi*. Members of this subgenus all share conserved cytoskeletal elements that provoke a strong and cross-reactive serological response. All diagnostic antigens and antisera currently available for use in serodiagnostic testing contain these conserved elements or antibodies to them, and thus none of the serological procedures described below is specific for dourine. Therefore, the diagnosis of dourine must include history, clinical, and pathological findings as well as serology to establish the definite confirmed case of the disease (Calistri *et al*., 2013). Significant improvements in dourine serodiagnosis will require development of more *T. equiperdum*-specific subunit antigens and antibodies to them.

2.1. Complement fixation test

Standard or microplate techniques may be used. Guinea-pig serum (available commercially) is used as a source of complement. Other reagents are sheep red blood cells (RBCs) washed in veronal buffer, and rabbit haemolytic serum (i.e. rabbit anti-sheep RBC) (commercial) as well as known negative and positive control sera.

2.1.1. Antigen production

Because of lack of solid serological or molecular markers to differentiate *T. equiperdum* from the other *Trypanozoon* taxa (Büscher *et al.,* 2019; Cuypers *et al*., 2017), it is important to indicate which *T. equiperdum* strain is used for any antigen preparation. Strains that easily grow in rodents are *T. equiperdum* OVI, BoTat, Dodola and TeAp-N/D1. Strains that are adapted to *in-vitro* culture are *T. equiperdum* OVI and IVM-t1. It should be kept in mind that crude antigen preparations such as described below, are not dourine-specific and will cross-react with sera from horses infected with *T. brucei* and *T. evansi*.

2.1.1.1 Antigen preparation from *in-vitro* propagated parasites

The procedure described below is based on Bassarak *et al.* (2016) with some modifications. *Trypanosoma equiperdum* OVI (ITMAS 241199C, purchased from Institute of Tropical Medicine, Antwerp, in 2008) was adapted to *in-vitro* culture conditions. Culture-adapted trypanosome stocks in liquid nitrogen are available on request[1].

i) Reagents and solutions to prepare medium

Substance	Identification System	Number
MEM powder for 1 litre with Earle's salts & L-glutamine, without $NaHCO_3$ (Sigma-Aldrich M0268)		
2-Mercapto-ethanol	CAS	60-24-2
Adenosine	CAS	58-61-7
Antibiotic-antimycotic solution (100×)		
Bathocuproine disulfonate	CAS	52698-84-7
Cysteine	CAS	52-90-4
D(+)-Glucose × 1 H_2O	CAS	50-99-7
Glycerol	CAS	51-81-5
HEPES	CAS	7365-45-9
Isopropanol	CAS	67-63-0
Hypoxanthine	CAS	68-94-0
New-born calf serum, heat-inactivated (NCS)		
Potassium chloride	CAS	7447-40-7
Magnesium sulfate × 7 H_2O	CAS	10034-99-8
MEM non-essential (100×)		

1 From the WOAH Reference Laboratory for dourine: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

Substance	Identification System	Number
Sodium pyruvate	CAS	113-24-6
Sodium chloride	CAS	7647-14-5
Sodium hydrogene carbonate	CAS	144-55-8
$Na_2HPO_3 \times 12\ H_2O$	CAS	10039-32-4
$NaH_2PO_3 \times 2\ H_2O$	CAS	10049-21-5
Ornithine/HCl	CAS	3184-13-2
Thymidine	CAS	50-89-5
Hypoxanthine 100× stock solution	225 ml H_2O, 340 mg hypoxanthine, 25 ml 1 M NaOH. Stir in water bath for 20 min at 55°C. Filter through 0.22 µm filter; store at 4°C.	
Cysteine/bathocuproine-disulfonate 100× stock solution	225 ml H_2O, 705 mg bathocuproine disulfonate, 4550 mg cysteine, 25 ml 2 M HCl. Stir for 20 min at 55°C. Filter through 0.22 µm filter. Store at 4°C.	

ii) Preparation of the culture medium with 15% NCS (example for 3 litre)

Using a fume hood, add 47 µl 2-mercapto-ethanol to 10 ml H_2O. In a 5 litre beaker with 2430 ml H_2O, add: 3 MEM powder packs, 6.6 g $NaHCO_3$, 17.85 g HEPES, 3 g glucose, 0.66 g sodium pyruvate, 0.15 g ornithine, 0.012 g thymidine, 0.039 g adenosine, 30 ml MEM non-ess 100× stock solution, 15 ml antibiotic-antimycotic 100× stock solution, and 10 ml of the 2-mercapto-ethanol dilution. Adjust to pH 7.4 with NaOH and HCl and stir for 10 minutes. Add 30 ml hypoxanthine 100× stock solution and 30 ml cysteine/bathocuproine-disulfonate 100× stock solution. Adjust to pH 7.4 with NaOH and HCl and add H_2O up to 2550 ml.

In three 1 litre flasks, dispense 150 ml NCS. Fill the flasks with 850 ml culture medium filtered over a 0.22 µm filter. Mix gently and store at 4°C. The culture medium is stable for at least 8 weeks.

iii) Preparation of the trypanosome dilution buffer (TDB), pH 7.7

Dissolve 3.23 g $Na_2HPO_4 \times 12\ H_2O$, 0.14 g $NaH_2PO_4 \times H_2O$, 0.19 g KCl, 2.34 g NaCl, 0.13 g $MgSO_4 \times 7\ H_2O$, 1.80 g D(+)-Glucose x H_2O in 450 ml H_2O. Adjust to pH 7.7 with NaOH & HCl. Adjust to 500 ml with H_2O. Filter through 0.22 µm filter. Store at 4°C (stable for at least at 8 weeks).

iv) Preparation of a 5% PVP (polyvinylpyrrolidone), 0.01% merthiolate-NaCl solution

Prepare a 1% merthiolate-NaCl solution by dissolving 4.25 mg NaCl and 5 mg sodium ethylmercurithiosalicylate in 0.5 ml H_2O. In a 50 ml beaker, dissolve 425 mg NaCl and 2.5 g PVP 25 in 40 ml H_2O. Add the 0.5 ml 1% merthiolate-NaCl solution and adjust to 50 ml with H_2O. Filter through 0.22 µm filter. Store at 4°C.

v) Prepare a trypanosome culture with 1×10^5 trypanosomes/ml respecting a surface-volume ratio of 3.25 cm^2 per ml, e.g. in three-level T-500 culture flasks filled with 154 ml culture medium, and incubate at 37°C in a CO_2 incubator.

vi) Harvest the trypanosomes at concentrations of $1.5–2 \times 10^6$/ml once or twice a week in batches of 400 ml cell culture medium. Keep trypanosomes on ice during the whole process. Trypanosome containing medium is filled in a set of 50 ml tubes and centrifuged (10 minutes, 4°C, 1300 ***g***). Pellets of 8 tubes are resuspended carefully with a small volume of ice-cold TDB and transferred to one new, sterile 50 ml tube. The trypanosomes are washed twice with TDB (10 minutes, 4°C, 1300 ***g***) and the supernatant is removed completely. Pellets are stored at –20°C. It is advisable to confirm sterility of preparations using blood agar plates.

vii) The total number of cells of all pellets is determined. Prepare a fresh PVP-merthiolate solution (1 ml per 1×10^9 trypanosomes). Thaw frozen pellets on ice, resuspend the pellets with 50% of the calculated volume of ice-cold 5% PVP in 0.01% merthiolate-NaCl solution and pool them in a new sterile 50 ml tube. Fill ice-cold 5% PVP in 0.01% merthiolate-NaCl

solution to 100% of the calculated volume. Fill 200 µl antigen solution each in sterile beaded rim bottles (mix thoroughly several times during process), and place them in the biosafety transport box on ice for transport to the –80°C freezer. The lyophilisation apparatus is started and after 90 minutes the frozen antigen containing bottles are placed into the lyophilisation apparatus. Lyophilisation is performed overnight. The next day, lyophilisation is completed and immediately the cap is closed tightly and the antigen stored at –20°C. Alternatively, the antigen solution can be stored in small volumes at –80°C. The working dilution of antigen is standardised by titration against a 1/5 dilution of a standard low-titre antiserum.

2.1.1.2 Antigen preparation from *in-vivo* propagated parasites

Taking into account the 3Rs principle, the *in-vivo* propagation procedure described below should be considered only if *in-vitro* propagation cannot be implemented.

i) Specific-pathogen free rats are anaesthetised with either CO_2 or isoflurane and inoculated with *T. equiperdum* cryopreserved stock after a blood smear has indicated viability. Adult rats receive 0.3–1.0 ml of rapidly thawed frozen stabilate intraperitoneally. At maximum parasitaemia, these starter rats are anaesthetised and a cardiac puncture is used to collect blood into an anticoagulant, such as heparin or Alsever's filled syringes, which will serve as a stock culture for the inoculation of additional rats.

ii) Twenty to 100 large rats are anaesthetised immediately prior to being inoculated intraperitoneally with 0.3–1.0 ml of this stock culture. All rats need to have a heavy infection concurrently. If necessary, the dose is adjusted and additional rats are inoculated to reach maximum parasitaemia at the desired time of 72–96 hours. Rats usually die within 3–5 days; prior to this, blood is taken daily from the tail for thin wet blood films and examined microscopically. When parasitaemia is maximal, the rats are euthanised via CO_2 and blood is collected via cardiac puncture for separation of the trypanosomes by one of the two protocols below: differential centrifugation or anion exchange chromatography.

iii) For differential centrifugation, infected rat blood is collected in Alsever's or acid–citrate–dextrose (ACD) saline solution. The bulk blood is thoroughly mixed, and then 45 ml volumes are aliquoted into 50 ml conical centrifuge tubes. The tubes are centrifuged at 2500 ***g*** for 10 minutes at 4°C. The contents of the tube should separate out into three parts (from top to bottom): serum/anticoagulant, trypanosomes/white blood cells (WBC), and red blood cells (RBCs).

iv) The serum/anticoagulant layer is removed and discarded. The trypanosomes/WBC layer is removed from each tube and placed in a vessel large enough to "re-bulk" all trypanosomes together. In addition, approximately 1–3 ml of the RBCs below the trypanosome/WBC layer will contain trypanosomes. Several ml of RBCs below the trypanosome/WBC layer should be removed and added to the bulk.

v) To wash trypanosomes to remove RBCs, the same anticoagulant that was used in the bulk blood collection should be used, and several washing steps should be performed. Add ~25 ml bulked trypanosome layer to 50 ml conical tubes, and top up each tube to 45 ml total volume of anticoagulant. The tubes are centrifuged at 2500 ***g*** for 10 minutes at 4°C. The trypanosome-containing layer is again removed and saved. Repeat the above washing steps until the RBC have been mostly removed from the trypanosome layer.

vi) To dilute the purified trypanosomes for antigen production, the purified trypanosome layer is mixed with lyophilisation media (5% PVP). Generally, the starting dilution factor is 1:5 trypanosome layer to lyophilisation media. Before use in CFTs, the antigen must be dispersed to a fine suspension with a hand-held or motorised ground glass homogeniser chilled in ice (Watson, 1920). This antigen may be divided into aliquots, frozen and lyophilised.

vii) For anion exchange chromatography, blood is taken on heparin and loaded on a DEAE (diethylaminoethyl) cellulose gel equilibrated with a solution of phosphate buffered saline (PBS) containing glucose, pH 8.0 (Lanham & Godfrey, 1970). Blood cells are retained on the gel and the eluted trypanosomes are centrifuged at 1000–1500 ***g*** for 15 minutes. One volume of sedimented trypanosomes is resuspended in ice-cold phosphate buffer (0.01 M, pH 8.0) and lysed by hypotonic shock for 15 minutes. Thereafter, the suspension is

centrifuged at 42,000 ***g*** for 1 hour and the supernatant is collected and filtered through a 0.22 μm filter. The cleared supernatant contains the hydro-soluble fraction of the trypanosomes. The protein content can be determined by UV spectrophotometry or similar method and this antigen preparation can be stored at –80°C in small volumes. The antigen is standardised by titration against a 1/5 dilution of a standard low-titre antiserum.

2.1.2. Sera

Positive and negative sera should be inactivated at 58°C for 30 minutes before being used in the tests. Mule and donkey sera are normally inactivated at 62°C for 30 minutes. The USDA complement fixation protocol calls for inactivation of sera for 35 minutes (United States Department of Agriculture [USDA], 2016). Dilutions of sera that are positive in the screening test are titrated against two units of antigen. Test sera are screened at a dilution of 1/5. Sera showing more than 50% complement fixation at this dilution are usually deemed to be positive.

2.1.3. Anticomplementary sera

If the anticomplementary control shows only a trace, this may be ignored. For all other anticomplementary sera, the activity can be titrated. A duplicate series of dilutions is made and the sample is retested using *T. equiperdum* antigen in the first row and veronal buffer only in the second. The second row gives the titre of the anticomplementary reaction. Provided the first row shows an end-point that is at least three dilutions greater than the second, the anticomplementary effect may be ignored and the sample reported as positive. If the results are any closer, a fresh sample of serum must be requested.

2.1.4. Buffers and reagents

i) Trypanosome dilution buffer (TDB), pH 7.7: 18 mM Na_2HPO_4 12 H_2O, 2 mM NaH_2PO_4 H_2O, 5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4$ 7 H_2O, and 18 mM glucose.

ii) 5% PVP in 1/10,000 merthiolate-NaCl solution: 145 mM NaCl, 1% 25 mM merthiolate-145 mM NaCl solution and 5% PVP.

0.15 M veronal buffered saline, pH 7.4, or any alternative commercially available CFT buffer, is used for diluting reagents and for washing sheep RBCs. Antigen is pretested by titration, and two units are used in the test. Guinea-pig complement (C) is tested for its haemolytic activity, and diluted to provide two units for the test. Sheep RBCs in Alsever's or ACD saline solution are washed three times. A 3% solution is used for the haemolytic system. The USDA protocol calls for 2% solution in the microtitration procedure (USDA, 2016). Titrated rabbit-anti-sheep RBCs – the rabbit haemolytic serum – is taken at double the concentration of its haemolytic titre (two units). All test sera, including positive and negative control sera, are inactivated at a 1/5 dilution before testing.

2.1.5. Primary dilutions

i) Test, positive and negative control sera are diluted 1 in 5 with veronal buffer.

ii) The solutions are incubated in a water bath at 58°C for 30 minutes to inactivate complement and destroy anticomplementary factors. Donkey and mule sera should be inactivated at 63°C for 30 minutes.

2.1.6. Screening test procedure

i) 25 μl of inactivated test serum is placed in each of three wells.

ii) 25 μl of inactivated control serum is placed in each of three wells.

iii) 25 μl of *T. equiperdum* antigen diluted to contain 2 units is placed in the first well only for each serum.

iv) 25 μl of complement diluted to contain 2 units is added to the first two wells only for each serum.

v) 25 μl veronal buffer, pH 7.4, is added to the second well for each serum (anticomplementary well).

vi) 50 µl veronal buffer, pH 7.4, is added to the third well for each serum (lysis activity well).

vii) The complement control is prepared.

viii) The plate is shaken on a microshaker sufficiently to mix the reagents.

ix) The plate is incubated for 1 hour in a water bath, incubator or in a humid chamber at 37°C.

x) The haemolytic system is prepared. After the first 50 minutes of incubation, the sheep RBCs are sensitised by mixing equal volumes of rabbit haemolytic serum, diluted to contain 2 units per 50 µl, and a 3% suspension of washed RBCs; the solution is mixed well and incubated for 10 minutes at 37°C.

xi) After incubation, 50 µl of haemolytic system is added to each well.

xii) The plate is shaken on a microshaker sufficiently to mix the reagents.

xiii) The plate is incubated for 30 minutes at 37°C. To aid in reading the results, the plates can be centrifuged after incubation.

xiv) *Reading the results:* the plate is viewed from above with a light source beneath it. The fixation in every well is assessed by estimating the proportion of cells not lysed. The degree of fixation is expressed as 0, 1+, 2+, 3+, 4+ (0%, 25%, 50%, 75% or 100% cells not lysed). Reactions are interpreted as follows: 4+, 3+, 2+ = positive, 1+ = suspicious, trace = negative, complete haemolysis = negative.

xv) *End-point titration:* All sera with positive reactions at 1/5 are serially double diluted and tested according to the above procedure for end-point titration.

2.2. Indirect fluorescent antibody test

An IFAT for dourine can also be used as a confirmatory test or to resolve inconclusive results obtained by the CFT. The test is performed as follows:

2.2.1. Antigen

(For method, see preparation of CFT antigen in Section B.2.1). Blood is collected into heparinised vacutainers or into a solution of acid–citrate–dextrose from an animal in which the number of trypanosomes is still increasing (more than 10 parasites per 10×40 microscope field should be present.)

i) The blood is centrifuged for 10 minutes at 800 ***g***.

ii) One to two volumes of PBS are added to the packed RBCs, the mixture is agitated, and smears that cover the whole slide evenly are made.

iii) The smears are air-dried and then wrapped in bundles of four, with paper separating each slide. The bundles of slides are wrapped in aluminium foil, sealed in an airtight container over silica gel, and stored at –20°C or –80°C.

iv) Slides stored at –20°C should retain their activity for about 1 year, at –80°C they should remain useable for longer.

2.2.2. Acid–citrate–dextrose solution

Use 15 ml per 100 ml of blood.

2.2.3. Conjugate

Fluorophore-labelled anti-horse immunoglobulins (commercially available).

2.2.4. Test procedure

i) The antigen slides are allowed to reach room temperature in a desiccator. An alternative method is to remove slides directly from the freezer and fix them in acetone for 15 minutes.

ii) The slides are marked out.

iii) Separate spots of test sera diluted in PBS are applied, and the slides are incubated in a humid chamber at ambient temperature for 30 minutes.

iv) The slides are washed in PBS, pH 7.2, three times for 5 minutes each, and air-dried.

v) Fluorescence-labelled conjugate is added at the correct dilution. Individual batches of antigen and conjugate should be titrated against each other using control sera to optimise the conjugate dilution. The slides are incubated in a humid chamber at ambient temperature for 30 minutes.

vi) The slides are washed in PBS, three times for 5 minutes each, and air-dried. An alternative method, to reduce background fluorescence, is to counter-stain, using Evans Blue (0.01% in distilled water) for 1 minute, rinse in PBS and then air dry

vii) The slides are mounted in glycerol/PBS (50/50), immersion oil (commercially available, non-fluorescing grade), or mounting reagent for fluorescent staining (commercially available).

viii) The slides are then examined under UV illumination. Incident light illumination is used with a suitable filter set. Slides may be stored at 4°C for 4–5 days. Sera diluted at 1/80 and above showing strong fluorescence of the parasites are usually considered to be positive. Estimating the intensity of fluorescence demands experience on the part of the observer.

Standard positive and negative control sera should be included in each batch of tests, and due consideration should be given to the pattern of fluorescence in these controls when assessing the results of test sera.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The ELISA has been developed and compared with other serological tests for dourine (Wassall *et al.,* 1991).

Carbonate buffer, pH 9.6, for antigen coating on to microtitre plates: Na_2CO_3 (1.59 g); $NaHCO_3$ (2.93 g); and distilled water (1 litre). Alternatively, PBS (KH_2PO_4 [0.2 g]; Na_2HPO_4 12 H_2O [2.94 g]; NaCl [8.0 g]; KCl [0.2 g in 1 litre distilled water]) can be used for preparation of the antigen solution.

2.3.1. Blocking buffer

Carbonate buffer + 3% fetal calf serum (FCS), or PBS + 1% (w/v) casein.

2.3.2. PBS, pH 7.4, with Tween 20 (PBST) for washing

PBS + 0.05% (v/v) Tween 20.

2.3.3. Sample and conjugate buffer

PBST + 6% FCS, or PBS + 1% (w/v) casein.

2.3.4. Substrate indicator system

ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) (commercially available).

2.3.5. Conjugate

Peroxidase-labelled anti-horse IgG (commercially available).

2.3.6. Antigen

For method, see preparation of CF test antigen in Section B.2.1.

2.3.7. Test procedure

i) Wells in columns 2, 4, 6, etc., are charged with 100 µl of antigen (2 µg/ml), columns 1, 3, 5, etc., are charged with the same amount of carbonate buffer or PBS. The plate is incubated for 40 minutes at 37°C (or overnight at 4°C) in a humid chamber, and 350 µl of blocking buffer

is added to each well. The plate is incubated for 1 hour at ambient temperature, washed three times with PBST, with soaking times of 3 minutes/cycle.

ii) 150 µl of test samples and equine control sera, prediluted 1/100 in sample/conjugate buffer, is added in parallel to wells with and without antigen. The plate is incubated for 1 hour and washed three times with PBST.

iii) Properly diluted conjugate in sample/conjugate buffer is added in volumes of 150 µl to all wells. The plate is incubated for 1 hour with subsequent washing as above.

iv) 150 µl of substrate indicator system is added to all wells and incubated for 1 hour.

v) The plate is shaken for 10 seconds, and the results are read photometrically at a wavelength of between 410 and 420 nm.

vi) *Calculation of results:* absorbance (with antigen) minus absorbance (without antigen) = net extinction. A reaction exceeding a net absorbance value extinction of 0.3 is regarded as a positive result.

Standard positive and negative control sera should be included in each batch of tests.

2.4. Other serological tests

Other serological tests have been used, including radioimmunoassay, counter immunoelectrophoresis and agar gel immunodiffusion but these formats are outdated. More recently, an immunochromatographic test with recombinant antigen derived from *T. evansi* and an immunoblotting test with native antigen from *T. equiperdum* OVI have been developed and deserve further evaluation (Davaasuren *et al.*, 2017; Luciani *et al*., 2018).

C. REQUIREMENTS FOR VACCINES

No vaccines are available for this disease. The only effective control is through the slaughter of infected animals. Good hygiene is essential during assisted mating because infection may be transmitted through contaminated fomites.

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NB: There is a WOAH Reference Laboratory for dourine (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for dourine

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.6.4.

EPIZOOTIC LYMPHANGITIS

SUMMARY

***Description and importance of the disease**: Epizootic lymphangitis is a contagious, chronic disease of horses and other Equidae characterised clinically by a spreading, suppurative, ulcerating pyogranulomatous dermatitis and lymphangitis. This is seen particularly in the neck, legs and chest but can occur anywhere on the body. It can also present as an ulcerating conjunctivitis, or more rarely, in a respiratory form with purulent nasal discharge, pyogranulomatous lesions around the nares with extension of involvement of the nasal lacrimal duct, and lower respiratory signs caused by multifocal pneumonia. Transmission is thought to occur through contact of infected material with traumatised skin, by biting flies, ticks or inhalation of spores. The causative agent,* Histoplasma capsulatum *var.* farciminosum*, is a thermally dimorphic, fungal soil saprophyte that persists in the environment providing a reservoir of infection. Differential diagnoses include glanders* (*farcy*), *caused by* Burkholderia mallei*, ulcerative lymphangitis due to* Corynebacterium pseudotuberculosis, *sporotrichosis caused by* Sporothrix schenckii, *and the skin lesions of histoplasmosis caused by* H. capsulatum *var.* capsulatum*.*

***Identification of the agent:** Identification of the agent is made by its appearance in smears of the exudate or in histological sections of the lesion material. The yeast form of the organism is present in large numbers in well established lesions, and appears as pleomorphic ovoid to globose structures, approximately 2–5 µm in diameter, located both extracellularly and intracellularly in macrophages and giant cells. Organisms are usually surrounded by a 'halo' when stained with Gram stain, haematoxylin and eosin, Giemsa, periodic acid–Schiff reaction or Gomori methenamine–silver stain. The mycelial form of the organism grows slowly under aerobic conditions at 25–30°C on a variety of media, including mycobiotic agar, enriched Sabouraud's dextrose agar, brain–heart infusion agar, and pleuropneumonia-like organism nutrient agar, however culture is challenging. Conversion to the yeast phase at 37°C must be demonstrated for confirmation of diagnosis.*

***Serological and other tests:** Antibodies to* H. capsulatum *var*. farciminosum *develop at or before the onset of clinical signs. Assays reported for detection of antibody include fluorescent antibody, enzyme-linked immunosorbent assay, and passive haemagglutination tests. In addition, a skin hypersensitivity test has been described.*

***Requirements for vaccines and diagnostic biologicals:** Killed and live vaccines have been trialled on a limited scale in endemic areas, but only within research settings and are not commercially available. Skin hypersensitivity tests can be used to detect cell-mediated immunity.*

A. INTRODUCTION

1. Description and impact of the disease

Epizootic lymphangitis is a contagious, chronic disease of horses, mules and donkeys. The disease is characterised clinically by a suppurative, ulcerating, and spreading pyogranulomatous, multifocal dermatitis and lymphangitis. It is seen most commonly in the extremities, chest wall and the neck, but it can also be present as an ulcerating conjunctivitis of the palpebral conjunctiva, or rarely as a multifocal pneumonia. The organism may also invade open lesions including ruptured strangles abscesses and castration wounds. It has also been called pseudofarcy or pseudoglanders. Another synonym is equine histoplasmosis, which may be a more accurate name for the disease, as not all clinical cases present obvious lymphangitis. The form that the disease takes seems to depend primarily on the route of entry (Singh, 1965a). The traumatised skin may be infected directly by infected

pus, nasal or ocular secretions or indirectly by soil or contaminated harnesses, grooming equipment, feeding and watering utensils, wound dressings or flies. It is also believed that ticks may play a role in the transmission of this agent (Ameni & Terefe, 2004). The conjunctival form of the disease is believed to be spread by flies of the *Musca* or *Stomoxys* genera (Singh, 1965a). The pulmonary form of the disease is less frequently observed than the cutaneous form, and is reported to occur after inhalation of the organism (Singh, 1965b). The incubation period is from around 3 weeks to 2 months (Ameni, 2006). In all cases, the lesions are nodular and granulomatous in character, and the organism, once established, spreads locally by invasion and then via the lymphatics. There is often thickening, or 'cording', of lymphatics, with the formation of pyogranulomatous nodules and infection can extend to regional lymph nodes, which become enlarged and inflamed. On occasion, and probably depending on host susceptibility and severity of infection, lesions may heal spontaneously after 2–3 months, resulting in stellate scar formation. Extensive lesions with high mortality rates can occur in areas where there is limited access to veterinary therapeutics and nutrition (Ameni, 2006).

2. Nature and classification of the pathogen

The causative agent, *Histoplasma capsulatum* var. *farciminosum*, is a thermally dimorphic fungus. The mycelial form is present in soil; the yeast form is usually found in lesions. *Histoplasma farciminosum* was formerly described as an independent species, but this assessment has been changed and it is now considered to be a variety of *H. capsulatum* due to the close morphological similarities of both the mycelial and yeast forms (Ueda *et al*, 2003). Antigenically, *H. capsulatum* var. *farciminosum* and *H. capsulatum* var. *capsulatum* are indistinguishable, however the latter is the cause of disseminated histoplasmosis, is endemic in North America and has a wide host range (Robinson & Maxie, 1993). An antigen detection test is commercially available for detection of *Histoplasma capsulatum* var. *capsulatum* from equine clinical samples, although its performance in detecting *Histoplasma capsulatum* var. *farciminosum* is unknown. DNA sequences of four protein-coding genes have been analysed to elucidate the evolutionary relationships of *H. capsulatum* varieties. This indicated that *H. capsulatum* var. *farciminosum* is deeply buried in the branch of SAm Hcc group A, (H60 to -64, -67, -71, -74 and -76) suggesting it may originate from an isolate of South American *H. capsulatum* var. *capsulatum* (Kasuga *et al*, 1999, Murata *et al.,* 2007). These molecular findings suggest that HCF and HCC are more closely related than previously thought, and future advances in whole genome sequencing may further develop understanding of the taxonomy, of various *Histoplasma* species that could have relevance for identifying epidemic strains or virulence factors. More recently, it was demonstrated that nested polymerase chain reaction can be used to detect the presence of HCF directly from equine clinical samples (Scantlebury *et al.,* 2016).

3. Differential diagnosis

The cutaneous form of the disease may be confused with farcy (the skin form of glanders), which is caused by *Burkholderia mallei*, ulcerative lymphangitis, which is caused by *Corynebacterium pseudotuberculosis*, indolent ulcers caused by *Rhodococcus equi*, sporotrichosis caused by *Sporothrix schenckii*, and histoplasmosis caused by *H. capsulatum* var. *capsulatum*, cryptococcosis, strangles, sarcoids and cutaneous lymphosarcomas (Jungerman & Schwartzman, 1972; Lehmann *et al*, 1996), therefore it is important to confirm the causative agent.

4. Epidemiology

The disease is currently endemic in regions of Sub-Saharan Africa, and historically cases were reported in North Africa, some parts of Asia, India, Pakistan, Japan, and some countries bordering the Mediterranean sea (Refai & Loot, 1970). Lack of diagnostic testing facilities, surveillance and reporting from endemic regions means that the disease's prevalence is unknown and thus co-ordinated efforts are required to identify the current location of disease to inform disease control strategies.

The disease is common in Ethiopia, especially in cart horses, affecting an average of 18.8% of horses in warm, humid areas between 1500 and 2300 metres above sea level (Ameni, 2006; Ameni & Terefe, 2004, Endebu & Roger, 2003). Reports from other parts of the world are sporadic and all cases must be verified by laboratory testing. The prevalence of the disease increases with assembling of animals; outbreaks have occurred in the past when large numbers of horses were stabled together for cavalry and other transportation needs e.g. during the Boer war (Pallin, 1904). Usually, it is horses, mules, and donkeys that are affected by the disease, although infection may occur in camels (Chandel & Kher, 1994; Purohit *et al.*, 1985), cattle, wildlife species, and dogs (Murata *et al.* 2007; Ueda *et al*, 2003). Experimentally, laboratory animal species such as mice, guinea-pigs and rabbits are also susceptible to infection (Herve *et al.*, 1994; Singh, 1965a).

5. Zoonotic risk and biosafety requirements

Infection in humans has been reported sporadically, but its zoonotic potential is not fully established (Al-Ani *et al*, 1998; Chandler *et al*, 1980; Guerin *et al*, 1992; Murata *et al.* 2007). All laboratory procedures should be conducted with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of epizootic lymphangitis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Direct microscopy	–	–	–	+	++	–
Culture	–	–	++	+++	+++	–
Nested PCR	++	++	++	++	++	–
Detection of immune response						
FAT	–	–	++	++	++	–
Indirect ELISA	–	–	++	++	++	–
Passive HA test	–	–	++	++	++	–
Hyper-sensitivity skin test	–	–	++	++	–	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; FAT = fluorescent antibody test,
ELISA = enzyme-linked immunosorbent assay; HA = haemagglutination.

1. Identification of the agent

Material should be aspirated asceptically from unruptured nodules using a needle and syringe. For microbiological isolation, the material should be placed in a liquid nutrient medium with antibacterials and kept refrigerated until culturing, which should be attempted as soon as possible. For direct examination, swabs of lesion material can be smeared on glass slides and fixed immediately. For histopathology, sections of lesion material, including both viable and nonviable tissue, should be placed in 10% neutral buffered formalin. Confirmation of the disease is dependent on the demonstration of *H. capsulatum* var. *farciminosum*.

1.1. Direct microscopic examination

1.1.1. Stained impression smears

Impression smears of purulent material can be stained directly with Gram's, Giemsa or Periodic Acid–Schiff reagent and examined for the typical yeast form of the organism, which will appear as Gram-positive, pleomorphic, ovoid to globose structures, approximately 2–5 μm in diameter (Al-Ani *et al.*, 1998). They may occur singly or in groups, and may be found either extracellularly or within macrophages. The intracellular cytoplasm may be variably stained depending upon the age of the lesion or sample handling, and a refractile halo around the organisms (unstained capsule) is frequently observed.

1.1.2. Histopathology

In haematoxylin and eosin (H&E)-stained histological sections, the appearance of the lesion is quite characteristic and consists of pyogranulomatous inflammation with fibroplasia. Langhans giant cells are common. The presence of numerous organisms, both extracellularly and intracellularly within macrophages or multinucleated giant cells in tissue sections stained with H&E, Periodic acid–Schiff reaction and Gomori methenamine–silver stain are observed (Robinson & Maxie, 1993). There is some indication that the number of organisms increases with chronicity. The organisms are pleomorphic, often described as slightly lemon-shaped basophilic masses, varying from 2 to 5 μm in diameter, that are surrounded by a 'halo' when stained with H&E or Gram's stain (Al-Ani, 1999).

1.1.3. Electron microscopy

Electron microscopy has been applied to skin biopsy samples of 1.5–2.0 mm immediately prefixed in phosphate buffered 2% glutaraldehyde solution at 4°C and post-fixed in 1% osmium tetroxide. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. Examination demonstrated the fine internal structure of the organism, *H. capsulatum* var. *farciminosum,* including the cell envelope, plasma membrane, cell wall, capsule and inner cell structures (Al-Ani, 1999).

1.2. Culture

The mycelial form of *H. capsulatum* var. *farciminosum* is challenging to culture and grows slowly on laboratory media (2–8 weeks at 26°C). Media that can be used include Mycobiotic agar (Al-Ani *et al.*, 1998), Sabouraud's dextrose agar enriched with 2.5% glycerol, brain–heart infusion agar supplemented with 10% horse blood, and pleuropneumonia-like organism (PPLO) nutrient agar enriched with 2% dextrose and 2.5% glycerol, pH 7.8 (Guerin *et al*, 1992; Robinson & Maxie, 1993). The addition of antibiotics to the media is recommended: cycloheximide (0.5 g/litre) and chloramphenicol (0.5 g/litre). Broad-spectrum antibacterial activity is obtained if gentamicin (50 mg/litre) and penicillin G (6×10^6 units/litre) are used instead of chloramphenicol. Colonies appear in 2–8 weeks as dry, grey-white, granular, wrinkled mycelia. The colonies become brown with aging. Aerial forms occur, but are rare. The mycelial form produces a variety of conidia, including chlamydospores, arthroconidia and some blastoconidia. However, the large round double-walled macroconidia that are often observed in *H. capsulatum* var. *capsulatum* are lacking.

As a confirmatory test the yeast form of *H. capsulatum* var. *farciminosum* can be induced by subculturing some of the mycelium into brain–heart infusion agar containing 5% horse blood or by using Pine's medium alone at 35–37°C in 5% CO_2. Yeast colonies are flat, raised, wrinkled, white to greyish brown, and pasty in consistency (Robinson & Maxie, 1993). However, complete conversion to the yeast phase may only be achieved after four to five repeated serial transfers on to fresh media every 8 days.

2. Serological tests

There are published reports of various tests to detect antibodies as well as a skin hypersensitivity test for detection of cell-mediated immunity. Antibodies usually develop at or just after the onset of clinical signs.

2.1. Fluorescent antibody tests

2.1.1. Indirect fluorescent antibody test

The following non-quantitative procedure is as described by Fawi (1969).

i) Slides containing the organisms are made by smearing the lesion contents on to a glass slide or by emulsifying the cultured yeast phase of the organism in a saline solution and creating a thin film on a glass slide.

ii) The slides are heat-fixed by passing the slide through a flame.

iii) The slides are then washed in phosphate buffered saline (PBS) for 1 minute.

iv) Undiluted test sera are placed on the slides, which are then incubated for 30 minutes at 37°C.

v) The slides are washed in PBS three times for 10 minutes each.

vi) Fluorescein isothiocyanate (FITC)-conjugated anti-horse antibody at an appropriate dilution is flooded over the slides, which are then incubated for 30 minutes at 37°C.

vii) Washing in PBS is repeated three times for 10 minutes each.

viii) The slides are examined using fluorescence microscopy.

2.1.2. Direct fluorescent antibody test

The following procedure is as described by Gabal *et al* (1983).

i) The globulin fraction of the test serum is precipitated using 35% saturated ammonium sulphate, and then re-suspended to its original serum volume in saline and purified using gel filtration. The serum is then conjugated to FITC.

ii) Small colony particles of the cultured mycelial form of the organism are suspended in 1–2 drops of saline on a glass slide. With a second slide, the colony particles are crushed and the solution is dragged across the slide to create a thin film. Smears are also made directly from pus from unruptured nodules.

iii) The smears are heat-fixed.

iv) The slides are incubated with dilutions of conjugated serum for 60 minutes at 37°C.

v) The slides are washed in PBS three times for 5 minutes each.

vi) The slides are examined using fluorescence microscopy.

2.2. Indirect enzyme-linked immunosorbent assay

The following procedure is as described by Gabal & Mohammed (1985).

2.2.1. Test procedure

i) The mycelial form of the organism is produced on Sabouraud's dextrose agar in tubes, and incubated for 4 weeks at 26°C. Three colonies are ground in 50 ml of sterile PBS. The suspension is diluted 1/100 and the 96-well microtitre plates are coated with 100 µl/well.

ii) The plates are incubated at 4°C overnight.

iii) The plates are washed with PBS containing Tween 20 (0.5 ml/litre) (PBS-T) three times for 3 minutes each.

iv) The plates are incubated with 5% bovine serum albumin, 100 µl/well, at 23–25°C for 30 minutes, with shaking.

v) The plates are washed with PBS-T three times for 3 minutes each.

vi) The sera are serially diluted using twofold dilution in duplicate in PBS-T, starting with a 1/50 dilution and incubated for 30 minutes at 23–25°C.

vii) The plates are washed with PBS-T three times for 3 minutes each.

viii) Peroxidase-labelled goat anti-horse IgG is diluted 1/800 and used at 100 µl/well, with incubation for 30 minutes at 23–25°C, with shaking.

ix) The plates are washed with PBS-T three times for 3 minutes each.

x) Finally, 100 µl/well of hydrogen peroxide and ABTS (2,2'-Azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) in a citric acid buffer, pH 4, is added.

xi) The plates are read at 60 minutes in a spectrophotometer at wavelength 405 nm.

xii) The absorbance values are obtained twice from each serum dilution and the standard deviation and average percentage of the absorbance values of the different serum samples are considered in the interpretation of the results.

2.3. Passive haemagglutination test

The following procedure is as described by Gabal & Khalifa (1983).

2.3.1. Test procedure

i) The organism is propagated for 8 weeks on Sabouraud's dextrose agar. Five colonies are scraped, ground, suspended in 200 ml of saline, and sonicated for 20 minutes. The remaining mycelial elements are filtered out, and the filtrate is diluted 1/160.

ii) Normal sheep red blood cells (RBCs) are washed, treated with tannic acid, washed, and re-suspended as a 1% cell suspension.

iii) Different dilutions of the antigen preparation are mixed with the tanned RBCs and incubated in a water bath at 37°C for 1 hour. The RBCs are collected by centrifugation, washed three times in buffered saline and re-suspended to make a 1% cell suspension.

iv) Test sera are inactivated by heating at 56°C for 30 minutes and then absorbed with an equal volume of washed RBCs.

v) Dilutions of serum (0.5 ml) are placed in test tubes with 0.05 ml of antigen-coated tanned RBCs.

vi) Agglutination is recorded at 2 and 12 hours.

vii) Agglutination is detected when the RBCs form a uniform mat on the bottom of the tube. A negative test is indicated by the formation of a 'button' of RBCs at the bottom of the tube.

2.4. Skin hypersensitivity tests

i) Skin test antigen is prepared by one of two methods as described in Section C.

ii) Animals are inoculated intradermally in the neck with 0.1 ml skin test antigen containing 0.2 mg/ml protein.

iii) The inoculation site is examined for the presence of a local indurated and elevated area at 24, 48 and 72 hours post-injection. An increase in skin thickness of > 4 mm is considered to be positive.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Vaccines

Depending on regulations in the region affected by disease, control of the disease is usually through elimination of the infection, although this may be limited by lack of resources and access to anti-fungals in regions where the disease is currently endemic. International guidelines recommend that control is achieved by culling infected horses and application of strict hygiene practices to prevent spread of the organism, however this may not be readily implemented in all regions for socio-economic reasons and lack of available compensation for loss of the animal. There are published reports on the use of killed (Al-Ani *et al*, 1998; Noskoav, 1960) and live attenuated vaccines (Zhang *et al*, 1986) in areas where epizootic lymphangitis is, or was previously endemic, apparently with

relatively good results, however these were from experimental studies and currently no vaccine is commercially available.

2. Skin test antigens

Skin test antigens for intradermal inoculation are prepared by one of two published methods:

2.1. Method 1 (Armeni *et al.,* 2006; Gabal & Khalifa, 1983)

A pure culture of *H. farcimonsum* is propagated for 8 weeks on Sabouraud's dextrose agar containing 2.5% glycerol. Five colonies are scraped, ground, suspended in 200 ml of saline, undergo five freeze–thaw cycles and are sonicated at an amplitude of 40° for 20 minutes. The remaining mycelial elements are removed by centrifugation at 1006 ***g*** at 4°C for 11 minutes. Sterility of the preparation is verified by incubating an aliquot on Sabouraud's dextrose agar at 26°C for 4 weeks.

2.2. Method 2 ('histofarcin'; Ameni *et al.,* 2006; Soliman *et al.,* 1985)

i) The mycelial form of the organism is grown on polystyrene discs floating on 250 ml of PPLO media containing 2% glucose and 2.5% glycerine at 23–25°C for 4 months.

ii) The fungus-free culture filtrate is mixed with acetone (2/1) and held at 4°C for 48 hours.

iii) The supernatant is decanted and the acetone is allowed to evaporate.

iv) Precipitate is suspended to 1/10 original volume in distilled water.

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NB: At the time of publication (2018) there were no WOAH Reference Laboratories for epizootic lymphangitis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018

CHAPTER 3.6.5.

EQUINE ENCEPHALOMYELITIS (EASTERN, WESTERN AND VENEZUELAN)

SUMMARY

***Description and importance of the disease**: Eastern, Western and Venezuelan equine encephalomyelitis (EEE, WEE and VEE), viruses belong to the genus* Alphavirus *of the family* Togaviridae*. EEE, WEE and VEE are alphaviruses present in the Americas and can cause disease in both humans and equids with encephalitis in most clinical cases. EEE, WEE, and VEE are typically maintained in nature by alternating between vertebrate hosts and vector mosquitoes. Encephalitis caused by these encephalitis viruses occurs sporadically in horses and humans from mid-summer to late autumn in temperate regions but can occur year-round in tropical regions, depending on climate conditions that support the presence of the mosquito vector. Clinical disease in horses is characterised by fever, anorexia, and severe depression. In severe cases, it can progress to hyperexcitability, blindness, ataxia, severe mental depression, recumbency, convulsions, and death.*

EEE virus infection in horses is often fatal, while WEE virus can cause a subclinical or mild disease with less than 30% mortality. The principal reservoir hosts for EEE and WEE are passerine birds. Most infections in birds are nonclinical, but EEE and WEE viruses have been reported to cause disease in poultry, game birds and ratites. Small mammals, such as rodents may also amplify EEE. Sporadic cases of EEE have been reported in cows, sheep, pigs, deer, and dogs. Horses and humans are incidental dead-end hosts for EEE and WEE. However some horses may develop a transient viraemia that has been suggested to be sufficient to transmit EEE virus to mosquitoes under the right conditions.

VEE viruses are considered to be one of the most important equine pathogens in Mexico, Central and South America. For example, one epidemic in Colombia associated with up to 100,000 equid deaths and 250,000 human cases. In contrast to EEE and WEE, horses play a key role in virus amplification, disease spread and maintenance of epizootics of VEE. The VEE complex of viruses includes six antigenic subtypes (I–VI). Within subtype I there are five antigenic variants (variants AB–F). Originally, subtypes I-A and I-B were considered to be distinct variants, but they are now considered to be identical (I-AB). Antigenic variants I-AB and I-C are associated with epizootic activity in equids and humans. Historically, severe outbreaks have involved many thousands of human and equine cases. The other three variants of subtype I (I-D, I-E, I-F) and the other five subtypes of VEE (II–VI) circulate in natural enzootic cycles. Equidae are not dead-end hosts for VEE virus; they serve as amplifying hosts for epizootic VEE strains while enzootic VEE viruses cycle primarily in sylvatic rodents and mosquitoes. Enzootic variants and subtypes have been considered to be nonpathogenic for equids, but can cause clinical disease in humans. During 1993 and 1996, limited outbreaks of encephalitis in horses in Mexico were shown to be caused by enzootic VEE viruses of subtype I-E. More recently, sporadic outbreaks have occurred in Mexico, Central America, and northern and western parts of South America. Human enzootic subtypes extend more broadly into northern Central America and South America (Weaver et al., *2012).*

***Detection of the agent:** A presumptive diagnosis of EEE, WEE or VEE can be made when susceptible horses display the characteristic somnolence and other signs of neurological disease in areas where haematophagous insects are active. There are no characteristic gross lesions. Histopathological lesions can provide a presumptive diagnosis. EEE virus can usually be isolated from the brain and sometimes other tissues of dead horses, however WEE and VEE viruses are rarely isolated. The viruses can be isolated from field specimens by inoculating embryonated chicken eggs or cell cultures. The virus can be identified by reverse-transcription polymerase chain reaction (RT-PCR), complement fixation (CF), immunofluorescence, or plaque reduction neutralisation (PRN) tests.*

Specific identification of epizootic VEE virus variants can be made by the indirect fluorescent antibody test, or a differential PRN test using subtype- or variant-specific monoclonal antibody, or by nucleic acid sequencing.

Serological tests: *Antibodies can be identified by PRN test, haemagglutination inhibition (HI), CF, or IgM capture enzyme-linked immunosorbent assay.*

Requirements for vaccines: *EEE and WEE vaccines are safe and immunogenic. The only recommended vaccines against VEE are an attenuated virus vaccine, made with strain TC-83, or inactivated virus preparations also made from this strain. Attenuated virus is immunogenic when given by intramuscular injection and can cause adverse reactions in the recipient.*

Formalin-inactivated virulent VEE virus preparations should not be used in equids, as residual virulent virus can remain after formalin treatment, causing severe illness in both animals and humans. Epizootics of VEE have occurred from the use of such formalin-treated viruses.

A. INTRODUCTION

Eastern equine encephalomyelitis (EEE), Western equine encephalomyelitis (WEE) and Venezuelan equine encephalomyelitis (VEE) viruses are members of the genus *Alphavirus* of the family *Togaviridae*. Although closely related, EEE, WEE and VEE are genetically and antigenically distinct (Arechiga-Ceballos & Aguilar-Setien, 2015, Kumar *et al.,* 2018). The natural ecology for virus maintenance typically occurs via alternating infection of birds and mosquitoes (EEE and WEE), mosquitoes and rodents (VEE enzootic cycle), or mosquitoes and horses (VEE epizootic cycle) (Arechiga-Ceballos & Aguilar-Setien, 2015; Go *et al.,* 2014; Salimi *et al.,* 2016). EEE virus has also been isolated from snakes, and these may have a role as reservoir hosts (Bingham *et al.,* 2012; White *et al.,* 2011). Clinical disease may be observed in humans and horses, both of which are incidental dead-end hosts for EEE and WEE virus (Kumar *et al.,* 2018; Zacks & Paessler, 2010). However some horses may develop a transient viraemia that has been suggested as potentially sufficient to transmit EEE virus to mosquitoes under the right conditions (Franklin, 2002).

All three viruses are classified as alphaviruses present in the Americas andare present in the Americas (Arechiga-Ceballos & Aguilar-Setien, 2015). EEE has lineages associated with both North America (predominately the central and eastern United States of America [USA], Canada, and Central America) and South America. The South American strains of EEE, previously known as lineages II, III, and IV, are now known as Madariaga virus (Arrigo *et al.*, 2010) after strains causing outbreaks in Darien, Panama in 2010 (Carrera *et al.*, 2013) led to the re-evaluation of viral sequencing and reclassification of the South American strain of EEE (Madariaga virus) as a separate divergent strain. WEE was historically detected primarily in the western USA and Canada, Mexico, and Central and South America (Arechiga-Ceballos & Aguilar-Setien, 2015; Kumar, 2018; Morris, 1989; Reisen & Monath, 1989; Walton, 1981). Highlands J virus, antigenically related to WEE virus, has been isolated in the eastern USA. Although Highlands J virus is generally believed not to cause disease in mammals, it has been isolated from the brain of a horse in Florida dying of encephalitis (Karabatsos *et al*., 1988). Epizootic VEE viruses have been identified in Central and South America with the most recent spread into Southern USA from Mexico in 1971 (Kumar, 2018; Oberste *et al.,* 1999; Salimi *et al.,* 2016; Sneider *et al.,* 1993; Wang *et al.,* 1999; Weaver *et al.,* 1999; Zehmer *et al.,* 1974).

The clinical signs of EEE, WEE and VEE can be identical. The disease caused by any of the three viruses is also known as sleeping sickness. Following an incubation period of 1–14 days, depending on the virus and strain, clinical signs include fever, anorexia, and depression. A presumptive diagnosis of equine viral encephalomyelitis in unvaccinated horses can be made if the characteristic somnolence is observed during the summer in temperate climates or the wet season in tropical and subtropical climates, when the mosquito vector is plentiful. However, a number of other diseases, such as West Nile virus (Chapter 3.1.25), rabies (Chapter 3.1.18), and other infectious, parasitic, or non-infectious agents can produce similar clinical signs and the diagnosis must be confirmed by the described diagnostic test methods.

EEE virus causes severe disease in humans with a mortality rate of 30–70% and a high frequency of permanent neurological sequelae in patients who survive. The North American variant is considered to be more pathogenic than South American strains (Madariaga virus) (Kumar *et al.,* 2018). EEE virus has been reported to cause disease in mammals other than equines and humans, including cows (McGee *et al*., 1992; Pursell *et al*., 1976), sheep (Bauer *et al*., 2005), pigs (Elvinger *et al*., 1996), white-tailed deer (Tate *et al*., 2005), and dogs (Farrar *et al*., 2005). EEE virus infections are usually observed in limited geographical areas. WEE is usually mild in adult humans, but can be a severe disease in children. The fatality rate is between 3 and 14%. WEE virus infection in horses has historically been

observed over a wide geographical area, e.g. sporadic cases over 2590 km^2 (1000 square miles), however, no disease outbreaks attributed to WEE have occurred since 1999.

Most encephalomyelitis infections in domestic fowl are caused by EEE virus and occur in the east coast states of the USA. Isolated events of high mortality in captive-raised game birds, primarily pheasants, chukars, aquarium penguins, and quail have been traced to EEE, WEE, or Highlands J virus infection (Morris, 1989; Reisen & Monath, 1989; Tuttle *et al*., 2005). Although virus is introduced by mosquitoes, transmission within the flocks is primarily by feather picking and cannibalism (Kumar *et al.,* 2018). Both EEE and WEE viruses have caused fatal disease in ratites. Haemorrhagic enteritis has been observed in emus infected with EEE and WEE viruses, and morbidity and mortality rates may be greater than 85%. EEE and Highlands J viruses have been found to produce depression, somnolence, decreased egg production, and increased mortality in turkeys (Guy, 1997).

The VEE virus complex is composed of six subtypes (I–VI). Subtype I includes five antigenic variants (AB–F), of which variants I-AB and I-C are associated with epizootic VEE in equids and concurrent epidemics in humans (Calisher *et al*., 1980; Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton *et al*., 1973; Walton & Grayson, 1989). Originally, subtypes I-A and I-B were considered to be distinct variants, but they are now considered to be identical (I-AB). The epizootic variants I-AB and I-C are thought to originate from mutations of the enzootic 1-D serotype (Weaver *et al*., 2004); I-AB and I-C isolates have only been obtained during equine epizootics. The enzootic strains include variants I-D, I-E and I-F of subtype I, subtype II, four antigenic variants (A–D) of subtype III, and subtypes IV–VI. Normally, enzootic VEE viruses do not produce clinical encephalomyelitis in the equine species (Walton *et al*., 1973), but in 1993 and 1996 in Mexico, the 1-E enzootic subtype caused limited epizootics in horses (Estrada-Franco *et al.,* 2004). The enzootic variants and subtypes can produce clinical disease in humans (Monath & Trent, 1981; Pan-American Health Organization, 1972; Powers *et al*., 1997; Walton, 1981; Walton & Grayson, 1989).

Historically, epizootic VEE was limited to northern and western South America (Pan-American Health Organization, 1972). From 1969 to 1972, however, epizootic activity (variant 1-AB) occurred in parts of North and Central America. Epizootics of VEE caused by I-AB or I-C virus have not occurred in North America since 1972. Equine and human isolations of epizootic VEE virus were subtype I-C strains from Venezuela in 1993, 1995,1996, 1999, 2000, 2003 (Navarro *et al*., 2005) and Colombia in 1995. Additionally, variant I-AB has been isolated from sentinel hamsters in Venezuela (Medina *et al*., 2015). For up-to-date information, consult WOAH WAHIS interface[1]).

The foci of enzootic variants and subtypes are found in areas classified as tropical wet forest, i.e. those areas with a high water table or open swampy areas with meandering sunlit streams. These are the areas of the Americas where rainfall is distributed throughout the year or areas permanently supplied with water. Enzootic viruses cycle among rodents, and perhaps birds, by the feeding of mosquitoes (Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989). Enzootic VEE virus strains have been identified in the Florida Everglades (subtype II), Mexico (variant I-E), Central American countries (variant I-E), Panama (variants I-D and I-E), Venezuela (variant I-D), Colombia (variant I-D), Peru (variants 1-D, III-C, and III-D), French Guiana (variant III-B and subtype V), Ecuador (variant I-D), Bolivia (variant I-D), Suriname (variant III-A), Trinidad (variant III-A), Brazil (variants I-F, III-A, and subtype IV), and Argentina (subtype VI). In an atypical ecological niche, variant III-B has been isolated in the USA (Colorado and South Dakota) in an unusual association with birds (Aguilar *et al*., 2009; Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989), while the Everglades virus is a subtype II VEE virus that infects rodents and dogs in Florida.

1. Biosafety

Severe clinical disease and death caused by EEE and WEE viruses have been reported in laboratory workers. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). It is recommended that personnel be immunised against EEE virus (United States Department of Health and Human Services, 2009). Precautions should also be taken to prevent human infection when performing post-mortem examinations on horses suspected of being infected with the equine encephalomyelitis viruses.

1 https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/world-animal-health-information-system/

Human VEE virus infections have originated by aerosol transmission from the cage debris of infected laboratory rodents and from laboratory accidents (Quiroz *et al.,* 2009). Infections with both epizootic and enzootic variants and subtypes have been acquired by laboratory workers (American Committee on Arthropod-Borne Viruses [ACAV], 1980). Severe clinical disease or death can occur in humans. Those who handle infectious VEE viruses or their antigens prepared from infected tissues or cell cultures should be vaccinated and shown to have VEE virus-specific neutralising antibody (Berge *et al*., 1961; Pan-American Health Organization, 1972). If vaccination is not a viable option, additional personal protective equipment to include respiratory protection is recommended for all procedures. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by risk analysis (see chapter 1.1.4).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of EEE, WEE and VEE and their purpose[2]

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
RT-PCR	–	++	–	+++	–	–
Isolation in cell culture	–	++	–	+++	–	–
Detection of immune response						
IgM capture ELISA	–	+	–	++	–	–
Plaque reduction neutralisation	+++	+	–	++	+++	+++
Haemagglutination inhibition (paired samples)	+	++	–	++	++	++
Complement fixation (paired samples)	–	+	–	++	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction;
IgM ELISA = immunoglobulin M enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended

1. Detection of the agent

1.1. *In-vitro* and *in-vivo* culture

The definitive method for diagnosis of EEE or WEE is virus isolation followed by typing. EEE virus can usually be isolated from the brains of horses, unless more than 5 days have elapsed between the appearance of clinical signs and the death of the horse. EEE virus can frequently be isolated from brain tissue even in the presence of a high serum antibody titre. WEE virus is rarely isolated from tissues of

2 For WEE and VEE, not all test modalities have been thoroughly evaluated in naturally infected horses

infected horses. Brain is the tissue of choice for virus isolation, but the virus has been isolated from other tissues, such as the liver and spleen.

During VEE infection, viraemia coincides with the onset of pyrexia within 12–24 hours of infection. Viraemia usually terminates 5–6 days after the onset of infection, and coincides with the production of neutralising antibodies and the appearance of clinical neurological signs. Frequently, VEE viruses cannot be isolated from the brains of infected equids. Blood samples for virus isolation should be collected from febrile animals that are closely associated with clinical encephalitic cases. It is recommended that a set of these tissues be collected in duplicate, one set for virus isolation and the other set in formalin for histopathological examination. Specimens for virus isolation should be sent refrigerated if they can be received in the laboratory within 48 hours of collection; otherwise, they should be frozen and sent with dry ice. A complete set of tissues will allow the performance of diagnostic techniques for other diseases. For isolation, a 10% suspension of tissue is prepared in phosphate buffered saline (PBS), pH 7.8, containing bovine serum albumin (BSA) (fraction V; 0.75%), penicillin (100 units/ml), and streptomycin (100 µg/ml). The suspension is clarified by centrifugation at 1500 ***g*** for 30 minutes.

EEE, WEE and VEE viruses can be isolated in a number of cell culture systems. The most commonly used cell cultures are primary chicken or duck embryo fibroblasts, continuous cell lines of African green monkey kidney (Vero), rabbit kidney (RK-13), or baby hamster kidney (BHK-21). Isolation is usually attempted in 25 cm^2 cell culture flasks. Confluent cells are inoculated with 1.0 ml of tissue suspension. Following a 1–2-hour absorption period, cell monolayer is rinsed twice with culture medium or PBS, and maintenance medium is added. Cultures are incubated for 6–8 days, and one blind passage is made. EEE, WEE and VEE viruses will produce a cytopathic change in cell culture. Cultures that appear to be infected are frozen. The fluid from the thawed cultures is used for virus identification.

Tissue suspensions can be inoculated by the yolk-sac route into 6–8-day-old embryonating chicken eggs. There are no diagnostic signs or lesions in the embryos infected with these viruses. Inoculated embryos should be incubated for 7 days, but deaths usually occur between 2 and 4 days post-inoculation. Usually only one passage is made unless there are dead embryos, from which virus cannot be isolated.

Viral isolates can be identified by reverse-transcription polymerase chain reaction (RT-PCR), by direct or indirect fluorescent antibody test, or by plaque reduction neutralisation (PRN) tests using polyclonal or monoclonal antibodies to specific viruses obtained commercially or prepared via hyperimmunisation of animals with further collection of their serum (or ascites fluid in case of mice) and purification of immunoglobulins.

1.2. Molecular methods – detection of nucleic acid

1.2.1. Conventional reverse-transcription PCR

Several methods of RT-PCR for detection of EEE, WEE or VEE viral RNA in mosquitoes and vertebrate tissues have been described, although few have been extensively validated for mammalian samples (Linssen *et al*., 2000; Monroy *et al*., 1996; Vodkin *et al*., 1993). A multiplex nested RT-PCR method was developed to expedite differential diagnosis in cases of suspected EEE or West Nile arboviral encephalomyelitis in horses (Johnson *et al*., 2003). The assay has enhanced speed and sensitivity compared with cell culture virus isolation and has been used extensively in the US NVSL[3] during several subsequent arbovirus seasons. Primer sequences used at NVSL for detection of EEE, WEE and VEE viral RNA are shown in Table 2. The first reaction is performed in one tube and starts with reverse transcription at 46–50°C (depending on the optimum temperature range of the enzyme used) for 30 minutes followed by polymerase activation at 95°C for 15 minutes. Then, 35 cycles consisting of denaturation at 94°C for 45 seconds; annealing at 58°C for 45 seconds, and elongation at 72°C for 1 minute are performed. An additional elongation step at 72°C for 9 minutes is applied at the end of the reaction. The nested PCR uses 1 µl of the first PCR product in 50 µl of reaction mix, and is performed under the following cycling conditions: polymerase activation at 95°C for 15 minutes, 35 cycles consisting of

3 NVSL: National Veterinary Service Laboratories (of the USA).

the steps described above with annealing temperature 46°C, followed by the final elongation at 72°C for 9 minutes.

An approach aimed at detecting variants of VEE virus using one pair of degenerated primers (Table 2) was also developed (Pisano *et al.,* 2012). Following reverse transcription at 46–50°C (depending on the enzyme used) for 30 minutes and denaturation at 94°C for 2 minutes, the authors recommend first PCR (40 cycles) consisting of denaturation at 94°C for 30 seconds; annealing at 64°C for 60 seconds; and elongation at 72°C for 30 seconds, supplemented with a final extension at 72°C for 5 minutes. For the nested reaction, 2 µl of the first PCR product is mixed in 50 µl of reaction mix and subjected to initial denaturation at 94°C for 2 minutes, followed by 40 cycles of amplification consisting of denaturation at 94°C for 30 seconds; annealing at 61°C for 40 seconds; and elongation at 72°C for 30 seconds. A final extension at 72°C for 5 minutes is then applied.

Table 2. Primers used for detection of EEE, WEE and VEE viral RNA via nested RT-PCR

Virus	Genome position	Amplicon length	Primer sequence (5'–3')	Source
EEE (1st stage)	9233–9797	565	F: AGG-GCT-TAC-CTG-ATT-GAC R: GTA-ACG-CCA-GGA-GTA-TTG	NVSL
EEE (nested)	9571–9710	140	F: GGC-TCA-AGA-GTC-AGG-AGA R: CGG-ATG-TGA-CAC-AAG-AGA	NVSL
WEE (1st stage)	9032–9621	590	F: TAA-GTG-TGG-CGA-CTA-CAG R: TCA-GGC-AGT-CTC-TTC-TTG	NVSL
WEE (nested)	9241–9575	335	F: CTC-ACA-CGC-CTA-CAG-TCA R: AGT-GCC-TAC-CAG-GAT-AGC	NVSL
VEE I-AB/C/D (1st stage)	9215–9776	562	F: AGC-CAG-TGC-ACA-AAG-AAG R: TAG-GTG-TTA-GCC-GGT-AAG	NVSL
VEE I-AB/C/D (nested)	9536–9671	136	F: GGG-TGG-GAG-TTT-GTA-TGG R: CCA-GGA-TGG-TGG-ACA-TAG	NVSL
VEE I-E (1st stage)	9611–10085	475	F: GTA-ATC-CAC-ACG-GAC-TAC R: GCA-TAA-CCC-GCT-CTG-TTG	NVSL
VEE I-E (nested)	9794–9955	162	F: GCA-TGC-CTC-TGT-GCT-TAG R: ATT-TCA-GCA-AGC-GGG-TAG	NVSL
VEE all (1st stage)	45–176	156	F: ATG-GAG-AAR-GTT-CAC-GTT-GAY-ATC-G R: YTC-GAT-YAR-YTT-NGA-NGC-YAR-ATG-C	Pisano *et al.*, 2012
VEE all (nested)	83–163	80	F: ARG-AYA-GYC-CNT-TCC-TYM-GAG-C R: CRT-TAG-CAT-GGT-CRT-TRT-CNG-TNA-C	Pisano *et al.*, 2012

F: forward; R: reverse.

A combination of an RT-PCR with an enzyme-linked immunosorbent assay (ELISA: RT-PCR-ELISA) was reported as a method to identify alphaviruses that are pathogenic to humans (Wang *et al*., 2006).

1.2.2. Real-time reverse-transcription PCR

A multiplexed detection of EEE and WEE viral RNA via real-time RT-PCR has been reported (Kang *et al.,* 2010), but the method has not been extensively evaluated in field samples. A sensitive real-time RT-PCR for detection of North American variants of EEE and WEE viral RNA has been developed and validated on a limited panel of field samples; it demonstrated a superior sensitivity compared with virus isolation in Vero cell culture (Lambert *et al*., 2003). The primers and probes (Table 3) were evaluated with a number of synthetic RNA constructs that incorporated various

substitutions present in North American EEE and WEE viral variants (Vina-Rodriguez *et al.,* 2016). This confirmed the high sensitivity and specificity of the reagents developed by Lambert *et al.* (2003). The real-time RT-PCR starts with reverse transcription at 46–50°C (depending on the enzyme used) for 30 minutes followed by *Taq*-polymerase activation at 95°C for 15 minutes. Then, 45 cycles consisting of denaturation at 95°C for 15 seconds; annealing at 55°C for 30 seconds; and elongation at 72°C for 30 seconds are performed.

Real-time RT-PCR for detection of VEE virus (Vina-Rodriguez *et al.,* 2016) was evaluated on a set of synthetic RNA oligonucleotides and needs further validation with field samples of VEE virus. This is particularly important because the nsP1 genome area where the primers and probes were selected is highly conserved in alphaviruses (Eshoo *et al.,* 2007), and the possibility of undesired cross-reactivity needs to be ruled out.

Table 3. Real-time RT-PCR primers and probes to EEE, WEE and VEE viruses. Both probes are labelled with FAM and quenched with BHQ1 (Lambert et al., 2003)

Virus	Reagent designation	Genome position	Sequence (5'–3')	Amplicon length
EEE	EEE 9391, primer F	9391–9411	ACA-CCG-CAC-CCT-GAT-TTT-ACA	69
	EEE 9459c, primer R	9459–9439	CTT-CCA-AGT-GAC-CTG-GTC-GTC	
	EEE 9414 Probe	9414–9434	TGC-ACC-CGG-ACC-ATC-CGA-CCT	
WEE	WEE 10,248 primer F	10,248–10,267	CTG-AAA-GTC-GGC-CTG-CGT-AT	67
	WEE 10,314c primer R	10,314–10,295	CGC-CAT-TGA-CGA-ACG-TAT-CC	
	WEE 10,271 Probe	10,271–10,293	ATA-CGG-CAA-TAC-CAC-CGC-GCA-CC	
VEE	AlphaVIR966 primer F	151-178	TCC-ATG-CTA-ATG-CYA-GAG-CGT-TTT-CGC-A	98
	AlphaVIR966 primer R	248-225	TGG-CGC-ACT-TCC-AAT-GTC-HAG-GAT	
	INEID-VEEV Probe	193-218	TGA-TCG-ARA-CGG-AGG-TRG-AMC-CAT-CC	

F: forward; R: reverse.

1.3. Antigen detection

Antigen-capture ELISA has been developed for EEE surveillance in mosquitoes. This can be used in countries that do not have facilities for virus isolation or RT-PCR (Brown *et al*., 2001). Immunohistochemical (IHC) procedures are very useful for diagnosis of EEE, as they are carried out on fixed tissues (Pennick *et al*., 2012). The envelope protein of EEE virus is targeted in IHC. Necrotic and inflamed areas of the brain are examined. In EEE virus infected horses, positive staining is observed most notably in neurons and associated dendritic processes. However, failure to identify viral antigen in equine central nervous system does not rule out infection.

2. Serological tests

Serological confirmation of EEE or WEE virus infection requires a four-fold or greater increase or decrease in antibody titre in paired serum samples collected 10–14 days apart. Most horses infected with EEE or WEE virus have a high antibody titre when clinical disease is observed. Consequently, a presumptive diagnosis can be made if an unvaccinated horse with appropriate clinical signs has antibody against only EEE or WEE virus. In contrast to EEE and WEE virus infection, onset of clinical signs and subsequent IgG antibody production against VEE virus occurs early post-infection. Development of encephalitis is variable, thus some neurologic horses may not exhibit a four-fold or greater increase in antibody titre in paired sera. Serum should be collected at onset of clinical signs and repeated in five to nine days for comparison. The detection of IgM antibody by the ELISA can be suggestive for an acute infection or a recent exposure to the virus, including recent vaccination, therefore interpretation of serological findings must be done in conjunction with clinical signs and the epizootic situation (Sahu *et al*., 1994). Vaccination history must be taken into account when interpreting results of any serological tests, particularly the PRN or virus

neutralisation (VN) tests. Although enzootic VEE subtypes and variants are non-pathogenic for equids, infection will stimulate antibody production that can cross-react on diagnostic tests with epizootic VEE virus variants. Furthermore, there may be cross-reactions between antibody against EEE and WEE virus in such tests as complement fixation (CF) and haemagglutination inhibition (HI) tests. CF antibodies against both EEE and WEE viruses appear later and do not persist; consequently, the CF test is less useful for the serological diagnosis of disease.

2.1. Complement fixation

The CF test is frequently used for the demonstration of antibodies, although the antibodies detected by the CF test may not persist for as long as those detected by the HI or PRN tests. A sucrose/acetone mouse brain extract is commonly used as antigen. The positive antigen is inactivated by treatment with 0.1% beta-propiolactone. The effectiveness of this treatment should be confirmed by viability testing using *in-vivo* or *in-vitro* culture (see Section B.1.1) prior to working with the antigen at the bench.

In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. The normal antigen, or control antigen, is mouse brain from uninoculated mice similarly extracted and diluted.

Sera are diluted 1/4 in veronal buffered saline containing 1% gelatin (VBSG), and inactivated at 56°C for 30 minutes. Titrations of positive sera may be performed using additional twofold dilutions. The CF antigens and control antigen (normal mouse brain) are diluted in VBSG to their optimal amount of fixation as determined by titration against the positive sera; guinea-pig complement is diluted in VBSG to contain 5 complement haemolytic units-50% (CH_{50}). Sera, antigen, and complement are reacted in 96-well round-bottom microtitre plates at 4°C for 18 hours. The sheep red blood cells (SRBCs) are standardised to 2.8% concentration. Haemolysin is titrated to determine the optimal dilution for the lot of complement used. Haemolysin is used to sensitise 2.8% SRBCs and the sensitised cells are added to all wells on the microtitre plate. The test is incubated for 30 minutes at 37°C. The plates are then centrifuged (200 ***g***), and the wells are scored for the presence of haemolysis. The following controls are used: (a) serum and control serum each with 5 CH_{50} and 2.5 CH_{50} of complement; (b) CF antigen and control antigen each with 5 CH_{50}, and 2.5 CH_{50} of complement; (c) complement dilutions of 5 CH_{50}, 2.5 CH_{50}, and 1.25 CH_{50}; and (d) cell control wells with only SRBCs and VBSG diluent. These controls test for anticomplementary serum, anticomplementary antigen, activity of complement used in the test, and integrity of the SRBC indicator system in the absence of complement, respectively.

To avoid anticomplementary effects, sera should be separated from the blood as soon as possible after collection and appropriate clotting has occurred. Positive and negative control sera should be used in the test.

2.2. Haemagglutination inhibition

The antigen for the HI test is the same as described above for the CF test. The antigen is diluted so that the amount used in each haemagglutinating unit (HAU) is from four to eight times that which agglutinates 50% of the RBCs in the test system. The haemagglutination titre and optimum pH for each antigen are determined with goose RBCs diluted in pH solutions ranging from pH 5.8 to pH 6.6, at 0.2 intervals.

Sera are diluted 1:10 in borate saline, pH 9.0, and then inactivated at 56°C for 30 minutes. Kaolin treatment is used to remove nonspecific serum inhibitors. Alternatively, nonspecific inhibitors may be removed by acetone treatment of serum diluted 1:10 in PBS followed by reconstitution in borate saline. Sera should be absorbed before use by incubation with a 0.05 ml volume of washed packed goose RBCs for 20 minutes at 4°C.

Following heat inactivation, kaolin treatment and absorption, twofold dilutions of the treated serum are prepared in borate saline, pH 9.0 with 0.4% bovalbumin. Serum dilutions (0.025 ml/well) are prepared in a 96-well round-bottom microtitre plate in twofold dilutions in borate saline, pH 9.0, with 0.4% bovalbumin. Antigen (0.025 ml/well) is added to the serum. Plates are incubated at 4°C overnight. RBCs

are derived from normal white male geese[4] and washed three times in dextrose/gelatin/veronal (DGV), and a 7.0% suspension is prepared in DGV. The 7.0% suspension is then diluted 1:24 in the appropriate pH solution, and 0.05 ml per well is added immediately to the plates. Plates are incubated for 30 minutes at 37°C. Positive and negative control sera are incorporated into each test. A test is considered to be valid only if the control sera give the expected results. Titres of 1:10 and 1:20 are suspect, and titres of 1:40 and above are positive.

2.3. Enzyme-linked immunosorbent assay

Several kits for IgM detection from equine specimens are commercially available. The ELISA is performed by coating flat-bottomed plates with anti-equine IgM capture antibody (Sahu *et al*., 1994). The example below provides a generic description of the procedure, which may vary depending on the recommendations developed by the manufacturer to achieve the optimum balance between sensitivity and specificity of the test.

The anti-equine IgM antibody is diluted in 0.5 M carbonate buffer, pH 9.6, and 50–100 µl of this solution is added to each well of a 96-well plate. The plates are incubated at 37°C for 1 hour, and then at 4°C overnight. Prior to use, the coated plates are washed three times with 200–300 µl/well of 0.01 M PBS containing 0.05% Tween 20. After the second wash, 200 µl/well of PBS/Tween/5% non-fat dried milk (or another blocking reagent) is added and the plates are incubated at room temperature for 1 hour. Following incubation, the plates are washed again three times with PBS/Tween. Test and control sera are diluted 1/400 in 0.01 M PBS, pH 7.2, containing 0.05% Tween 20, and 50 µl is added to each well. The plates are incubated at 37°C for 90 minutes and then washed three times. Next, 50 µl of viral antigen is added to all wells. The plates are incubated overnight at 4°C, and washed three times. Then, 50 µl of horseradish-peroxidase-conjugated monoclonal antibody (MAb) specific to the viral antigen used is added. The plates are incubated for 60–90 minutes at 37°C and then washed six times. Finally, 50 µl of freshly prepared ABTS (2,2'-azino-bis-[3-ethylbenzo-thiazoline-6-sulphonic acid]) substrate and hydrogen peroxide (0.1%) is added, and the plates are incubated at room temperature for 15–40 minutes. The light absorbance is measured at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing negative control antigen. To ensure specificity, each serum sample is tested for reactivity with both virus antigen and control antigen.

2.4. Plaque reduction neutralisation

The PRN test is very specific and can be used to differentiate between EEE, WEE and VEE virus infections. The PRN test is performed in duck embryo fibroblast, Vero, or BHK-21 cell cultures in 25 cm^2 flasks or six-well plates. Volumes listed below are for the flasks, and should be halved if test is performed in six-well plates. Prior to testing, serum is heat-inactivated at 56°C for 30 minutes. It can be screened at a 1/10 and 1/100 final dilution. Endpoints can be established using the PRN or HI tests if serial serum dilutions (e.g. 2-fold, 5-fold, 10-fold, etc.) are tested. This is particularly useful for paired samples obtained from one animal and separated by several days or weeks. Serum used in the PRN test is tested against 100 plaque-forming units (PFU) of virus (50 PFU for six-well plates). The virus/serum mixture is incubated at 37°C for 75 minutes before inoculation onto confluent cell culture monolayers in 25 cm^2 flasks. The inoculum is adsorbed for 1 hour, followed by the addition of 6 ml of overlay medium. The overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle's Basic Salts Solution without phenol red, 4% fetal bovine serum, 100 µg/ml gentamicin, 200 µg/ml nystatin, 0.45% solution of sodium bicarbonate, and 0.002% neutral red. When duck embryo fibroblasts are used, Solution 1 also contains 6.6% yeast extract lactalbumin hydrolysate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 48–72 hours, and endpoints are based on a 90% reduction in the number of plaques compared with the virus control flasks, which should have about 100 plaques.

4 RBCs from adult domestic white male geese are preferred, but RBCs from other male geese can be used. If cells from female geese are used, there may be more test variability. It has been reported that rooster RBCs cause a decrease in the sensitivity of the test.

C. REQUIREMENTS FOR VACCINES

1. Background

Inactivated vaccines against EEE and WEE viruses are commercially available. Attenuated EEE and WEE virus vaccines have not proven satisfactory. The vaccines licensed for use in the USA are prepared using the following combinations: EEE and WEE; EEE, WEE, and VEE; and EEE and VEE. In addition, tetanus toxoid, inactivated influenza virus, inactivated herpes viruses (EHV-1 and EHV-4) and inactivated West Nile virus have been combined with EEE and WEE or EEE, WEE, and VEE. Current vaccines are prepared from virus propagated in cell culture, and inactivated with formalin (Maire *et al*., 1970).

The recommended vaccines against VEE infection are an attenuated virus vaccine, strain TC-83, and an inactivated virus preparation made from that strain (Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989). Directions for use provided with commercial products should be followed.

Inactivated vaccine should be administered in two doses with an interval of 2–4 weeks between doses. Annual revaccination is recommended unless otherwise is indicated on the manufacturer's label.

Attenuated vaccine should be reconstituted with physiological saline and used immediately. Multidose vials are kept on ice while the vaccine is being used. Any vaccine not used within 4 hours of reconstitution should be safely discarded. Animals over 3 months of age are vaccinated subcutaneously in the cervical region with a single dose. Annual revaccination is recommended. Recommendations specific to local environs may vary based on risk. Consultation with an equine veterinarian may be beneficial for determining the appropriate schedule.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

See chapter 1.1.8 for general requirements for Master Seeds and allowable passages for vaccine production. Suitable seed lots should be maintained at –70°C in a lyophilised state.

2.1.1. Biological characteristics of the master seed

Standard strains of EEE and WEE viruses were isolated over 20 years ago, have been used for vaccine production and have been proven to produce a protective immunity. Strains of EEE virus that differ antigenically and in molecular structure have been identified from different geographical regions. However, the North American and Caribbean isolates appear to be similar (Weaver *et al*., 1994). Strains of WEE virus isolated from different countries have been found to be similar both by MAb testing and RNA oligonucleotide fingerprinting analysis (Reisen & Monath, 1989). A recent well-characterised isolate from the country where the vaccine is to be used would be advantageous. Selected viruses must be immunogenic and replicate to high titres in cell culture.

The VEE virus vaccine strain TC-83 originated from the Trinidad donkey strain (a variant of I-AB) of epizootic VEE virus isolated in 1944. This strain was derived by serial passage of the Trinidad donkey strain in fetal guinea-pig heart cells. It is safe and immunogenic at the established passage levels, and induces protective immunity in vaccinated equids, although adverse reactions can sometimes occur. The vaccine was originally developed for use in personnel involved in high-risk VEE virus research.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The MSV must be tested for purity, identity, and freedom from extraneous agents at the time before it is used in the manufacture of vaccine. The MSV must be free from bacteria, fungi and mycoplasma. The MSV is cultured on a Vero cell line and an embryonic equine cell type with confirmation by the fluorescent antibody technique to demonstrate freedom from equine

herpesvirus, equine adenovirus, equine arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus extraneous agents. The MSV must also be free from extraneous virus by cytopathic effect (CPE) and haemadsorption on cell culture on the Vero cell line and an embryonic equine cell type.

2.1.3. Validation as a vaccine strain

In an immunogenicity trial, the MSV at the highest passage level intended for production must prove its efficacy (protection) in the guinea-pig vaccination/serology potency test.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic (with pathogens with many serotypes)

In an emergency epizootic situation, there may be not enough time to fully test a new MSV for all extraneous agents; in such a situation, provisional acceptance of the new strain could be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account the characteristics of the process, including the nature and concentration of the inactivant for inactivated vaccines, before allowing release of any new product.

2.2. Method of manufacture

2.2.1. Procedure

The MSV should be propagated in cell lines known to support the growth of EEE, WEE and VEE viruses. See chapter 1.1.8 for additional guidance on the preparation and testing of master cell stocks. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the MSV, unless further passages prove to provide sufficient serological titres in guinea-pigs.

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum, may be used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with EEE, WEE or VEE working virus stock, which is generally 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–3 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Inactivated vaccines may be chemically inactivated with formalin and mixed with a suitable adjuvant. The duration of the inactivation period is based on demonstrated inactivation kinetics.

The preservatives used are thimerosal at a 1/1000 dilution and antibiotics (neomycin, polymyxin, amphotercin B, gentamicin, and others).

2.2.2. Requirements for ingredients

All ingredients used in the manufacture of EEE, WEE and VEE vaccines should be defined in approved manufacturing protocols and consistent from batch to batch. See chapter 1.1.8 for general guidance on ingredients of animal origin. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

2.2.3. In-process controls

Production lots should be examined daily for cytopathic changes. After harvesting, the virus suspension should be tested for the presence of microbial contaminants. Production lots must be titrated in cell culture before inactivation to standardise the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Inactivated lots must be tested for completeness of inactivation in 6- to 12-hour old chicks.

2.2.4. Final product batch tests

i) Sterility

Inactivated and live vaccine samples are examined for bacterial and fungal contamination. The volume of medium used in these tests should be enough to nullify any bacteriostatic or fungistatic effects of the preservatives in the product. To test for bacteria, ten vessels, each containing a minimum of 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing a minimum of 40 ml soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth. Individual countries may have other requirements.

ii) Identity

Separate batch tests for identity should be conducted if the batch potency test, such as cell culture titrations of live virus vaccines, does not sufficiently verify the identity of the agent in the vaccine. Identity tests may include fluorescent antibody or serum neutralisation assays.

iii) Safety

Batch safety testing for virus inactivation is conducted in 6- to 12-hour old chicks. Ten chicks are inoculated subcutaneously with 0.5 ml of product and observed daily for 10 days. If unfavourable reactions occur, the batch is unacceptable.

iv) Batch potency

Potency testing is performed by inoculating each of ten guinea-pigs with EEE, WEE or VEE virus vaccine stock, using one-half the horse dose on two occasions, 14–21 days apart, by the route recommended for the horse. Serum samples from each vaccinate and each control are tested 14–21 days after the second dose using the PRN test. The EEE titres should be ≥ 1/40, the WEE titres should be ≥ 1/40 and the VEE titres should be ≥ 1/4 (US Code of Federal Regulations, 2000), using Vero cells. If duck embryo fibroblasts are used in the PRN test, the titres will be lower. The use of animal batch release tests should be avoided wherever possible.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For regulatory approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

2.3.2. Safety requirements

The final inactivated vaccine formulation should be tested in a limited number of target animals prior to a larger-scale field study. The final vaccine formulation should not cause adverse reactions.

Field safety studies should be conducted before any vaccine receives final approval. Generally, two serials should be used, in three different geographical locations under typical animal husbandry conditions, and in a minimum of 600 animals. The vaccine should be administered according to label recommendations (including booster doses) and should contain the maximum permissible amount of viral antigen. (If no maximum antigen content is specified, serials should be of anticipated typical post-marketing potency.) About one-third of the animals should be at the minimum age recommended for vaccination.

i) Precautions (hazards)

Vaccine should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection) in the guinea-pig vaccination/serology potency test; each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

None known.

2.3.5. Duration of immunity

Comprehensive studies on duration of immunity are not available. An annual revaccination is recommended for the inactivated vaccine. Foals vaccinated under 1 year of age should be revaccinated before the next vector season.

2.3.6. Stability

The inactivated vaccine is stable and immunogenic for 2 years if kept refrigerated at 2–7°C. After 2 years, vaccine should be discarded. Follow recommended expiration dates on packaging.

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NB: At the time of publication (2022) there were no WOAH Reference Laboratories for equine encephalomyelitis (Eastern, Western and Venezuelan) (please consult the WOAH Web site for the current list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: EQUINE ENCEPHALOMYELITIS (EASTERN AND WESTERN) FIRST ADOPTED IN 1991; VENEZUELAN EQUINE ENCEPHALOMYELITIS FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.6.6.

EQUINE INFECTIOUS ANAEMIA

SUMMARY

Equine infectious anaemia (EIA) is a persistent viral infection of equids. The causative agent, EIA virus (EIAV) is a lentivirus in the family Retroviridae*, subfamily* Orthoretrovirinae*. Other members of the genus* Lentivirus *include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; simian immunodeficiency virus and maedi/visna virus. Although EIA may be suspected on the basis of clinical signs and pathological lesions, confirmation of infection requires further serological and molecular-based testing. Infected horses remain viraemic carriers for life and, with very rare exceptions, yield a positive serological test result. Although antibody levels fluctuate, EIA infection generates a persistent antibody response. All equids older than 12 months that test seropositive are identified as virus carriers. In young equids less than 12 months of age, positive serological reactions can be due to maternal antibodies; therefore the EIA status may have to rely solely on molecular techniques. As virus reservoirs, infected equids are a transmission risk to other equids. The virus is primarily blood-borne. Biting flies are mechanical vectors for the virus in nature and infection is often spread via iatrogenic routes.*

Identification of the agent: *Virus can be isolated by inoculating suspect blood into a susceptible horse or onto leukocyte cultures prepared from susceptible horses. Recognition of infection in experimentally challenged horses may be made on the basis of clinical signs, haematological changes, positive serological reactions and/or detection of the virus by molecular techniques. Successful virus isolation in horse leukocyte cultures is confirmed by the detection of specific EIA antigen, by immunofluorescence assay, polymerase chain reaction based techniques, or by the inoculation of culture fluids into susceptible horses. Virus isolation is rarely attempted due to the time, difficulty and expense involved.*

Serological tests: *Agar gel immunodiffusion (AGID) tests and enzyme-linked immunosorbent assays (ELISAs) are simple, reliable serological tests for the demonstration of EIAV infection. The AGID test should be used to confirm positive ELISA results. Antibody levels are highly variable, and fluctuate due to the changing nature of the virus. EIA antigens can be prepared from infected tissue cultures or by using recombinant DNA technology. A variety of licensed and validated commercial test kits is available.*

Requirements for vaccines: *An attenuated live vaccine was developed in the early 1970s and used extensively in China (People's Rep. of) between 1975 and 1990. Numerous other methods have since been attempted with variable results. The strategy for EIA control has shifted from vaccination to quarantine to avoid the interference of vaccinal antibodies with diagnostic tests. There are no vaccines currently available.*

A. INTRODUCTION

Equine infectious anaemia (EIA) occurs world-wide. The infection, formerly known as swamp fever, is limited to equids. Many cases remain clinically unapparent. The disease is characterised by recurrent febrile episodes, thrombocytopenia, anaemia, rapid loss of weight and oedema of the lower parts of the body. If death does not result from one of the acute clinical attacks, a chronic stage develops and the infection tends to become inapparent. The incubation period is normally 1–3 weeks, but may be as long as 3 months. In acute cases, lymph nodes, spleen and liver are hyperaemic and enlarged. Histologically these organs are infiltrated with nests of immature lymphocytes

and plasma cells. Kupffer cells in the liver often contain haemosiderin or erythrocytes. The enlarged spleen may be felt on rectal examination. Differential diagnoses include equine viral arteritis (Chapter 3.6.10), *Anaplasma phagocytophilum,* and other causes of oedema, fever, anaemia, or thrombocytopaenia/ecchymoses.

EIA virus (EIAV) is in the genus *Lentivirus* in the family *Retroviridae*, subfamily *Orthoretrovirinae*. Other members of the genus include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; simian immunodeficiency virus; and maedi/visna virus.

Once a horse is infected with EIAV, its blood remains infectious for the remainder of its life and the horse can potentially transmit the infection to other horses (Cheevers & McGuire, 1985). Transmission occurs by transfer of blood or contaminated secretion from an infected horse. In nature, spread of the virus is most likely via interrupted feeding of bloodsucking horseflies (*Tabanidae*) on a clinically ill horse and then on susceptible horses. Transmission can also occur by the iatrogenic transfer of blood through the use of contaminated blood products, needles, syringes, IV administration sets or other equipment. *In utero* infection of the fetus may occur (Kemen & Coggins, 1972). The virus titre is higher in horses with clinical signs and the risk of transmission is higher from these animals than the carrier animals with a lower virus titre. However, studies in mules indicate infected animals with positive enzyme-linked immunosorbent assay (ELISA) and indeterminate agar gel immunodiffusion (AGID) test results can have viral loads at the same level as animals with strong antibody responses, and are therefore equally likely as a potential source of transmission (Scicluna *et al*., 2013).

EIAV is not considered a risk for human health. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

AGID tests (Coggins *et al*., 1972) and ELISAs (Suzuki *et al*., 1982) are accurate, reliable tests for the detection of EIA in horses, except for animals in the early stages of infection and foals of infected dams (McConnico *et al*., 2000; USDA 2007). In other rare circumstances, misleading results may occur when the level of virus circulating in the blood during an acute episode of the disease is sufficient to bind available antibody, and if initial antibody levels never rise high enough to be detectable (Toma, 1980). Although the ELISA will detect antibodies somewhat earlier and at lower concentrations than the AGID test, positive ELISAs are confirmed using the AGID test. This is due to false-positive results that have been noted with ELISAs. The AGID test is specific, thus has the advantage of distinguishing between EIA and non-EIA antigen–antibody reactions. Discrepancies between testing methods or tests with questionable results can be further evaluated by immunoblot testing (Issel *et al.,* 1999; 2013; Rusvai *et al*., 2009).

Table 1. Test methods available for the diagnosis of equine infectious anaemia and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
PCR	–	+/–	–	+/–	–	–
Virus isolation	–	–	–	+	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
AGID	++	++	++	++	++	–
ELISA	++	++	++	+	+	–
Immunoblot	–	++	++	++	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Virus isolation and identification

Virus isolation is usually not necessary to make a diagnosis.

Isolation of the virus from suspect horses may be made by inoculating their blood onto leukocyte cultures prepared from horses free of infection. Virus production in cultures can be confirmed by detection of specific EIA antigen by ELISA (Shane *et al*., 1984), by immunofluorescence assay (Weiland *et al*., 1982), or by molecular tests. Virus isolation is rarely attempted because of the difficulty of growing horse leukocyte cultures.

1.2. Polymerase chain reaction

A nested polymerase chain reaction (PCR) assay to detect EIA proviral DNA from the peripheral blood of horses has been described (Nagarajan & Simard, 2001). The nested PCR method is based on primer sequences from the *gag* region of the proviral genome. It has proven to be a sensitive technique to detect field strains of EIAV in white blood cells of EIA infected horses; the lower limit of detection is typically around 10 genomic copies of the target DNA (Nagarajan & Simard, 2001; 2007). A real-time reverse-transcriptase PCR assay has also been described (Cook *et al*., 2002). To confirm the results of these very sensitive assays, it is recommended that duplicate samples of each diagnostic specimen be processed. Because of the risk of cross contamination, it is also important that proper procedures are followed (see Chapter 1.1.5 *Quality management in veterinary testing laboratories* and Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*). It should be noted that primer mismatches with circulating virus, possibly caused by the high rate of mutation in the virus, may cause a failure of PCR to detect virus (Cappelli *et al.,* 2011; Quinlivan *et al*., 2007).

The following are some of the circumstances where the PCR assay maybe used for the detection of EIAV infection in horses:

i) Conflicting results on serologic tests;

ii) Suspected infection but negative or questionable serologic results;

iii) Complementary test to serology for the confirmation of positive results;

iv) Confirmation of early infection, before serum antibodies to EIAV develop;

v) Ensuring that horses that are to be used for antiserum or vaccine production or as blood donors are free of EIAV;

vi) Confirmation of the status of a foal from an infected mare.

2. Serological tests

Due to the persistence of EIAV in infected equids, detection of serum antibody to EIAV confirms the diagnosis of EIAV infection.

2.1. Agar gel immunodiffusion test

The AGID test detects precipitating antibody produced in response to EIAV infection. Specific reactions are indicated by precipitation lines between the EIA antigen and the test serum and confirmed by their identity with the reaction between the antigen and the positive standard serum.

Reagents for AGID are available commercially from several companies. Alternatively, AGID antigen and reference serum may be prepared as described below.

2.1.1. Preparation of antigen

Specific EIA antigen may be prepared from the spleen of acutely infected horses (Coggins *et al*., 1973), from infected equine tissue culture (Malmquist *et al*., 1973), from a persistently infected canine thymus cell line (Bouillant *et al*., 1986), or from proteins expressed in bacteria or baculovirus using the recombinant DNA technique (Archambault *et al*., 1989; Kong *et al*., 1997). Preparation from infected cultures or from recombinant DNA techniques gives a more uniform result than the use of spleen cells and allows for better standardisation of reagents.

To obtain a satisfactory antigen from spleen, a horse must be infected with a highly virulent strain of EIAV. The resulting incubation period should be 5–7 days, and the spleen should be collected 9 days after inoculation, when the virus titre is at its peak and before any detectable amount of precipitating antibody is produced. Undiluted spleen pulp is used in the immunodiffusion test as antigen (Coggins *et al*., 1973). Extraction of antigen from the spleen with a saline solution and concentration with ammonium sulphate does not give as satisfactory an antigen as selection of a spleen with a very high titre of EIA antigen.

Alternatively, equine fetal kidney or dermal cells or canine thymus cells are infected with a strain of EIAV adapted to grow in tissue culture (American Type Culture Collection, or Chinese strain adapted to equine fetal dermal cells). Virus is collected from cultures by precipitation with 8% polyethylene glycol or by pelleting by ultracentrifugation. The diagnostic antigen, p26, is released from the virus by treatment with detergent or ether (Malmquist *et al*., 1973). EIAV core proteins, expressed in bacteria or baculovirus, are commercially available and find practical use as high quality antigens for serological diagnosis.

The p26 is an internal structural protein of the virus that is coded for by the *gag* gene. The p26 is more antigenically stable among EIAV strains than the virion glycoproteins gp45 and gp90 (Montelaro *et al*., 1984). There is evidence of strain variation in the p26 amino acid sequence; however there is no evidence to indicate that this variation influences any of the serological diagnostic tests (Zhang *et al*., 1999).

2.1.2. Preparation of standard antiserum

A known positive antiserum may be collected from a horse previously infected with EIAV. This serum should yield a single dense precipitation line that is specific for EIA, as demonstrated by a reaction of identity in comparison with a known standard serum. It is essential to balance the antigen and antibody concentrations in order to ensure the optimal sensitivity of the test. Reagent concentrations should be adjusted to form a narrow precipitation line approximately equidistant between the two wells containing antigen and serum.

2.1.3. Test procedure (Association Française de Normalisation [AFNOR], 2000; Coggins *et al*., 1973; Pearson & Coggins, 1979)

i) Immunodiffusion reactions are carried out in a layer of agar in plastic Petri dishes as glass dishes can result in slippage. For Petri dishes that are 100 mm in diameter, 15–17 ml of 1% Noble agar in 0.145 M borate buffer (9 g H_3BO_3, plus 2 g NaOH per litre), pH 8.6 (± 0.2) is used. A metal punch is used to create several "rosettes," each of six wells surrounding a centre well

of the same diameter. The wells are 5.3 mm in diameter and 2.4 mm apart. Each well must contain the same volume of reagent and should be completely but not over-filled.

ii) The antigen is placed in the central well and the standard antiserum is placed in alternate exterior wells. Serum samples for testing are placed in the remaining three wells.

iii) The dishes are maintained at room temperature in a humid environment (18°C–26°C recommended).

iv) After 24–48 hours the precipitation reactions are examined over a narrow beam of intense, oblique light and against a black background. The reference lines should be clearly visible at 24 hours, and at that time any test sera that are strongly positive may also have formed lines of identity with those between the standard reagents. A weakly positive reaction may take 48 hours to form and is indicated by a slight bending of the standard serum precipitation line between the antigen well and the test serum well. For EIA AGID, the bending caused by a weak positive reaction looks like a very small hook or rounding into the sample well. Sera with high precipitating antibody titres will form a complete line of identity or may form broader precipitin bands that tend to be diffuse. Such reactions can be confirmed as specific for EIA by dilution at 1/2 or 1/4 prior to retesting; these then give a more distinct line of identity. Sera devoid of EIA antibody will not form precipitation lines and will have no effect on the reaction lines of the standard reagents. Nonspecific precipitation lines may occur. These nonspecific lines can cross the control lines, typically showing no line of identity with the control lines.

v) Interpretation of the results: Horses that are in the early stages of an infection may not give a positive serological reaction in an AGID test. Such animals should be bled again after 3–4 weeks. To make a diagnosis in a young foal, it may be necessary to determine the antibody status of the dam. If the mare passes EIA antibody to the foal through colostrum, then a period of 6 months or longer after birth must be allowed for the maternally-derived antibody to wane. Sequential testing of the foal at monthly intervals may be useful to observe the decline in maternal antibody. To conclude that the foal is not infected, a negative result must be obtained (following an initial positive result) at least 2 months after separating the foal from contact with the EIA positive mare or any other positive horse. It should be noted that maternal antibodies can often be detected for up to 12 months of age, therefore alternative diagnostic methods should be considered, for example PCR could be used to determine the presence/absence of EIA virus in the blood of the foal.

2.2. Enzyme-linked immunosorbent assay

Several diagnostic test kits for EIA, including AGID and ELISA, are licensed in various countries for the diagnosis of equine infectious anaemia and are available internationally. The ELISAs generally target antibody produced against the p26 core protein antigen but may also have a second target antibody produced against the gp45 antigen. These antigens may also be synthetic fusion proteins or recombinant antigens. Typical ELISA protocols are used. If commercial ELISA materials are not available, a non-competitive ELISA using p26 antigen purified from cell culture material may be employed (Shane *et al*., 1984).

A positive test result by ELISA should be confirmed using the AGID test because false-positive results have been noted with the ELISA. The results can also be confirmed by the immunoblot technique. A standard antiserum for immunodiffusion, which contains detectable antibody, is available from the WOAH Reference Laboratories[1]. This standard should not be used as the reference for minimum detection limits for the ELISA reaction. Uniform methods for EIA control have been published (USDA, 2007).

C. REQUIREMENTS FOR VACCINES

Inactivated and subunit EIAV vaccines were tested in different laboratories and proved to protect infections of homologous prototype strains only. An attenuated live vaccine, developed in the early 1970s, was extensively used

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

in China (People's Rep. of) between 1975 and 1990 and was effective in controlling the prevalence of EIA. With low prevalence since 1990, the strategy for EIA control has shifted from vaccination to quarantine to avoid the interference of vaccine antibodies with diagnostic tests.

Although no safety concerns arose with the use of attenuated EIAV vaccine in China, it should be noted that, like other lentiviruses, EIAV is highly mutable and can integrate into host genomes. The use of a live EIAV vaccine should be considered only after a thorough risk assessment.

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NB: There are WOAH Reference Laboratories for equine infectious anaemia:
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for equine infectious anaemia

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2019.

CHAPTER 3.6.7.

EQUINE INFLUENZA (INFECTION WITH EQUINE INFLUENZA VIRUS)

SUMMARY

Description of the disease: Equine *influenza is an acute respiratory infection of horses, donkeys, mules and zebras caused by two distinct subtypes (H7N7, formerly equi-1, and H3N8, formerly equi-2) of influenza A virus within the genus* Influenzavirus *A of the family* Orthomyxoviridae. *Viruses of the H7N7 subtype have not been isolated since the late 1970s. Equine influenza viruses of both subtypes are considered to be of avian ancestry and highly pathogenic avian H5N1 has been associated with an outbreak of respiratory disease in donkeys in Egypt. In fully susceptible equidae, clinical signs include pyrexia and a harsh dry cough followed by a mucopurulent nasal discharge. In partially immune vaccinated animals, one or more of these signs may be absent. Vaccinated infected horses can still shed the virus and serve as a source of virus to their cohorts. Characteristically, influenza spreads rapidly in a susceptible population. The disease is endemic in many countries with substantial equine populations.*

While normally confined to equidae, equine H3N8 influenza has crossed the species barrier to dogs. Extensive infection of dogs has been reported in North America where it normally produces mild fever and coughing but can cause fatal pneumonia. While equine influenza has not been shown to cause disease in humans, serological evidence of infection has been described primarily in individuals with an occupational exposure to the virus. During 2004–2006 influenza surveillance in central China (People's Rep. of) two equine H3N8 influenza viruses were also isolated from pigs.

Identification of the agent: *Embryonated hens' eggs and/or cell cultures can be used for virus isolation from nasopharyngeal swabs or nasal and tracheal washes. Isolates should always be sent immediately to a WOAH Reference Laboratory. Infection may also be demonstrated by detection of viral nucleic acid or antigen in respiratory secretions using the reverse-transcription polymerase chain reaction (RT-PCR) or an antigen-capture enzyme-linked immunosorbent assay (ELISA), respectively.*

Serological tests: *Diagnosis of influenza virus infections is usually only accomplished by tests on paired sera; the first sample should be taken as soon as possible after the onset of clinical signs and the second approximately 2 weeks later. Antibody levels are determined by haemagglutination inhibition (HI), single radial haemolysis (SRH) or ELISA.*

Requirements for vaccines: *Spread of infection and severity of disease may be reduced by the use of potent inactivated equine influenza vaccines containing epidemiologically relevant virus strains. Inactivated equine influenza vaccines contain whole viruses or their subunits. The vaccine viruses are propagated in embryonated hens' eggs or tissue culture, concentrated, and purified before inactivation with agents such as formalin or beta-propiolactone. Inactivated vaccines provide protection by inducing humoral antibody to the haemagglutinin protein. Responses are generally short-lived and multiple doses are required to maintain protective levels of antibody. An adjuvant is usually required to stimulate durable protective levels of antibody. Live attenuated virus and viral vectored vaccines have been licensed in some countries.*

Vaccine breakdown has been attributed to inadequate vaccine potency, inappropriate vaccination schedules, and outdated vaccine viruses that are compromised as a result of antigenic drift. An in-vitro potency test (single radial diffusion) can be used for in-process testing of the antigenic content of inactivated products before addition of an adjuvant. In process testing of live and vectored vaccines relies on titration of infectious virus. International surveillance programmes monitor antigenic drift among equine influenza viruses and each year the Expert Surveillance Panel (ESP) for Equine

Influenza makes recommendations for suitable vaccine strains. Following a change in recommendations, vaccines should be updated as quickly as possible to ensure optimal protection. This is particularly important for highly mobile horse populations and for any horse travelling internationally.

A. INTRODUCTION

Equine influenza is caused by two subtypes: H7N7 (formerly subtype 1) and H3N8 (formerly subtype 2) of influenza A viruses (genus *Influenzavirus* A of the family *Orthomyxoviridae*); however there have been very few reports of H7N7 subtype virus infections in the last 30 years (Webster, 1993).

In fully susceptible equidae, clinical signs include pyrexia, nasal discharge and a harsh dry cough; pneumonia in young foals and donkeys and encephalitis in horses have been described as rare events (Daly *et al*., 2006; Gerber, 1970). Clinical signs associated with infection in dogs also include fever and a cough; occasionally infection results in suppurative bronchopneumonia and peracute death (Crawford *et al.,* 2005). Characteristically, influenza spreads rapidly in a susceptible population. The virus is spread by the respiratory route, and indirectly by contaminated personnel, vehicles and fomites. The incubation period in susceptible horses may be less than 24 hours. In partially immune vaccinated animals the incubation period may be extended, one or more clinical signs may be absent and spread of the disease may be limited. This makes clinical diagnosis of equine influenza more difficult as other viral diseases, such as equine herpesvirus-associated respiratory disease, may clinically resemble a mild form of influenza. Horses infected with equine influenza virus become susceptible to secondary bacterial infection and may develop mucopurulent nasal discharge, which can lead to diagnosis of bacterial disease with the underlying cause being overlooked.

Equine influenza viruses are believed to be of avian ancestry, and more recent transmission of avian viruses to horses and donkeys has been recorded. The sequence analysis of an H3N8 virus isolated in 1989 from horses during a limited influenza epidemic in North Eastern China (People's Rep. of) established that the virus was more closely related to avian influenza viruses than to equine influenza viruses (Guo *et al*., 1992). Avian H5N1 has been associated with respiratory disease of donkeys in Egypt (Abdel-Moneim *et al*., 2010).

Equine influenza viruses have the potential to cross species barriers and have been associated with respiratory disease in dogs primarily in North America (Crawford *et al*., 2005). Isolated outbreaks of equine influenza have also occurred in dogs within the UK but the virus has not become established in the canine population. Close contact with infected horses was thought to be involved in each outbreak in the UK. Equine influenza viruses have also been isolated from pigs in central China (People's Rep. of) (Tu *et al*., 2009). Despite the occasional identification of seropositive persons with occupational exposure there is currently little evidence of zoonotic infection of people with equine influenza (Alexander & Brown, 2000).

In endemic countries the economic losses due to equine influenza can be minimised by vaccination and many racing authorities and equestrian bodies have mandatory vaccination policies. Vaccination does not produce sterile immunity; vaccinated horses may shed virus and contribute silently to the spread of the disease. Appropriate risk management strategies to deal with this possibility should be developed.

For recent information on distribution at the country level please consult the WAHIS interface (https://wahis.woah.org/#/home).

B. DIAGNOSTIC TECHNIQUES

Test methods available for the diagnosis of equine influenza and their purpose are summarised in Table 1. Laboratory diagnosis of acute equine influenza virus infections is based on virus detection in nasal swabs collected from horses with acute respiratory illness. Alternatively, the demonstration of a serological response to infection may be attempted with paired serum samples. Ideally, both methods are used. Equine influenza virus may be isolated in embryonated hens' eggs or cell culture. Infection may also be demonstrated by detection of viral antigen in respiratory secretions using an antigen capture enzyme-linked immunosorbent assay (ELISA) or of viral genome using reverse-transcription polymerase chain reaction (RT-PCR) assays. All influenza viruses are highly contagious for susceptible hosts and care must therefore be taken during the handling of infected eggs or cultures to avoid accidental cross-contamination. Standard strains should not be propagated in the diagnostic laboratory, at least

never at the same time or in the same place where diagnostic samples are being processed. All working areas must be efficiently disinfected before and after virus manipulations, which should preferably be conducted within biohazard containment level 2 and class II safety cabinets.

It is important to obtain samples as soon as possible after the onset of clinical signs, preferably within 3–5 days. These samples include nasopharyngeal swabs and nasal or tracheal washings, the latter taken by endoscopy. Swabs may consist of absorbent cotton wool sponge/gauze on wire, and should be long enough to be passed via the ventral meatus into the nasopharynx. Swabs should be transferred to a tube containing transport medium immediately after use. This medium consists of phosphate buffered saline (PBS) containing either 40% glycerol or 2% tryptose phosphate broth with 2% antibiotic solution (penicillin [10,000 units], streptomycin [10,000 units] in sterile distilled water [100 ml]), and 2% fungizone (250 mg/ml stock). If the samples are to be inoculated within 1–2 days they may be held at 4°C, but, if kept for longer, they should be stored at –70°C or below. Samples should be kept cool during transport to the laboratory.

Sample processing should follow the quality procedures outlined in Chapter 1.1.5 *Quality management in veterinary testing laboratories*, taking measures to prevent cross contamination. The liquid is expelled from the swab by squeezing with forceps and the swab is then disposed of suitably. Further antibiotics may be added if samples appear to be heavily contaminated with bacteria. The remainder of the fluid is stored at –70°C. Samples treated with antibiotics are allowed to stand on ice for 30–60 minutes and are then centrifuged at 1500 ***g*** for 15 minutes to remove bacteria and debris; the supernatant fluids are used for inoculation. The remainder of the fluid is stored at –70°C. Filtration of samples is not advised as influenza virus may adsorb on to the filter and be lost from the sample.

Table 1. Test methods available for the diagnosis of equine influenza

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[a]						
Virus isolation	–	+	–	++	–	–
Real-time RT-PCR	–	+++	+++	+++	+++	–
RAD	–	+	–	++	+	–
Antigen-capture ELISA	–	++	++	+++	++	–
Detection of immune response						
HI	++	++[b]	–	+++[b]	+++	++
SRH	++	++[b]	–	+++[b]	+++	+++
ELISA	++	+	++[c]	+[b]	+++	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; RAD = rapid antigen detection;
HI = haemagglutination inhibition; SRH = single radial haemolysis; ELISA = enzyme-linked immunosorbent assay.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]Testing of paired samples required;
[c]May be useful for DIVA (detection of infection in vaccinated animals) when used with appropriate vaccines.

1. Identification of the agent

Isolation of infectious virus may be carried out in embryonated hens' eggs or cell cultures. Traditionally, eggs have been preferred for isolation of equine influenza as many clinical isolates do not grow well in cells without serial passage. Comparison of H3N8 viruses isolated in eggs and Madin–Darby canine kidney (MDCK) cells indicated that MDCK cells are capable of selecting variant viruses that are not representative of the predominant virus in clinical specimens (Ilobi *et al.*, 1994). However, some viruses have been successfully isolated in MDCK cells but not in eggs and selection of variants has also occurred as a result of culture in eggs (Oxburgh & Klingborn, 1999). Ideally, isolation should be attempted using both substrates.

RT-PCR and real-time RT-PCR assays are being widely used in diagnostic laboratories as a more sensitive alternative to virus isolation (Quinlivan *et al.*, 2005). Influenza virus antigen in nasal secretions may also be detected directly by a sensitive antigen-capture ELISA for the H3N8 virus using a monoclonal antibody (MAb) against the equine influenza virus nucleoprotein (Livesay *et al.*, 1993).This assay is not commercially available, other than as a diagnostic service, but commercial self-contained kits for detecting human influenza are available and have been shown to detect equine influenza antigen (Chambers *et al.*, 1994; Yamanaka *et al.*, 2008). This approach is less sensitive than RT-PCR but provides a rapid result on which management decisions may be based. It should not be used to the exclusion of virus isolation. It is essential that new viruses be isolated and sent to reference laboratories for characterisation as part of the surveillance programme to monitor antigenic drift and emergence of new viruses and to provide isolates for inclusion in updated vaccines. Positive RT-PCR and ELISA results are useful in the selection of samples for virus isolation attempts if resources are limited, or for the selection of specimens to be sent to a reference laboratory for virus isolation and characterisation.

1.1. Virus isolation in embryonated hens' eggs

Fertile eggs are set in a humid incubator at 37–38°C and turned twice daily; after 10–11 days, they are examined by candling and live embryonated eggs are selected for use. The area above the air sac is cleansed with alcohol and a small hole is made through the shell. Inoculum may be introduced into the amniotic or the allantoic cavity. Several eggs/sample are inoculated (0.1 ml) in the amniotic cavity with no additional dilution of the sample (sample may also be diluted 1/10 and 1/100 in PBS containing antibiotics). The syringe is withdrawn approximately 1 cm and a further 0.1 ml is inoculated into the allantoic cavity. Alternatively, many laboratories opt to inoculate into the allantoic cavity alone through a second hole drilled just below the line of the air sac. The hole(s) is/are sealed with wax or Sellotape, and the eggs are incubated at 34–35°C for 3 days. The embryos that die within 24 hours following inoculation should be discarded. The eggs that contain embryos that die more than 24 hours after inoculation or contain live embryos after 3 days are examined for the presence of equine influenza virus.

The eggs are transferred to 4°C for 4 hours or overnight to kill the embryos and to reduce bleeding at harvest. The shells are disinfected, and the amniotic and/or allantoic fluid is harvested by pipette, each harvest being kept separate. These are tested for haemagglutination (HA) activity by mixing twofold dilutions of the harvested fluid in equal volumes (0.025 ml) with chicken red blood cells (RBCs) (0.5% [v/v] packed cells in PBS) in V- or U-bottomed microtitre plates or 0.4% guinea-pig RBCs (0.4% [v/v] packed cells in PBS) in V- or U-bottomed plates. Plates are incubated for approximately 30 minutes preferably at 4°C to prevent neuraminidase activity. If chicken RBCs are used, the plates may be read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. Non-agglutinated guinea-pig cells appear as a button at the bottom of the well and may take longer to settle. If there is no HA activity, aliquots of each harvest are pooled and passaged into further eggs. All HA positive samples are divided into aliquots and stored at –70°C; one aliquot is titrated for HA immediately. The HA titre is the reciprocal of the greatest dilution to show agglutination. If the HA titre is 1/16 or more, the isolate is characterised immediately. If titres are low, positive samples should be passaged. Care should be taken to avoid generation of defective interfering particles by prediluting the inoculum 1/10, 1/100, 1/1,000. Positive samples arising from the highest dilution should be selected as stocks for storage. It may be necessary to undertake as many as five passages to isolate the virus, particularly from vaccinated horses. If virus has not been recovered by the fifth passage, further passages are unlikely to be successful.

1.2. Virus isolation in cell cultures

Cultures of the MDCK cell line (MDCK, ATCC CCL34) may be used to isolate equine influenza viruses. The cells are grown to confluence in tubes and then infected in triplicate with 0.25–0.5 ml of each sample, processed as described above. Prior to inoculation, the cell monolayer is washed at least once with tissue

culture medium containing trypsin (2 μg/ml) without serum. The cultures are maintained with serum-free medium containing 0.5–2 μg/ml trypsin (treated with TPCK [L-1-tosylamine-2-phenylethyl chloromethyl ketone] to remove chymotrypsin, available pretreated, e.g. from Sigma), and examined daily for evidence of cytopathic effects (CPE). If positive, or after 7 days in any case, the supernatant fluids are tested for HA. Fluids with titres of ≥1/16 are characterised immediately. Negative fluids and those with titres <1/16 are repassaged up to five passages.

Alternatively, the cells are screened for evidence of haemadsorption (HAD). This procedure detects expression of viral antigens at the cell surface. The medium is removed from the cultures and the tubes are washed with PBS. One or two drops of a 50% suspension of chicken or guinea-pig RBCs are added, the tubes are rotated carefully, and kept at room temperature (23°C ± 2°C) for 30 minutes. Unbound RBCs are washed off with PBS, and the cultures are examined microscopically for evidence of HAD.

1.3. Haemagglutinin subtyping

The HA subtype of new isolates of equine influenza viruses may be determined by haemagglutination inhibition (HI; Section B.2.1) using H7N7- and H3N8-specific antisera. Isolates may first be treated with Tween 80/ether, which destroys viral infectivity and reduces the risk of cross-contamination. In the case of H3N8 viruses particularly, this treatment enhances the HA activity (John & Fulginiti, 1966). However, treatment with Tween 80/ether also decreases specificity and may increase the variability of the results obtained. Standard antigens must be titrated in parallel with tests to identify viruses and should include H7N7 strains (e.g. A/eq/Prague/56, A/eq/Newmarket/77) and H3N8 strains (e.g. A/eq/Newmarket/2/93, and A/eq/South Africa/4/03 and A/eq/Richmond/1/07). Virus strains may be obtained from WOAH Reference Laboratories (see Table given in Part 4 of this *Terrestrial Manual*). Additionally, recent isolates from the same geographical area should be included if available. The standard antigens should be treated with Tween 80/ether to avoid cross-contamination. Test antigens and standard antigens are always back-titrated to confirm their antigen content.

Since the 1980s, only subtype H3N8 viruses have been isolated from horses. The HA sequence of equine H3 isolates can be determined rapidly by RT-PCR and sequencing, as described by Rash *et al.* (2014), and is encouraged for surveillance purposes.

New isolates of equine influenza viruses may be further characterised by HI using strain-specific antisera. The species in which antibodies are raised will influence the cross-reactivity of the antiserum, with ferrets providing the most strain-specific antibody (Mumford, 1992). The specificity and cross reactivity of the sera are also influenced by the immunisation schedule. Sera obtained 3 weeks after a single antigen application are considered to be most discriminative.

All isolates should be sent immediately to an International Reference Laboratory designated by WOAH or the World Health Organization (WHO) for inclusion in the strain surveillance programme to monitor antigenic drift and emergence of new viruses.

1.4. Neuraminidase subtyping

Subtyping of neuraminidase requires specific antisera and no routine technique is available. Subtyping can also be done using specific PCR primers. Since the 1980s only subtype H3N8 viruses have been isolated from horses. The NA sequence of equine H3 isolates can be determined rapidly by RT-PCR and sequencing, as described by Rash *et al.* (2014), and is encouraged for surveillance purposes.

1.5. Polymerase chain reaction

RT-PCR assays both conventional and real-time, are routinely used for the detection of equine influenza genome in nasal secretions as they are more sensitive than virus culture in eggs or detection of nucleoprotein using rapid antigen detection (RAD) kits for the detection of human influenza. A probe-based real-time RT-PCR assay based on the matrix gene and developed for the detection of a wide range of influenza type A strains including avian H5N1 (Heine et al., 2007) was combined with an automated DNA extraction system to establish a high throughput assay used in the mass screening of horses during the eradication programme in Australia in 2007 (Foord *et al.*, 2009). This type of paninfluenza assay has been used effectively for diagnosis and surveillance of equine influenza by WOAH Reference Laboratories (Gildea *et al.,* 2013a). Real-time RT-PCR assays specific for equine-2 influenza (H3N8) and

equine-1 (H7N7) viruses have also been described and a commercial RT-PCR kit for the detection of equine influenza has become available (Lu *et al.*, 2009).

The WOAH Reference Laboratories selected a pan-reactive influenza type A real-time RT-PCR assay targeting the matrix gene based on the assay described by Heine *et al*. (2007) for validation in line with the WOAH Validation Template. The assay has been validated for the intended purpose of certifying freedom from infection in individual animals for trade or movement.

The sequences of the PCR primers used are:

Forward: 5'-AGA-TGA-GYC-TTC-TAA-CCG-AGG-TCG-3'

Reverse: 5'-TGC-AAA-NAC-ATC-YTC-AAG-TCT-CTG-3'

Probe: 6-FAM-5'-TCA-GGC-CCC-CTC-AAA-GCC-GA-3'-TAMRA

N=A, G, C or T and Y=C or T

The protocol below may require modification to accommodate individual laboratory or different RT-PCR kit requirements. Strong positive, weak positive and negative reference samples are available from the WOAH Reference Laboratory at the Irish Equine Centre to facilitate the setting up, validation and monitoring of the assay.

i) RNA is extracted from 100 µl nasopharyngeal secretions. Nucleic acid is eluted in 80 µl of elution buffer and 5 µl is used as the template in each 25 µl one-step RT-PCR reaction.

   Commercial extraction kits are widely available; the viral nucleic acid extraction step can be performed according to the procedures specified in each kit. A synthetic internal positive control is introduced at this stage and is included throughout the process to monitor for PCR inhibitors, i.e. to reduce the likelihood of false negatives.

ii) Kits for the one-step real-time RT-PCR are available commercially. Below are some basic steps that can be modified depending on local requirements, kits used and equipment available.

iii) Primers and probes are stored at a working dilution of 10 µM.

iv) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared, following the manufacturer's instructions. Typically, 20 µl per sample consists of 12.5 µl 2 × RT buffer, 2.0 µl tRNA, 0.8 µl of each primer (final concentration 0.32 µM), 0.2 µl of probe (final concentration 0.08 µM), 1.0 µl 25 × RT-PCR enzyme mix, 1.25 µl IPC (internal positive control) primer/probe mix and 1.45 µl nuclease free water.

v) 5 µl of RNA sample including test and positive and negative controls, are added to 20µl of the master mix in the appropriate wells of the PCR plate.

vi) The plate is placed in a real-time thermal cycler programmed for reverse transcription and cDNA amplification or fluorescence detection as suggested by the manufacturers. The following thermal profile is an example: reverse transcription at 45°C for 10 minutes followed by denaturation at 95°C for 10 minutes. cDNA is amplified by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds

Note: times and temperatures may vary and should be optimised for the reagents or kit used.

1.5.1. Interpretation of the results

The results are expressed at cycle threshold (Ct) values representing the number of cycles necessary for a statistically significant rise in reporter dye emission. A threshold bar used to determine these values is set manually to a consistent ΔRn value about halfway along the linear portion of an amplification plot. Ct values up to 40 are considered positive. Samples are classified as negative if the IPC Ct is within the range acceptable to the manufacturer and the Ct for influenza is 40 or above.

The real-time RT-PCR assay outlined above is not specific to equine influenza virus and will also detect avian and swine influenza virus. It is possible that it will also detect human influenza virus that may incidentally contaminate either the equine nasopharynx or the sample, although it is considered that the risk of this is very low. To further investigate any samples that test positive by RT-PCR, attempts should

be made to sequence the HA gene. The assay described below is based on that described by Rash *et al.* (2014) and has been used for both Florida clade 1 and Florida clade 2 isolates. However, with the continued divergence of the two clades, primer sequences may need to be modified to remain optimal and should be compared against recent equine influenza virus sequences available on public sequence databases. The HA gene is amplified as a set of four overlapping PCR products, whilst three overlapping amplicons are produced from the NA gene. Each amplicon is between 400 and 600 bp in length. Equine influenza H3N8-specific PCR primers are tagged at the 5' end with M13 forward or reverse primer sequences, simplifying subsequent sequencing reactions. The protocol below describes a general method and may need adapting for use with different enzymes and commercial kits.

1.5.2. cDNA synthesis

cDNA is transcribed from viral RNA (extracted as described above) by reverse transcription (RT). Reverse transcriptase enzymes should be used following the manufacturer's instructions. Firstly, 4 µl RNA is denatured in the presence of a modified UNI-12 primer (5'-AGT-AGC-RAA-AGC-AGG-3', final concentration 1 µM) and 7 µl water at 70°C for 10 minutes before cooling on ice. Following this, dNTP mix (final concentration 0.5 µM each dNTP), DTT (10 mM final concentration), 1 × reaction buffer and 200 U reverse transcriptase enzyme are added to a final reaction volume of 20 µl. The reaction is incubated at 42°C for 1 hour followed by an inactivation step at 65°C for 20 minutes. A negative RT control should also be included where water is substituted for the RNA.

1.5.3. PCR amplification of HA and NA gene segments

A high-fidelity DNA polymerase enzyme is used to amplify the HA and NA gene segments by PCR. For each amplicon, 50 µl PCR reactions are made consisting of 2 µl cDNA, dNTP mix (0.2 µM each dNTP), 1 × reaction buffer, 1U DNA polymerase, water and primer pairs (final concentration each of 0.2 µM) as listed in Table 2. The following cycling conditions may need adjusting depending on the DNA polymerase used: initial denaturation at 98°C for 30 seconds, followed by 30 cycles of 98°C for 10 seconds, 50°C for 30 seconds and 72°C for 30 seconds and a final extension at 72°C for 2 minutes. A negative control should be included for each primer pair where the product from the negative RT control is substituted for the cDNA.

Table 2. Primer sequences for amplifying the HA and NA gene segments of H3N8 equine influenza virus by PCR for sequencing

Primer name	Primer sequence (5'–3')	Approximate nucleotide coverage
HA/AF	GC-GTAAAACGACGGCCAGT AGCAAAAGCAGGGGACGATATT	1–515
HA/AR	GC-AACAGCTATGACCATG GATTTGTTAGCCAATTCAG	
HA/BF	GC-GTAAAACGACGGCCAGT CAGGTGTCACTCAAAACG	428–1032
HA/BR	GC-AACAGCTATGACCATG GGATTTGCTTTTCTGGTAC	
HA/CF	GC-GTAAAACGACGGCCAGT GGTTACATATGGAAAATGCC	939–1336
HA/CR	GC-AACAGCTATGACCATG GAGCCACCAGCAATTCT	
HA/DF	GC-GTAAAACGACGGCCAGT GAAGGAAGAATTCAGGA	1251–1733
HA/DR	GC-AACAGCTATGACCATG GAGTAGAAACAAGGGTGTTTTTAAC	
NA/AF	GC-GTAAAACGACGGCCAGT AGCAAAAGCAGGAGTTT	1–508
NA/AR	GC-AACAGCTATGACCATG GCCCTATTTTGACACTC	
NA/BF	GC-GTAAAACGACGGCCAGT CACACAGGGCTCATTAC	417–1049
NA/BR	GC-AACAGCTATGACCATG CCGAAACCTTTTACACCG	
NA/CF	GC-GTAAAACGACGGCCAGT CACAGTTGGATATTTGTG	951–1461
NA/CR	GC-AACAGCTATGACCATG AGTAGAAACAAGGAGTT	

The PCR products can be visualised following electrophoresis on a 0.8% agarose gel stained with a nucleic acid stain. PCR products may be purified using a commercial kit or by ethanol precipitation. Sequencing reactions are carried out using chain-terminating dideoxynucleotides and the M13 forward (5'-GTA-AAA-CGA-CGG-CCA-GT-3') and reverse (5'-AAC-AGC-TAT-GAC-CATG-3') primers for each PCR product to determine the sequence on both DNA strands. The final sequences can be assembled from the overlapping amplicons and any primer-derived sequence can then be removed.

Although the genetic sequence of isolates can also be derived from PCR assays it remains essential to isolate infectious virus in order to examine the antigenic properties of new isolates and evaluate antigenic drift in the field.

2. Serological tests

Influenza infections can be detected by performing serological tests on paired sera to show a rise in specific antibody. These tests should be carried out whether virus isolation has been attempted or not. They are robust and may prove positive in the absence of virus isolation. Two simple methods exist, HI and single radial haemolysis (SRH), each equally efficient and widely used. ELISAs for antibodies against influenza nucleoprotein are available but while less commonly used have been shown to be robust and are useful for testing large numbers of samples (Galvin *et al.,* 2013, Kittleberger *et al.,* 2011, Sergeant *et al.,* 2011). The complement fixation (CF) test can also be applied, but is not in general use. The paired serum samples, i.e. the acute and convalescent samples, should be tested together to minimise the impact of inter-assay variability. The standard antigens are described above (Section B.1.3). If available, isolates from recent cases should be included. Freeze-dried post-infection equine antisera to A/eq/Newmarket/77 (H7N7), A/eq/Newmarket/1/93 (American lineage H3N8), A/eq/Newmarket/2/93 (European lineage H3N8), A/eq/South Africa/4/03 (Florida Clade 1, sublineage of American lineage), A/eq/Richmond/07 (Florida Clade 2, sublineage of American lineage) and an influenza-negative equine serum, are available from the European Directorate for the Quality of Medicines (EDQM)[1]. These sera have been assigned SRH values through an international collaborative study and can be used as primary reference sera for the assay (Daly *et al.*, 2007; Mumford, 2000).

2.1. Haemagglutination inhibition test

The antigen is first treated with Tween 80/ether in order to increase the sensitivity of the test, particularly for H3N8 viruses. The HI test can also be performed without ether treatment although at reduced sensitivity. The test is best done in microtitre plates using the appropriate dilution equipment. A macrotest may be used, for which antigen is diluted to a final HA titre of 1/8 per well and the volumes for PBS, sera and antigen are 0.5 ml. Sera are pretreated to remove nonspecific haemagglutinins, then inactivated at 56°C for 30 minutes. Pretreatments include the use of one of the following: (a) kaolin and RBCs absorption, not recommended for H7N7 HI, (b) potassium periodate, or (c) *Vibrio cholerae* receptor-destroying enzyme. Potassium periodate or *V. cholerae* receptor-destroying enzyme is the treatment of choice. The treated sera are diluted in PBS, a standard dose of antigen is added (HA titre of 1/4 per well for microtitration assay), and these are kept at room temperature (23°C ± 2°C) for 30 minutes. After gentle mixing, RBCs are added and the test is read 30 minutes later. The HI titres are read as the highest dilution of serum giving complete inhibition of agglutination. Either chicken RBCs (1% [v/v] packed cells) in V-bottomed microtitre plates or guinea-pig RBCs (0.5% [v/v] packed cells) in V- or U-bottomed plates may be used. If chicken RBCs are used, the plates may read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. Non-agglutinated guinea-pig cells appear as a 'button' in the bottom of the well and may take longer to settle. The HI titre is the reciprocal of the greatest dilution showing complete inhibition of agglutination. Currently, a cut-off point for positive samples has not been determined for the HI test and thus, low titres should be investigated further. Titre increases of fourfold or more between paired sera indicate recent infection.

2.1.1. Tween 80/ether treatment of the virus

i) To 39.5 ml of infective allantoic fluid, add 0.5 ml of a 10% (v/v) suspension of Tween 80 in PBS to give a 0.125% (v/v) concentration of Tween 80.

ii) After mixing gently at room temperature for 5 minutes, add 20 ml of diethyl ether to give a final concentration of 33.3% by volume, and mix the suspension well at 4°C for 15 minutes.

1 Headquarters: EDQM – Council of Europe, 7 allée Kastner, CS 30026, F-67081 Strasbourg, France.

iii) After allowing the layers to separate by standing, remove the aqueous layer containing the disrupted virus particles to a glass bottle with a loose lid and allow the excess ether to evaporate off overnight (John & Fulginiti, 1966). Safety precautions while handling ether must be strictly observed and work should be confined to a fume hood.

iv) Store treated virus in aliquots at –70°C.

2.1.2. Titration of haemagglutination

i) Add 25 µl of PBS to all wells in a row of a microtitre plate.

ii) Add 25 µl of virus to first well then generate a twofold dilution series across the plate leaving the last well as a negative control.

iii) Add an extra 25 µl of PBS to all wells.

iv) Add 50 µl of RBCs to all wells. Leave at room temperature or at 4°C (particularly if ambient temperatures are high), for 30 minutes. The HA titre is taken as the last virus dilution giving partial HA.

2.1.3. Potassium periodate pretreatment of sera

i) Mix one volume (150 µl) of serum with two volumes (300 µl) of freshly prepared 0.016 M potassium periodate (0.38 g in 100 ml PBS), and leave at 22°C (± 2°C) for 15 minutes.

ii) Add a further one volume of 3% glycerol in PBS to neutralise any excess periodate solution, mix and leave at room temperature (23°C ± 2°C) for 15 minutes.

iii) Inactivate in a 56°C water bath for 30 minutes.

2.1.4. Test procedure

i) Dispense 25 µl of PBS to all wells of a microtitre plate.

ii) Add serum (25 µl) to the first well of a row of 12, then generate twofold serial dilutions (1/8 to at least 1/512, allowing for dilution of 1/4 from treatment of serum), leaving the last well as a control.

iii) Dilute the antigen to give a dose of 4 HA units (4 × minimum agglutinating dose, i.e. titre/4).

iv) Add 25 µl to each well, and incubate at room temperature (22°C ± 2°C) for 30 minutes.

v) Add 50 µl of RBCs to each well. Leave at room temperature or at 4°C (particularly if ambient temperatures are high), for 30 minutes.

vi) The plates may be read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. No agglutination is recorded as a positive result.

2.2. Single radial haemolysis

In this test, viral antigens are coupled to fixed RBCs that are suspended in agarose containing guinea-pig complement (C'). Wells are punched in the agarose and filled with test sera. Influenza antibodies and C' lyse the antigen-coated RBCs, resulting in a clear, haemolytic zone around the well; the size of this zone is directly proportional to the level of strain-specific antibody in the serum sample (Morley *et al.*, 1995).

Special immunodiffusion plates (MP Biomedical) may be used for the assay, but simple Petri dishes are also suitable. Sheep RBCs collected into Alsever's solution are washed three times. The C' can be obtained commercially, or normal guinea-pig serum can be used. The viral antigens are egg-grown stocks or purified preparations; the strains used are the same as for the HI tests. The viruses are coupled to RBCs by potassium periodate or by chromic chloride. The coupled antigen/RBCs preparations are mixed with C', together with a 1% solution of agarose (low melting grade) in PBS. Care must be taken to ensure that the temperature is not allowed to rise above 42°C at any time. The mixture is poured into plates and left to set. Wells of 3 mm in diameter and 12 mm apart are punched in the solidified agarose, at least 6 mm from the edge of the plates. Such plates may be stored at 4°C for 12 weeks. Plates are prepared for each antigen.

Sera are inactivated at 56°C for 30 minutes, but no further treatment is necessary. Paired sera should be assayed on the same plate. As a minimum, a subtype-specific antiserum should be included as a control serum in one well on each plate. All sera are tested in a control plate containing all components except

virus to check for nonspecific lysis. Alternatively, an unrelated virus, such as A/PR/8/34 (H1N1), may be used in the control plate. Sera that show haemolytic activity for sheep RBCs must be pre-absorbed with sheep RBCs. Zones of lysis should be clear and not hazy or translucent. All clear zones should be measured and the area of haemolysis calculated.

2.2.1. Preparation of reagents

i) *Saline/HEPES:* 0.85% NaCl (4.25 g/500 ml); 0.05 M HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid; 5.95 g/500 ml); and 0.02% sodium azide. Make to pH 6.5 with NaOH.

ii) *Saline/HEPES/BSA:* as saline/HEPES with 0.2% (w/v) bovine serum albumin (BSA).

iii) *$CrCl_3$ stock solution (2.25 M) 6 g/10 ml:* Make fresh 1/400 dilution in 0.85% NaCl for each assay.

iv) *PBS 'A':* NaCl (10.00 g); KCl (0.25 g); Na_2PO_4 (1.45 g); KH_2PO_4 (0.25 g); and Na azide (0.20 g). Make up to 1 litre with distilled water.

v) *Agarose in PBS:* Place flask containing PBS 'A' on a stirrer. Slowly add 10 g agarose to the stirring solution. Liquefy in a pressure cooker. Dispense into glass bottles for storage at 22°C (± 2°C).

vi) *Virus antigen:* Allantoic fluid containing infectious virus is harvested and stored at –70°C. A short titration curve determines the optimum ratio of virus antigen to RBCs to be used when preparing sensitised sheep RBCs. The H7N7 influenza strains always produce clear zones; the H3N8 strains sometimes produce hazy zones, in which case it is necessary to concentrate the virus by centrifugation.

vii) *Sheep blood:* Collect blood into an equal volume of Alsever's solution and store at 4°C. It may be necessary to test bleed several sheep, as characteristics of RBCs from individual sheep vary. Keep the blood for 2 days before use, it may then be usable for up to 3 weeks, providing sterility is maintained.

viii) *Complement:* Use commercially available guinea-pig complement or collect serum from young guinea-pigs of 300–350 g body weight and store in small volumes at –70°C. For use, thaw in cold water and hold at 4°C prior to mixing.

ix) *Treatment of sera:* Use undiluted sera heat inactivated at 56°C for 30 minutes. Avoid repeated freeze–thaw cycles.

2.2.2. Test procedure

i) Wash sheep RBCs at least three times in saline/HEPES.

ii) Prepare an appropriate volume of 8% RBCs (v/v packed cells) in saline/HEPES, having first calculated the number of plates required and allowing 1 ml per 6 × 11 cm immunoplate and 1–2 ml extra.

iii) Add a predetermined volume[2] of virus antigen to the 8% RBCs solution. Hold the mixture at 4°C for 10 minutes. Haemagglutination may be observed.

iv) SLOWLY add CrCl3 (1/400 in 0.85% NaCl) at half the total volume of virus/RBCs suspension. Hold at 22°C (±2°C) for 5 minutes with occasional mixing.

v) Sediment the sensitised RBCs by centrifugation at 1500 ***g*** for 5 minutes.

vi) Gently resuspend in saline/HEPES/BSA and centrifuge at 1500 ***g*** for 5 minutes.

vii) Resuspend RBCs to an 8% suspension in PBS 'A'.

During the sensitisation process, melt the agarose. Shortly before use, pipette 7.8 ml volumes to Universal bottles and retain at 42°C. Check that the agar has cooled to 42°C before use.

2 Prepare three plates by adding 0.6, 1.2 or 1.8 ml of virus antigen to 2 ml RBCs. Add 1.3, 1.6 and 1.9 ml $CrCl_3$ respectively and resuspend to 2 ml in PBS 'A'. Optimum volume of virus antigen is that which results in the largest and clearest zones with appropriate reference serum.

2.2.3. Preparation of plates

i) Add 0.9 ml of virus-sensitised sheep RBCs to 7.8 ml of agarose (42°C). Mix quickly, but gently.

ii) Add 0.3 ml of undiluted guinea-pig serum. Mix again and pour into immunoplates on a levelling table. Allow to set and air dry without a lid for 5 minutes.

iii) Place lids on plates and store at 4°C in a humid box until used.

iv) Prepare control plates with unsensitised cells or cells sensitised with an unrelated virus. Batches of prepared plates can be stored for several weeks.

v) Punch 3 mm holes in the set gels to a prepared template, allow for 16 test sera and a positive control serum. On antigen control plates, prepare five rows of eight wells.

vi) Pipette 10 µl of heat-inactivated (56°C for 30 minutes) test sera and a positive control serum to appropriate wells. Incubate at 34°C for 20 hours in a humid box.

vii) Measure zone diameters, and calculate areas of haemolysis after the area of the well has been deducted.

2.2.4. Interpretation of the results

For results to be valid, positive and negative control sera should give results assigned through the international collaborative study (Daly *et al.*, 2007; Mumford, 2000) or if not using these international standards, results consistent with those expected on the basis of prior experience. Areas of haemolysis for the control sera should be clear and intra-laboratory variation should be no more than 5% for the control serum. Results may be expressed as mm^2 or as a ratio of the control serum value. Sera giving positive results in the control plate should be adsorbed with sheep RBCs. For diagnostic purposes, acute and convalescent sera should be tested in duplicate on the same plate. Increase in zone areas produced by convalescent serum compared with acute serum is evidence of infection. An increase in area of 25 mm^2 or 50% whichever is smaller, is routinely deemed to be significant but this depends on the reproducibility of the test within the laboratory, and should be equivalent to a twofold or more increase in antibody concentration. This area can be calculated from a standard curve generated from a dilution series of a standard antiserum.

2.3. Competitive/blocking ELISA

Several competitive/blocking ELISAs have been described, based on influenza A virus nucleoprotein and a monoclonal anti-nucleoprotein horseradish peroxidase conjugated antibody. Four readily available ELISAs, originally developed for avian influenza, have been evaluated and shown to be effective in the detection of influenza A antibodies in horses (Kittleberger *et al.,* 2011). The specific method supplied with each assay should be followed.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Equine influenza is a self-limiting disease and its economic significance is primarily due to the contagious nature of the virus and the disruption to equestrian activities. Rapid diagnosis, movement restrictions and vaccination are the key control measures for equine influenza. Vaccination decreases clinical signs and virus shedding. Influenza vaccines are widely available and are routinely used in competition horses in Europe, the Americas, and Asia. In some countries, vaccination is mandatory for sport horses and racehorses that are competing under rules of equestrian organisations. Following a primary course of three doses at intervals of around 0, 1 and 6 months, an annual booster is the usual minimum requirement. More frequent vaccination is recommended for young horses and some equestrian organisations require biannual boosters.

Equine influenza virus vaccines typically consist of inactivated whole viruses or their glycoprotein subunits, with or without adjuvant. Live attenuated virus and canary pox vectored vaccines have become

available commercially in some countries. Immunity generated by inactivated vaccines administered via the intramuscular route is reliant on stimulation of circulating antibody to the HA, which neutralises virus; some products have been shown to stimulate antibody in respiratory secretions. Critically the integrity and conformation of the HA should be maintained during inactivation procedures to ensure that the vaccine stimulates appropriate neutralising antibody. This can be tested by use of an immunological assay such as SRD (single radial diffusion), which measures immunologically active HA capable of reacting with specific anti-HA antibodies. The immunogenicity of the vaccine can be confirmed by measurement of HA antibody stimulated in small animal models or the target species. Several equine influenza vaccines including inactivated whole virus, subunit and a canary pox-vectored vaccine have also been shown to have the potential for cell-mediated immunity (CMI) priming (Gildea *et al.,* 2013b).

Live virus-vectored vaccines, which contain the influenza virus gene encoding HA rather than antigen, cannot be potency tested by SRD. Instead, the infectious titre of the recombinant is used as an *in-vitro* measure of potency and immunogenicity is assessed by measurement of antibody stimulated in the target species.

Antibody to HA as measured by SRH, stimulated by inactivated whole virus, subunit or canary pox-vectored vaccine correlates well with protection against infection in an experimental challenge model system (Edlund Toulemonde *et al*., 2005; Mumford, 1983; 1994). In contrast, a cold-adapted temperature-sensitive mutant used as a live attenuated vaccine replicates in the upper respiratory tract and does not stimulate high levels of circulating antibody to HA but nevertheless provides protection against challenge infection. Immunity is presumed to be mediated through mucosal or cellular responses rather than circulating antibody. As with the vectored vaccine, in-process control testing is reliant on measurement of the infectious virus titre.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature as manufacturers are obliged to meet European Pharmacopeia, USDA or other national and regional requirements.

All equine influenza vaccines should contain epidemiologically relevant strains

A formal equine influenza global surveillance programme has been in place since 1995. The WOAH Reference Laboratories and other collaborating laboratories collect data on outbreaks of equine influenza and strain variation that is reviewed annually by an Expert Surveillance Panel (ESP) including representatives from WOAH and WHO. This panel makes recommendations on the need to update vaccines, and these are published annually in the WOAH *Bulletin* and on the WOAH website. The criteria for updating equine influenza vaccines are similar to those for human influenza vaccines and based on analysis of antigenic changes, genetic changes and, when possible, supporting experimental challenge data.

H7N7: Many vaccines still contain an H7N7 strain. However, the Expert Surveillance Panel has recommended that the H7N7 component should be omitted as reports of infections with this subtype have not been substantiated during the past 30 years.

H3N8: Antigenic and genetic variants of H3N8 viruses co-circulate (Bryant *et al.*, 2009; Favaro *et al.,* 2018) and it is important to include a strain or strains that are epidemiologically relevant as recommended by the WOAH Expert Surveillance Panel. WOAH Reference Laboratories can provide help in selecting suitable vaccine strains.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

For each vaccine strain, a prototype batch should be prepared to establish its suitability as a vaccine strain, i.e. purity and safety should be confirmed by standard techniques. The ability of seed-lot viruses to grow to high titre and generate sufficient antigenic mass to stimulate adequate

antibody responses in the target species, should be confirmed. Additionally, vaccine virus derived in MDCK cells should be fully characterised to ensure that antigenic variants have not arisen during the culture process, such that the vaccine virus is no longer representative of the original isolate.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Virus strains may be obtained from WOAH Reference Laboratories[3]. Viruses selected as vaccine strains should be described in terms of origin and passage history. The strains are propagated in the allantoic cavity of 10-day-old embryonated hens' eggs or cell cultures, such as MDCK. All manipulations must be conducted separately for each strain. Viral growth is monitored by HA tests. Passaged virus is identified by serological tests, such as HI or SRH or by PCR using specific primers. If vaccine virus is grown in cell culture, antigenic studies with ferret sera and MAbs should be undertaken to ensure that variant viruses have not been selected during passage to prepare master and working seed viruses. Master and working seed viruses are divided into aliquots and stored in freeze-dried form at –20°C or at –70°C following testing for extraneous agents. Records of storage conditions should be maintained.

The master seed lot of each vaccine strain selected should be processed at one time to assure a uniform composition, tested for extraneous agents, and fully characterised. Antisera or MAbs for use in HI tests to characterise vaccine strains may be obtained from WOAH and WHO Reference Laboratories.

Working seed lots are derived from a master seed lot and should be of uniform composition, free from extraneous agents, and fully characterised. Aliquots of the working seed are used for production of vaccine.

Master and working seed lots should be prepared in specific pathogen free eggs or, as a minimum, in eggs derived from a healthy flock.

If MDCK cells are used to propagate vaccine virus, master cell lines should be established and stored in liquid nitrogen, and should be tested for freedom from extraneous agents according to National Control Authority Guidelines.

Examination of seed viruses for extraneous agents including mycoplasmas and other equine viruses should be performed by appropriate techniques, including inoculation of susceptible tissue cultures and examination for cytopathic effect or application of fluorescent antibodies for antigen detection.

The presence of other common equine respiratory pathogens, e.g. equine herpesviruses 1, 2, 4, equine picornaviruses, equine viral arteritis, and equine adenoviruses, should be specifically excluded.

The absence of bacteria should be confirmed by standard sterility tests and toxicity tests in small animals.

2.2. Method of manufacture

2.2.1. Procedure

Production is based on a seed-lot system that has been validated with respect to the characteristics of the vaccine strains. Where eggs are used, each strain of virus is inoculated separately into the allantoic cavity of 9–11-day-old embryonated hens' eggs from a healthy flock. The eggs are incubated at a suitable temperature for 2–3 days, and the allantoic fluid is collected. Alternatively, each strain is inoculated separately into MDCK cell cultures. The viral suspensions of each strain are collected separately and inactivated. If necessary, they may be purified. Suitable adjuvants and antimicrobial preservatives may be added.

3 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

Monovalent virus pools should be inactivated as soon as possible after their preparation, by a method approved by the National Control Authority. If formalin (37% formaldehyde) or beta-propiolactone (2-oxetanone) is used, the concentration by volume should not exceed 0.2%. Ideally, pools should be held at 4°C and should be inactivated within 5 days of harvest. Inactivation of the vaccine must be demonstrated. A suitable method consists of inoculating 0.2 ml of undiluted monovalent pool and 1/10 and 1/100 dilutions of the monovalent pool into the allantoic cavities of groups of fertile eggs (10 eggs in each group), and incubating the eggs at 33–37°C for 3 days. At least 8 of the 10 eggs should survive at each dosage level. A volume of 0.5 ml of allantoic fluid is harvested from each surviving egg. The fluid harvested from each group is pooled, and 0.2 ml of each of the three pools is inoculated, undiluted, into a further group of 10 fertile eggs. Haemagglutinin activity should not be detected in these new groups of eggs. In some countries, the requirement that 80% of the eggs should survive during incubation may be impossible to satisfy, in which case the National Control Authority should then specify a modified requirement to be satisfied. Before inactivation, samples should be collected for bacterial and fungal sterility tests.

Monovalent material may be concentrated and purified by high-speed centrifugation or other suitable methods approved by the National Control Authority, either before or after the inactivation procedure. It is important to concentrate and purify the virus under optimum conditions, e.g. temperatures that preserve its antigenic properties.

The monovalent virus pool shall be shown not to contain viable influenza virus when tested by inoculation of embryonated hens' eggs, by a method approved by the National Control Authority. Alternatively, the satisfactory inactivation can also be demonstrated by inoculating monolayers of MDCK cells.

2.2.2. Requirements for substrates and media

Vaccines should be produced in eggs or in a cell line that meets the requirements of the National Control Authority. Wherever practicable the use of substances of animal origin for example, serum and trypsin should be kept to a minimum. Substances of animal origin used during production should be subjected to a suitable, validated sterilisation or inactivation procedure or be tested for the absence of extraneous organisms.

2.2.3. In-process controls

Relevant in-process controls should be applied before and after inactivation and before and after concentration and purification.

In-process controls include: (a) identity of virus strains (tested by HI); (b) sterility; (c) virus titre; (d) haemagglutinin content (tested by chicken RBCs agglutinating units, CCA [chick cell agglutination]); and (e) immunologically active HA (tested by SRD or another suitable immunochemical method).

2.2.4. Single radial diffusion test

SRD is a reliable method for measuring immunologically active HA in terms of μg HA, and is used routinely for potency testing of human influenza vaccines (Wood *et al.*, 1983b).

The potency of inactivated equine influenza vaccine depends on the concentration of immunologically active haemagglutinin (Wood *et al.*, 1983a).

Assessment of the antigenic content of the vaccine by CCA alone may be misleading, as the sensitivity of this assay is a reflection of the ability of virus strains to agglutinate RBCs. Disruption of virus may lead to an apparent increase in HA as measured by CCA. The CCA assay does not provide a measure of the antigenic properties of the HA (HA may retain its properties to bind to RBCs while losing its ability to stimulate antibody).

Most equine influenza vaccines contain more than one variant of the H3N8 subtype. In this situation, it is not possible to judge the potency of individual H3N8 components from serological tests performed on sera collected from horses or small animals vaccinated with the final product, because of cross-reactivity between the two isolates of the same subtype. Thus, it is important

that a reliable method, such as SRD, be used to measure the potency of individual components before and after inactivation and prior to mixing and formulation with adjuvant.

In the SRD test, virus preparations are compared with a calibrated reference preparation of known HA content. Antigens are allowed to diffuse through a gel containing an antiserum specific for a particular HA. The distance diffused by the antigen before precipitation by the antibody incorporated in the gel is directly related to the concentration of haemagglutinin in the antigen preparations.

Standard reagents for SRD testing are available from the WHO International Laboratory for Biological Standards[4]. Reagents for the H3N8 strains A/eq/Miami/63, A/eq/Kentucky/81, A/eq/Newmarket/1/93 (American lineage) and A/eq/Newmarket/2/93 (European lineage) are currently available.

2.2.5. Final product batch tests

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

a) For inactivated or subunit vaccines absence of viral infectivity should be demonstrated by two passages in 10 embryonated hen eggs. The allantoic fluid should have no haemagglutinating activity.

b) Using no fewer than three horses, each horse is inoculated intramuscularly (at two different sites) with the dose of vaccine specified by the manufacturer; these inoculations are repeated 2–4 weeks later. The animals are kept under observation for 10 days after the second set of injections. No abnormal local or systemic reaction should ensue.

c) If vaccine is to be used in mares, safety should be demonstrated by giving two doses of vaccine to no fewer than two pregnant mares at the prescribed interval within the trimester for which the vaccine is recommended. Once safety has been demonstrated on a prototype batch, safety testing in pregnant mares may be omitted for routine testing of subsequent batches of the final product.

iii) Batch potency

Following mixing of viral antigens and adjuvants, aliquots should be potency tested *in vivo* using horses and guinea-pigs or a suitable alternative immunochemical assay. Adjuvants cause interference in quantitative *in-vitro* tests, such as CCA and SRD, although SRD may be used on the final product as a qualitative assay to demonstrate the presence of antigen for each vaccine strain. For repeated batch tests, only guinea-pigs or a suitable alternative immunochemical assay are used, subject to agreement of the National Control Authority.

a) Serological responses in horses

For a valid *in-vivo* potency test, naive seronegative horses must be selected for vaccination. Young horses or ponies (not less than 6 months old) should be screened for the presence of antibody using H7N7 and H3N8 viruses including recently isolated viruses relevant to the area in which the horses were reared. If HI tests are used for screening, H3N8 viruses should be treated with Tween 80/ether to maximise the sensitivity of the test. Alternatively, SRH may be used to establish the seronegative status of animals.

To test a vaccine for efficacy in horses, inject a volume corresponding to one vaccine dose by the recommended route into each of five susceptible seronegative horses. After the period recommended between the first and second doses, as stated on the label, a volume of vaccine corresponding to the second dose of vaccine is injected into each horse.

4 National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.

Three blood samples are collected from each animal, the first at the time of the first vaccination, the second 1 week after the first vaccination to check for anamnestic response, and the third 2 weeks after the second vaccination.

The serological assay used to measure the antibody response to the viruses contained in the vaccine must be standardised for a valid *in-vivo* potency test. The SRH assay (see Section B.2.2) is preferred as standard reference sera are available for quality control purposes from the European Pharmacopoeia[5]. These sera should be tested in parallel with the test sera to ensure that the test is valid with respect to sensitivity; the values obtained should not vary by more than 20% from the SRH values assigned in an international collaborative study (Daly *et al.*, 2007; Mumford, 2000). Due to poor repeatability and reproducibility of the HI test between different laboratories, no HI titre could be assigned to these sera.

Following vaccination the antibody value measured by SRH should not be less than 150 mm^2 This is higher than the value required in the European Pharmacopoeia Monograph for inactivated equine influenza vaccines (85 mm^2) as this value is not considered to be protective. If the value found for any horse after the first vaccination indicates that there has been an anamnestic response, the result is not taken into account. A supplementary test is carried out, as described above, replacing the horses that showed an anamnestic response with an equal number of new animals.

If the HI test is used, the antibody titre of each serum taken after the second vaccination in each test should not be less than 1/64 (calculated for the original serum, taking into account the predilution of 1/8). Alternatively, the antibody levels stimulated by the vaccine under test should be shown to be at least equal to the antibody levels stimulated by a standard vaccine tested in parallel that has been shown previously to protect horses against challenge infection.

b) Challenge studies in horses

Challenge studies may be carried out by exposing vaccinated horses/ponies to an aerosol of virulent influenza virus no fewer than 2 weeks and preferably more than 3 months after the second dose of vaccine. Comparisons of clinical signs, virus excretion and serological responses are made with a group of unvaccinated control animals challenged at the same time. The timing of the challenge procedure will reflect the claims to be made on the data sheet regarding duration of immunity. Protection at 2 weeks post-vaccination when antibody levels are at their peak level does not necessarily indicate good duration of immunity under field conditions.

If tests for potency in horses have been carried out with satisfactory results on a representative batch of vaccine, these tests may be omitted as a routine control on other batches of vaccine prepared using the same seed-lot system, subject to agreement by the National Control Authority.

c) Serological responses of guinea-pigs

Inject each of no fewer than five guinea-pigs free from specific antibodies with one vaccine dose. Collect blood samples 21 days later, and test the serum by SRH or HI (see Sections B.2.1 and B.2.2). Perform the tests of each serum using, respectively, the antigen(s) prepared from the strain(s) used in the production of the vaccine. The antibody titre of each serum in each test should not be less than the titre stimulated by a standard vaccine that has been shown to stimulate protective levels of antibody in horses.

2.3. Requirements for authorisation of vaccines

2.3.1. Safety requirements

The safety requirements may vary with the National Authority but usually include the assessment of the administration of an overdose and of the repeated administration of one dose to young

5 Serum to A/eq/Newmarket/1/77 (Catalogue number E0850010), A/eq/Newmarket/1/93 (E0850021) and A/eq/Newmarket/2/93 (E0850022).

horses and pregnant mares, if the vaccine is intended for use in pregnant mares. For live vaccines the dissemination by the vaccinated horse and the spread of the vaccine strain from vaccinated to unvaccinated horses along with the possible consequences must be investigated. Reversion to virulence studies should be performed by serial passaging of the live vaccine.

2.3.2. Efficacy requirements

The efficacy requirements may vary with the National Authority but usually include the assessment of the serological response in horses and virus challenge studies in susceptible horses (see Section C.2.2.5.iii.b).

Where claims for duration of immunity are made on the data sheet, these should be supported with data on the duration of protective levels of antibody maintained in horses vaccinated according to the recommended schedule. Antibody levels quoted as protective should be validated in challenge studies or by comparison with published reports.

2.3.3. Stability

Vaccines should be stored at 5±3°C and protected from light. The shelf life quoted on the data sheet should be demonstrated by testing the potency of aliquots over time using the guinea-pig potency test (see Section C.2.2.5.iii.c).

2.4. Requirements for authorisation of strain updates to vaccines

To enable vaccine manufacturers to respond quickly to recommendations from the Expert Surveillance Panel, existing vaccines that have been updated in accordance with the WOAH recommendations should be authorised if they meet the requirements for final product batch testing (see Section C.2.2.5).

The Committee for Veterinary Medicinal Products (CVMP) for the European Agency for the Evaluation of Medicinal Products (EMEA) has developed Guidelines on the compliance of authorised equine influenza vaccines with WOAH recommendations. In the USA, viral strain changes in equine influenza vaccines are addressed in the USDA/CVB/Veterinary Services Memorandum No. 800.111, which heed WOAH recommendations.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

A canary pox recombinant vaccine is available in some countries and was used in the equine influenza control and eradication programme in Australia in 2007. A nucleoprotein ELISA was used to differentiate horses vaccinated with the recombinant vaccine from horses that had been exposed to virus by natural infection (DIVA) This DIVA was possible because the canary pox recombinant expresses only the haemagglutinin gene of equine influenza. There is some evidence that this vaccine may be used to prime the immune system in the face of maternally derived antibodies (Minke *et al.*, 2008).

3.2. Special requirements

National Authorities frequently require an environmental risk assessment. This may include an assessment of the potential direct and indirect, immediate or delayed adverse effects of the genetically modified vaccine on human and animal health and on the environment. The phenotypic and genetic stability of the vaccine and its potential interactions with other organisms may need to be evaluated.

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NB: There are WOAH Reference Laboratories for equine influenza (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for equine influenza

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2019.

CHAPTER 3.6.8.

EQUINE PIROPLASMOSIS

SUMMARY

Equine piroplasmosis is a tick-borne protozoal disease of horses, mules, donkeys and zebra. The aetiological agents are blood parasites named Theileria equi *and* Babesia caballi*. Infected animals may remain carriers of these parasites for long periods and act as sources of infection for ticks, which act as vectors. These parasites are also easily spread by blood contaminated instruments.*

The introduction of carrier animals into areas where tick vectors are prevalent can lead to an epizootic spread of the disease.

Detection of the agent: *Infected horses can be identified by demonstrating the parasites in stained blood or organ smears during the acute phase of the disease. Romanovsky-type staining methods, such as Giemsa, give the best results. In carrier animals, low parasitaemias make it extremely difficult to detect parasites, especially in the case of* B. caballi *infections, although they may sometimes be demonstrated by using a thick blood smear technique.*

Paired merozoites joined at their posterior ends are a diagnostic feature of B. caballi *infection. The parasites in the erythrocytes measure 2 × 5 μm. The merozoites of* T. equi *are less than 2–3 μm long, and are pyriform, round or ovoid. A characteristic of* T. equi *is the arrangement of four pear-shaped merozoites forming a tetrad known as a 'Maltese cross'.*

Molecular techniques for the detection of T. equi *and* B. caballi *based on species-specific polymerase chain reaction (PCR) assays, targeting the 18S rRNA gene as well as BC48* (B. caballi) *and EMA-1* (T. equi) *genes, have been developed and continue to expand. These tests have been shown to be highly specific and sensitive and promise to play an increasing role in the diagnosis of infections. Importantly, the specificity of PCR can be defined beyond evaluation of the molecular mass of amplicons. Hybridisation with specific probes, restriction endonuclease analysis and sequencing of amplicons are also available.*

Serological tests*: Currently, the indirect fluorescent antibody test (IFAT) and the competitive enzyme-linked immunosorbent assay (C-ELISA) are the primary tests used for qualifying horses for importation. The complement fixation test (CFT), for many years the primary test, has been replaced by the IFA and C-ELISA; animals may be CF negative but still be infected. The IFAT and C-ELISA have been shown to be highly specific for each of the two species of piroplasmosis agents involved. One challenge with the IFAT is the need to dilute sera to reduce nonspecific binding and subsequent background, which may preclude binding to the intra-erythrocytic parasites. Sera dilutions to enhance specificity lead to a decrease in sensitivity of the IFAT and a specific cut-off should be determined. Indirect ELISAs using recombinant* T. equi *and* B. caballi *merozoite proteins in diagnostic assays appear to be very promising in the accurate determination of equine piroplasmosis infection.*

Requirements for vaccines: *There are no vaccines available.*

A. INTRODUCTION

Equine piroplasmosis is a tick-borne protozoal disease of horses, mules, donkeys and zebra. The aetiological agents of equine piroplasmosis are *Theileria equi* and *Babesia caballi*. Approximately fourteen species of Ixodid ticks in the genera *Dermacentor, Rhipicephalus* and *Hyalomma* have been identified as transstadial vectors of *B. caballi* and *T. equi*, while eight of these species were also able to transmit *B. caballi* infections transovarially (De Waal, 1992). Other genera such as *Ambloyomma* have also been identified as competent vectors (Scoles *et al.*, 2011). Infected animals may remain carriers of these blood parasites for long periods and act as sources of infection for

tick vectors. DNA of some of these parasites has also been detected in camels and dogs without clinical disease (Onyiche *et al.,* 2019). The role of non-equid species in the epidemiology of the disease is unclear.

The parasites occur in southern Europe, Asia, countries of the Commonwealth of Independent States, Africa, Cuba, South and Central America, and certain parts of the southern United States of America. *Theileria equi* has also been reported from Australia (but never established itself in this region), and is now believed to have a wider general distribution than *B. caballi*.

During the life cycle of *Babesia*, sporozoites initially invade red blood cells (RBCs) where they transform into trophozoites. In this situation the trophozoites grow and divide into two round, oval or pear-shaped merozoites. The mature merozoites are capable of infecting new RBCs and the division process is then repeated.

For *Babesia caballi,* the merozoites in the RBCs are pear-shaped, 2–5 μm long and 1.3–3.0 μm in diameter (Levine, 1985). The paired merozoites joined at their posterior ends are considered to be a diagnostic feature of *B. caballi* infection.

For *Theileria equi,* the merozoites are relatively small, less than 2–3 μm long (Levine, 1985), and are pyriform, round or ovoid. A characteristic of *T. equi* is the arrangement of four pear-shaped merozoites, measuring about 2 μm in length, forming a tetrad known as the 'Maltese cross' arrangement (Holbrook *et al*., 1968).

In *T. equi* infection, it has been shown that sporozoites inoculated into horses via a tick bite invade the lymphocytes (Schein *et al*., 1981). The sporozoites undergo development in the cytoplasm of these lymphocytes and eventually form *Theileria*-like schizonts. Merozoites released from these schizonts enter RBCs. Vertical transmission of *T. equi* from mare to foal has also been reported (Allsopp *et al*., 2007). In experimental infection, *T. equi* was detected not only in the blood but also in the other tissues such as livers, spleens, lungs, and bone marrows (Alhassan *et al*., 2007).

The taxonomic position of *T. equi* has been controversial and only relatively recently it has been redescribed as a *Theileria* (Mehlhorn & Schein, 1998). Further support for the close relation with *Theileria* spp. also comes from the homology found between 30 and 34 kDa *T. equi* surface proteins and similar sized proteins of various *Theileria* spp. (Knowles *et al*., 1997). However, the position of *T. equi* in phylogenetic trees based on the small subunit ribosomal RNA genes is variable and mostly appear as a sister clade of the Theilerids (Criado-Fornelio *et al*., 2003) leading some to suggest that *T. equi* is ancestral to the Theilerids (Criado-Fornelio *et al*., 2003) or a different group altogether (Allsopp *et al*., 1994). Completion of the *T. equi* genome supported its phylogenetic position as a sister taxon to *Theileria* spp. (Kappmeyer *et al.,* 2012). Sequence heterogeneity exists within both *B. caballi and T. equi*. In particular, the unusual high sequence diversity of *T. equi* 18S rDNA and the recent discovery of a new *Theileria* species, *Theileria haneyi*, closely related to *T. equi,* strongly indicate that various cryptic species are now collectively referred to as *T. equi* (Knowles *et al*., 2018). These sequence heterogenicities and cryptic species could potentially impact the interpretation of molecular diagnostic tests.

The clinical signs of equine piroplasmosis are often nonspecific, and the disease can easily be confused with other conditions. Piroplasmosis can occur in peracute, acute and chronic forms. The acute cases are more common, and are characterised by fever that usually exceeds 40°C, reduced appetite and malaise, elevated respiratory and pulse rates, congestion of mucous membranes, and faecal balls that are smaller and drier than normal.

Clinical signs in subacute cases are similar. In addition, affected animals show loss of weight, and fever is sometimes intermittent. The mucous membranes vary from pale pink to pink, or pale yellow to bright yellow. Petechiae and/or ecchymoses may also be visible on the mucous membranes. Normal bowel movements may be slightly depressed and the animals may show signs of mild colic. Mild oedematous swelling of the distal part of the limbs sometimes occurs.

Chronic cases usually present nonspecific clinical signs such as mild inappetence, poor performance and a drop in body mass. The spleen is usually found to be enlarged on rectal examination.

A rare peracute form where horses are found either dead or moribund has been reported.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of equine piroplasmosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Microscopic examination	–	+	–	++	+	–
PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
IFAT	++	++	++	–	++	–
C-ELISA	+++	++	+++	–	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test;
C-ELISA = competitive enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

Negative results in agent identification or serological tests do not necessarily mean that the animals are free from infections. In persistently infected carrier animals, the parasites may be sequestered in organs such as spleen and bone marrow, while the parasites and their genetic materials are undetectable in the general circulation (Pitel *et al*., 2010; Ribeiro *et al*., 2013). Similarly, during the early stage of the infections, horses may be seronegative until the antibodies reach the levels detectable by the serodiagnostic tools, while such animals may be positive by PCR assays (Abedi *et al*., 2014; Posada-Guzman *et al*., 2015). Therefore, PCR and serological tests are essential to determine whether an individual animal is free from infection. On the other hand, microscopy and PCR, which may be used in combination, are essential for confirming clinical cases associated with current infection. Treatment with antiparasitic drugs may mask infection and give rise to false negative results.

1. Detection of the agent

1.1. Microscopic examination

Infected horses may be identified by demonstrating the parasites in stained blood, optimally collected from superficial skin capillaries, or organ smears during the acute phase of the disease. Romanovsky-type staining methods, such as the Giemsa method, usually give the best results. However, even in acute clinical cases of *B. caballi* infection, the parasitaemia is very low and difficult to detect. Experienced workers sometimes use a thick blood smear technique to detect very low parasitaemia. Thick films are made by placing a small drop (approximately 50 µl) of blood on to a clean glass slide, which is then air-dried, heat-fixed at 80°C for 5 minutes, and stained in 5% Giemsa for 20–30 minutes.

An accurate identification of the parasite species is sometimes desirable, as mixed infections of *T. equi* and *B. caballi* probably occur frequently.

Identification of equine piroplasmosis in carrier animals by blood smear examination is not only very difficult but also inaccurate and therefore serological methods are preferred (see below). Serological tests however, may give false-negative or false-positive reactions (Tenter & Freidhoff, 1986).

1.2. *In-vitro* culture

Success in the establishment of *in-vitro* cultures of *T. equi* and *B. caballi* may be one alternative to supplement the methods described above, in order to identify carriers of the parasites. *Babesia caballi* parasites were successfully cultured from the blood of two horses that tested negative by the complement fixation test (CFT) (Holman *et al*., 1993). Similarly, *T. equi* could be cultured from horses that did not show any patent parasitaemia at the time of the initiation of the cultures (Zweygarth *et al*., 1997). This technique is largely superseded by molecular methods.

1.3. Molecular methods

Molecular techniques for the detection of *T. equi* and *B. caballi* have been described. These methods are based on species-specific polymerase chain reaction (PCR) assays, which mainly target the 18S rRNA gene. PCR assays for the specific detection of *T. equi* (forward primer: CAT-CGT-TGC-GGC-TTG-GTT-GG; reverse primer: CCA-AGT-CTC-ACA-CCC-TAT-TT) and *B. caballi* (forward primer: TTC-GCT-TCG-CTT-TTT-GTT-TTT-ACT; reverse primer: GTC-CCT-CTA-AGA-AGC-AAA-CCC-AA) based on the 18S rRNA gene have previously been described (Bashiruddin *et al.,* 1999). In one 18S rRNA-based multiplex PCR, 3 primers including a common forward primer for both *T. equi* and *B. caballi* (TCG-AAG-ACG-ATC-AGA-TAC-CGT-CG), a *T. equi*-specific reverse primer (TGC-CTT-AAA-CTT-CCT-TGC-GAT), and a *B. caballi*-specific reverse primer (CTC-GTT-CAT-GAT-TTA-GAA-TTG-CT) were used for simultaneous detection and identification of *T. equi* and *B. caballi* (Alhassan *et al.,* 2005). In addition to the PCR assays, other molecular diagnostic tests such as the highly sensitive loop-mediated isothermal amplification (LAMP) have been reported (Alhassan *et al*., 2007). The acquisition of the *T. equi* genome provides additional opportunities to improve and broaden diagnostic modalities for this parasite (Kappmeyer *et al.,* 2012). As mentioned above, there is significant sequence heterogenicity within both *T. equi* and *B. caballi.* As a result, molecular assays designed to detect some isolates may have reduced sensitivity for the detection of heterogenous isolates.

2. Serological tests

It is extremely difficult to diagnose the organisms in carrier animals by means of the microscopic examination of blood smears. Furthermore, it is by no means practical on a large scale. The serological testing of animals is therefore recommended as a preferred method of diagnosis, especially when horses are destined to be imported into countries where the disease does not occur, but the vector is present.

Sera should be collected and dispatched to diagnostic laboratories in accordance with the specifications of that laboratory. Horses for export that have been subjected to serological tests and shown to be free from infection, should be kept free of ticks to prevent accidental infections.

A number of serological techniques have been used in the diagnosis of piroplasmosis, such as the CFT, the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA).

2.1. Indirect fluorescent antibody test

The IFAT has been successfully applied to the differential diagnosis of *T. equi* and *B. caballi* infections (Madden & Holbrook, 1968). The recognition of a strong positive reaction is relatively simple, but any differentiation between weak positive and negative reactions requires considerable experience in interpretation. A detailed description of the protocol of the IFAT has been given (Madden & Holbrook, 1968). One challenge with the IFAT is the need to dilute sera to reduce non-specific binding and subsequent background, which may preclude binding of the intra-erythrocytic parasites. Sera dilutions to enhance specificity lead to a decrease in sensitivity of the IFAT. An example of an IFA protocol is given below.

2.1.1. Antigen production

Blood for antigen is obtained from horses with a rising parasitaemia, ideally 2–5%. Carrier animals that have already produced antibodies are not suitable for antigen production. Alternatively, parasites cultured *in vitro* can be used for the preparation of slide antigens to avoid contamination of antibodies to infected RBCs and for constant supply of infected RBCs, especially for *B. cabalii*. Blood (about 15 ml) is collected into 235 ml of phosphate-buffered saline (PBS), pH 7.2. The RBCs

are washed three times in cold PBS (1000 ***g*** for 10 minutes at 4°C). The supernatant fluid and the white cell layer are removed after each wash. After the last wash, the packed RBCs are reconstituted to the initial volume with 4% bovine serum albumin fraction V made up in PBS, i.e. the original packed cell volume = 30% so that one-third consists of RBCs. If the original RBC volume is 15 ml, then 5 ml of packed RBCs + 10 ml of 4% bovine albumin in PBS constitutes the antigen. After thorough mixing, the antigen is placed on to prepared wells on a glass slide using a template or a syringe. Alternatively, the cells can be spread smoothly on to microscope slides, covering the entire slide with an even, moderately thick film. These slides are allowed to dry, wrapped in soft paper and sealed in plastic bags or wrapped in aluminium foil, and stored at –20°C for up to 1 year.

2.1.2. Test procedure

i) Each serum sample is tested against an antigen of *B. caballi* and of *T. equi*.

ii) Prior to use, the frozen antigen slides are removed from storage at –20°C and incubated at 37°C for 10 minutes.

iii) The antigen smears are then removed from their protective covering and fixed in cold dry acetone (–20°C) for 1 minute. Commercially produced slides are available that are pre-fixed.

iv) If smears were prepared on the whole slide surface, squares (14–21 in number, i.e. 2–3 rows of 7 each) are formed on the antigen smears with nail varnish or rapidly drying mounting medium.

v) Test, positive and negative control sera are diluted from 1/80 to 1/1280 in PBS. Negative and positive control sera are included in each test.

vi) Sera are applied (10 µl each) at appropriate dilutions to the different wells or squares on the antigen smear, incubated at 37°C for 30 minutes, and washed several times in PBS and once in water.

vii) An anti-horse immunoglobulin prepared in rabbits and conjugated with fluorescein isothiocyanate (this conjugate is available commercially) is diluted in PBS and applied to the smear, which is then incubated and washed as before.

viii) After the final wash, two drops of a solution containing equal parts of glycerin and PBS are placed on each smear and mounted with a cover-slip.

ix) The smear is then examined under the microscope for the fluorescing parasites. Sera diluted 1/80 or more that show strong fluorescence are usually considered to be positive, although due consideration is also given to the patterns of fluorescence of the positive and negative controls.

2.2. Competitive enzyme-linked immunosorbent assay

A number of recombinant antigens for the use in ELISAs have been described. Recombinant *T. equi* (EMA-1; EMA-2) and *B. caballi* proteins (RAP-1; Bc48) have been produced in *Escherichia coli* (Huang *et al*., 2003; Kappmeyer *et al*., 1999; Knowles *et al*., 1992) or in insect cells by baculovirus (Xuan *et al*., 2001). Recombinant antigens produced in *E. coli* or by baculovirus have the obvious advantage of avoiding the need to infect horses for antigen production, and of eliminating the cross-reactions that have been experienced in the past with the crude ELISA antigens. They also provide a consistent source of antigen for international distribution and standardisation.

Indirect ELISAs using EMA-2 and BC48 have shown high sensitivity and specificity in detecting antibodies in infected horses (Huang *et al*., 2003; Ikadai *et al*., 1999; Kumar *et al.*, 2013). Initial results from these tests are promising and further validation of the assays is underway.

A competitive inhibition ELISA (C-ELISA) using EMA-1 protein and a specific monoclonal antibody (MAb) that defines this merozoite surface protein epitope, have been used in a C-ELISA for *T. equi* (Knowles *et al*., 1992). This C-ELISA overcomes the problem of antigen purity, as the specificity of this assay depends only on the specificity defined by the MAb *T. equi* epitope. A 94% correlation was shown between the C-ELISA and the CFT in detecting antibodies to *T. equi*. Sera that gave discrepant results were evaluated for their ability to immunoprecipitate 35S-methionine-labelled *in-vitro* translated products of *T. equi*

merozoite mRNA. Samples that were C-ELISA positive and CFT negative clearly precipitated multiple *T. equi* proteins. However, immunoprecipitation results with serum samples that were C-ELISA negative and CFT positive were inconclusive (Knowles *et al*., 1991). This C-ELISA for *T. equi* was also validated in Morocco and Israel, giving a concordance of 91% and 95.7% with the IFAT, respectively (Rhalem *et al*., 2001; Shkap *et al*., 1998). A similar C-ELISA has been developed using the recombinant *B. caballi* rhoptry-associated protein 1 (RAP-1) and a MAb reactive with a peptide epitope of a 60 kDa *B. caballi* antigen (Kappmeyer *et al*., 1999). The results of 302 serum samples tested with this C-ELISA and the CFT showed a 73% concordance. Of the 72 samples that were CFT negative and C-ELISA positive, 48 (67%) were shown to be positive on the IFAT, while four of the five samples that tested CFT positive and C-ELISA negative were positive on the IFAT (Kappmeyer *et al*., 1999).

A test protocol for an equine piroplasmosis C-ELISA has been described and used for additional validation studies (United States Department of Agriculture [USDA], 2005). The apparent specificity of the *T. equi* and *B. caballi* C-ELISAs lay between 99.2% and 99.5% using sera from 1000 horses presumed to be piroplasmosis free. One thousand foreign-origin horses of unknown infection status were tested by the C-ELISA and the CFT with an apparent greater sensitivity of the C-ELISA. The results were 1.1% (*T. equi*) and 1.3% (*B. caballi*) more seropositive animals detected by C-ELISA than by the CFT; the additional positive results were confirmed by IFAT. A similar study of 645 foreign-origin horses tested for import and pre-import purposes used heat-treated sera (58°C for 30 minutes), and resulted in 3.6% (*T. equi*) and 2.1% (*B. caballi*) more seropositive animals detected by the C-ELISA than by the CFT. Both C-ELISAs were highly reproducible well-to-well, plate-to-plate, and day-to-day, with overall variances of ± 1.2% and ±1.6% for the *T. equi* and *B. caballi* tests, respectively.

The C-ELISA protocol is given below.

A detailed description of antigen production and a test protocol has been given by the National Veterinary Services Laboratories (NVSL) of the USDA (2005). A commercial kit is now available that is based on the same antigens and monoclonal antibodies.

2.2.1. Solutions

i) Antigen-coating buffer

Prepare the volume of antigen-coating buffer required using the following amounts of ingredients per litre: 2.93 g sodium bicarbonate; 1.59 g sodium carbonate; sufficient ultra-pure water to dissolve, and make up to 1 litre with ultra-pure water. Adjust to pH 9.6.

ii) C-ELISA wash solution (high salt diluent)

Prepare the volume of C-ELISA wash solution required by using the following amounts of ingredients per litre: 29.5 g sodium chloride; 0.22 g monobasic sodium phosphate; 1.19 g dibasic sodium phosphate; 2.0 ml Tween 20; sufficient ultra-pure water to dissolve, and make up to 1 litre with ultra-pure water. Mix well. Adjust pH to 7.4. Sterilise by autoclaving at 121°C.

iii) Chromogenic substrate

0.1% (w/v) stock solution of C-ELISA substrate is prepared by dissolving 3,3',5,5'-tetramethylbenzidine (TMB) in dimethyl sulphide at 1 mg/ml. 10% (v/v) working solution is prepared by diluting TMB stock solution with phosphate–citrate buffer at pH 5.0. Fresh 30% hydrogen peroxide is added to TMB working solution at 0.02% (v/v) just before use.

2.2.2. Antigen production

Frozen transformed *E. coli* culture is inoculated at a 1/10,000 dilution into any standard non-selective bacterial growth broth (e.g. Luria broth) containing added carbenicillin (100 μg/ml) and isopropyl-thiogalactoside (IPTG, 1 mM). Cultures are incubated on an orbital shaker set at 200 rpm at 37°C overnight. Cells grown overnight are harvested by centrifugation (5000 ***g*** for

10 minutes), washed in 50 mM Tris/HCl and 5 mM ethylene diamine tetra-acetic acid (EDTA) buffer, pH 8.0, and harvested again as before[1].

Cells are resuspended to 10% of the original volume in the Tris/EDTA buffer to which 1 mg/ml of lysozyme has been added, and incubated on ice for 20 minutes. Nonidet P-40 detergent (NP-40) is then added to a final 1% concentration (v/v), vortexed, and the mixture is incubated on ice for 10 minutes. The material is next sonicated four times for 30 seconds each time at 100 watts, on ice, allowing 2 minutes between sonications for the material to remain cool. The sonicate is centrifuged at 10,000 ***g*** for 20 minutes. The resulting supernatant is dispensed in 0.5 ml aliquots in microcentrifuge tubes and may then be stored at –70°C for several years. The presence of heterologous host bacterial antigens does not interfere with the binding of specific equine anti-piroplasma antibodies or the binding of the paired MAbs to their respective expressed recombinant antigen epitopes, and is confirmed by the following procedures. The antigen-containing supernatants are quality controlled by titrating them with their paired MAbs and with reference monospecific equine antisera to verify both an adequate level of expression and complete specificity for the homologous species of piroplasmosis agent. Normal serum (negative serum) controls must not interfere with binding of the MAbs or positive equine reference sera to the expressed antigen preparation.

2.2.3. Test procedure

i) Microtitration plates are prepared by coating the wells with 50 µl of either *T. equi* antigen or *B. caballi* antigen diluted in antigen-coating buffer. The dilution used is determined by standard serological titration techniques. The plate is sealed with sealing tape, stored overnight at 4°C, and frozen at –70°C. Plates can be stored at –70°C for up to 6 months.

ii) The primary anti-*T. equi* or anti-*B. caballi* MAb and secondary antibody-peroxidase conjugate is diluted as directed by the manufacturer at the time of use in the C-ELISA, with antibody-diluting buffer (supplied with the test kit).

iii) Plates are thawed at room temperature, the coating solution is decanted, and the plates are washed twice with C-ELISA wash solution.

iv) The serum controls and test serum samples are diluted 1/2 with serum-diluting buffer before 50 µl of sera is added to wells. Each unknown serum sample is tested in single or duplicate wells. Positive control sera and blanks are tested in duplicate while negative controls are tested in triplicate on different parts of the plate. Plates are incubated covered, at room temperature (21–25°C) for 30 minutes in a humid chamber, and then washed three times in C-ELISA wash solution.

v) All wells then receive 50 µl/well of diluted primary anti-*T. equi* or anti-*B. caballi* MAb. (The MAb is produced in a cell culture bioreactor and is available from the NVSL, P.O. Box 844, Ames, Iowa 50010, USA.) Plates are incubated covered for 30 minutes at room temperature (21–25°C) in a humid chamber, and then washed three times in C-ELISA wash solution.

vi) Diluted secondary peroxidase anti-murine IgG (50 µl/well) conjugate is added to each well. Plates are incubated covered for 30 minutes at room temperature (21–25°C) in a humid chamber, and then washed three times in C-ELISA wash solution.

vii) Chromogenic enzyme substrate (50 µl/well) is added to all wells, and plates are incubated for 15 minutes at room temperature (21–25°C) during colour development.

viii) The colour development is stopped by adding 50 µl of stop solution to all wells and the plates are read immediately on a plate reader.

ix) The plates are read at 620, 630 or 650 nm wavelength (OD). The average OD is calculated for the duplicate wells for all control sera and blank wells. For a valid test, the mean of the negative controls must produce an OD >0.300 and <2.000. The mean positive control sera must produce an inhibition of ≥40%.

1 Antigen is available from the NVSL, P.O. Box 844, Ames, Iowa 50010, USA.

x) Per cent inhibition [%I] is calculated as follows: %I = 100 – [(Sample OD × 100) ÷ (Mean negative control OD)].

xi) If a test samples produces ≥40% inhibition it is considered positive. If the test sample produces <40% inhibition it is considered negative.

2.3. Complement fixation test

The CFT has been used in the past by some countries and is still widely used in some regions, but is no longer recommended to qualify horses for importation. The CFT is accurate for detection of early (acute) infections only, for which purpose it shows good sensitivity and specificity, but it may not identify all infected animals, especially those that have been drug-treated or that produce anti-complementary reactions, or because of the inability of IgG(T) (the major immunoglobulin isotype of equids) to fix guinea-pig complement. Antigen for the CFT is prepared by the experimental infection of horses, which raises animal welfare concerns. Therefore, it is likely that the CFT will be discontinued in the future; the IFAT and C-ELISA have replaced it as the tests that are most suitable for certifying individual animals prior to movement, including international trade.

C. REQUIREMENTS FOR VACCINES

No commercial vaccines are available currently.

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NB: There is a WOAH Reference Laboratory for equine piroplasmosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for equine piroplasmosis

NB: FIRST ADOPTED IN IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.6.9.

EQUINE RHINOPNEUMONITIS (INFECTION WITH EQUID HERPESVIRUS-1 AND -4)

SUMMARY

Equine rhinopneumonitis (*ER*) *is a collective term for any one of several contagious, clinical disease entities of equids that may occur as a result of infection by either of two closely related herpesviruses, equid herpesvirus-1 and -4* (*EHV-1 and EHV-4*). *Infection with EHV-1 is listed by WOAH. EHV-1 and EHV-4 are endemic in most domestic equine populations worldwide.*

Primary infection by either EHV-1 or EHV-4 is characterised by upper respiratory tract disease of varying severity that is related to the age and immunological status of the infected animal. EHV-1 also causes the more serious complications of abortion, perinatal foal death, or paralytic neurological disease (*equine herpesvirus myeloencephalopathy*). *EHV-4 has been associated with sporadic cases of abortion, but not the large outbreaks associated with EHV-1. Like other herpesviruses, EHV-1 and 4 induce long-lasting latent infections and can be reactivated following stress or pregnancy. Most horses are likely to be re-infected multiple times during their lifetime, often mildly or subclinically. Detection of viral DNA or anti-EHV antibodies should therefore be interpreted with care.*

Identification of the agent: *The standard method of identification of EHV-1 and EHV-4 from appropriate clinical or necropsy material is by polymerase chain reaction* (*PCR*), *followed by laboratory isolation of the virus in cell culture. Positive identification of viral isolates as EHV-1 or EHV-4 can be achieved by type-specific PCR. Viruses can be isolated in equine cell culture from nasal or nasopharyngeal swab extracts taken from horses during the febrile stage of respiratory tract infection, from the placenta and liver, lung, spleen, or thymus of aborted fetuses and early foal deaths, and from the leukocyte fraction of the blood of animals with acute EHV-1 infection. Unlike EHV-4, EHV-1 will also grow in various non-equine cell types such as the RK-13 cell line and this property can be used to distinguish between the two viruses.*

A rapid presumptive diagnosis of abortion induced by EHV-1 or (*infrequently*) *EHV-4 can be achieved by direct immunofluorescent detection of viral antigen in cryostat sections of placenta and tissues from aborted fetuses, using a conjugated polyclonal antiserum.*

Post-mortem demonstration of histopathological lesions of EHV-1 in placenta and tissues from aborted fetuses, cases of perinatal foal death or in the central nervous system of neurologically affected animals complements the laboratory diagnosis.

Serological tests: *Most horses possess some level of antibody to EHV-1/4, the demonstration of specific antibody in the serum collected from a single blood sample is therefore not confirmation of a positive diagnosis of recent infection. Paired, acute and convalescent sera from animals suspected of being infected with EHV-1 or EHV-4 should be tested for a four-fold or greater rise in virus-specific antibody titre by either virus neutralisation* (*VN*) *or complement fixation* (*CF*) tests. *Neither of these assays is type-specific but both have proven useful for diagnostic purposes especially since the CF antibody response to recent infection is relatively short-lived. Limited use has also been made of a type-specific enzyme-linked immunosorbent assay* (*Crabb* et al., *1995; Hartley* et al., *2005*).

Requirements for vaccines: *Both live attenuated and inactivated viral vaccines are available for use in assisting in the control of EHV-1/4. Vaccination is helpful in reducing the severity of respiratory infection in young horses and the incidence of abortion in mares, however current vaccines are not licenced to protect against neurological disease. Vaccination should not be considered a substitute*

for sound management practices known to reduce the risk of infection. Revaccination at frequent intervals is recommended in the case of each of the products, as the duration of vaccine-induced immunity is relatively short.

Standards for production and licensing of both attenuated and inactivated EHV-1/4 vaccines are established by appropriate veterinary regulatory agencies in the countries of vaccine manufacture and use. A single set of internationally recognised standards for EHV vaccines is not available. In each case, however, vaccine production is based on the system of a detailed outline of production employing a well characterised cell line and a master seed lot of vaccine virus that has been validated with respect to virus identity, safety, virological purity, immunogenicity and the absence of extraneous microbial agents.

A. INTRODUCTION

Equine rhinopneumonitis (ER) is a historically-derived term that describes a constellation of several disease entities of horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or myeloencephalopathy (Allen & Bryans, 1986; Allen *et al*., 1999; Bryans & Allen, 1988; Crabb & Studdert, 1995). The disease has been recognised for over 60 years as a threat to the international horse industry, and is caused by either of two members of the *Herpesviridae* family, equid herpesvirus-1 and -4 (EHV-1 and EHV-4). EHV-1 and EHV-4 are closely related alphaherpesviruses of horses with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (Telford *et al*., 1992; 1998). The two herpesviruses are enzootic in all countries in which large populations of horses are maintained as part of the cultural tradition or agricultural economy. There is no recorded evidence that the two herpesviruses of ER pose any health risks to humans working with the agents. Infection with EHV-1 is listed by WOAH.

Viral transmission to cohort animals occurs by inhalation of aerosols of virus-laden respiratory secretions. Morbidity tends to be highest in young horses sharing the same air space. Aborted tissues and placental fluids from infected mares can contain extremely high levels of live virus and represent a major source of infection. Extensive use of vaccines has not eliminated EHV infections, and the world-wide annual financial impact from these equine pathogens is immense.

In horses under 3 years of age, clinical ER usually takes the form of an acute, febrile respiratory illness that spreads rapidly through the group of animals. The viruses infect and multiply in epithelial cells of the respiratory mucosa. Signs of infection become apparent 2–8 days after exposure to virus, and are characterised by fever, inappetence, depression, and nasal discharge. The severity of respiratory disease varies with the age of the horse and the level of immunity resulting from previous vaccination or natural exposure. Fever and complications are more likely with EHV-1 than EHV-4. Subclinical infections with EHV-1/4 are common, even in young animals. Although mortality from uncomplicated ER is rare and complete recovery within 1–2 weeks is the normal outcome, respiratory infection is a frequent and significant cause of interrupted schedules among horses assembled for training, racing, or other equestrian events. Fully protective immunity resulting from infection is of short duration, and convalescent animals are susceptible to reinfection by EHV-1/4 after several months. Although reinfections by the two herpesviruses cause less severe or clinically inapparent respiratory disease, the risks of subsequent abortion or neurological disease remain. Like other herpesviruses, EHV-1/4 cause long-lasting latent infections and latently infected horses represent a potential infection risk for other horses. Virus can be reactivated as a result of stress or pregnancy. The greatest clinical threats to individual breeding, racing, or pleasure horse operations posed by ER are the potential abortigenic and neurological *sequelae* of EHV-1 respiratory infection.

Neurological disease, also known as equine herpesvirus myeloencephalopathy, remains an infrequent but serious complication of EHV-1 infection. A single mutation in the DNA polymerase gene (ORF30) has been associated with increased risk of neurological disease, however strains without this change can also cause paralysis (Nugent *et al.,* 2006; Goodman *et al*., 2007). Strain typing techniques have been employed to identify viruses carrying the neuropathic marker, and it can be helpful to be aware of an increased risk of neurological complications. However, for practical purposes strain-typing is not relevant for agent identification, or international trade. Strain typing may be beneficial for implementation of biosecurity measures in the management of outbreaks of equine herpesvirus myeloencephalopathy.

B. DIAGNOSTIC TECHNIQUES

Both EHV-1 and EHV-4 have the potential to be highly contagious viruses and the former can cause explosive outbreaks of abortion or neurological disease. Rapid diagnostic methods are therefore useful for managing the disease. Polymerase chain reaction (PCR) assays are widely used by diagnostic laboratories and are both rapid and sensitive. Real-time PCR assays that allow simultaneous testing for EHV-1 and EHV-4 and quantification of viral load have been developed. Virus isolation can also be useful, particularly for the detection of viraemia. This is also true of EHV-1 associated abortions and neonatal foal deaths, when the high level of virus in the tissues usually produces a cytopathic effect in 1–3 days. Immunohistochemical or immunofluorescent approaches can be extremely useful for rapid diagnosis of EHV-induced abortion from fresh or embedded tissue and are relatively straightforward. Several other techniques based on enzyme-linked immunosorbent assay (ELISA) or nucleic acid hybridisation probes have also been described, however their use is often restricted to specialised laboratories and they are not included here.

Table 1. Test methods available for the diagnosis of equine rhinopneumonitis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	–	+++	–	+++	–	–
PCR	–	+++	–	+++	–	–
Detection of immune response						
VN	+	+	+	+++	+++	+++
ELISA	+	+	+	++	+++	+
CFT	–	–	–	+++	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; VN = virus neutralisation;
ELISA = enzyme-linked immunosorbent assay; CFT = complement fixation test.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Collection and preparation of specimens

Nasal/nasopharyngeal swabs: swab extract can be used for DNA extraction and subsequent virus detection by PCR using one of a variety of published techniques or commercially available kits (see below). Virus isolation can also be attempted from the swab extracts. To increase the chances of isolating live virus, swabs are best obtained from horses during the very early, febrile stages of the respiratory disease, and are collected via the nares by sampling the area with a swab of an appropriate size and length for horses. After collection, the swab should be removed and transported immediately to the virology laboratory in 3 ml of cold (not frozen) virus transport medium (e.g. PBS or serum-free MEM [minimal essential medium] with antibiotics). Virus infectivity can be prolonged by the addition of bovine serum albumin, fetal calf serum or gelatine to 0.1% (w/v).

Tissue samples: total DNA can be extracted using a number of commercially available kits and used in PCR to detect viral DNA (described below in Section B.1.2.1). Virus isolation from placenta and fetal tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically

collected samples of placenta, liver, lung, thymus and spleen. Virus may be isolated from post-mortem cases of EHV-1 neurological disease by culture of samples of brain and spinal cord but such attempts to isolate virus are often unsuccessful; however, they may be useful for PCR testing and pathological examination. Tissue samples should be transported to the laboratory and held at 4°C until inoculated into tissue culture. Samples that cannot be processed within a few hours should be stored at –70°C.

Blood: for virus isolation from blood leukocytes, collect a 20 ml sample of blood, using an aseptic technique in citrate, heparin or EDTA [ethylene diamine tetra-acetic acid] anticoagulant. EDTA is the preferred anticoagulant for PCR testing. The samples should be transported without delay to the laboratory on ice, but not frozen.

1.2. Virus detection by polymerase chain reaction

PCR has become the primary diagnostic method for the detection of EHV-1 and EHV-4 in clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (Borchers & Slater, 1993; Lawrence *et al*., 1994; O'Keefe *et al*., 1994; Varrasso *et al*., 2001). A variety of type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (Varrasso *et al*., 2001). Diagnosis by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample.

For diagnosis of active infection by EHV, PCR methods are most reliable with tissue samples from aborted fetuses and placental tissue or from nasopharyngeal swabs of foals and yearlings. They are particularly useful in explosive outbreaks of abortion, respiratory or neurological disease in which a rapid identification of the virus is critical for guiding management strategies, including movement restrictions. PCR examination of spinal cord and brain tissue, as well as peripheral blood mononuclear cells (PBMC), are important in seeking a diagnosis on a horse with neurological signs.

Several PCR assays have been published. A nested PCR procedure can be used to distinguish between EHV-1 and EHV-4. A sensitive protocol suitable for clinical or pathological specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) has been described by Borchers & Slater (1993). However, nested PCR methods have a high risk of laboratory cross-contamination, and sensitive rapid one-step PCR tests to detect EHV-1 and EHV-4 (e.g. Lawrence *et al.,* 1994) are preferred. The WOAH Reference Laboratories use quantitative real-time PCR assays such as those targeting heterologous sequences of major glycoprotein genes to distinguish between EHV-1 and EHV-4. A multiplex real-time PCR targeting glycoprotein B gene of EHV-1 and EHV-4 was described by Diallo *et al.* (2007). PCR protocols have been developed that can differentiate between EHV-1 strains carrying the ORF30 neuropathogenic marker, using both restriction enzyme digestion of PCR products (Fritsche & Borchers, 2011) or by quantitative real-time PCR (Allen *et al,* 2007, Smith *et al*., 2012). Methods have also been developed to type strains for epidemiological purposes, based on the ORF68 gene (Nugent *et al.,* 2006). The WOAH Reference Laboratories employ in-house methods for strain typing, however these protocols have not yet been validated between different laboratories at an international level.

Real-time (or quantitative) PCR has become the method of choice for many diagnostic tests and provides rapid and sensitive detection of viral DNA. Real-time PCR assays have been described for EHV-1 and EHV-4. The real-time PCR test outlined below has been validated to ISO 17025 and is designed for use in a 96-well format. This can be readily combined with automatic nucleic acid extraction methods. This multiplex assay amplifies viral DNA sequences specific to either EHV-1 or EHV-4 in equine tissue samples, nasal swabs, or respiratory washes. It has not been validated for use with whole blood or buffy coat. The target region for amplification of each virus is in a conserved type-specific area of the gene for glycoprotein B (gB) for EHV-1 and ORF17 (encoding UL43) for EHV-4. Discrimination between EHV-1 and EHV-4 is carried out by the incorporation of type-specific dual labelled probes. The method uses in-house designed primers and probes, based on methods published by Hussey *et al.* (2006) and Lawrence *et al.* (1994). To establish such a real-time PCR assay for diagnostic purposes, validation against blinded samples is required. Sensitivity and specificity for the assay should be determined against each target. Support for development of assays and appropriate sample panels can be obtained from the WOAH Reference Laboratories.

Alternative validated protocols may be used, with appropriate optimisation of thermocycler times and temperatures, for example the methods of Diallo *et al.* (2006; 2007), Allen (2007).

1.2.1. Test protocol

i) Suitable specimens

Equine post-mortem tissues from newborn and adult animals or equine fetal tissue from abortions (tissues containing lung, liver, spleen, thymus, adrenal gland and placental tissues) can be used. For respiratory samples, equine nasopharyngeal swabs or deep nasal swabs (submitted in a suitable viral transport medium), tracheal wash (TW) or broncho-alveolar lavage (BAL) are all suitable. DNA should be extracted using an appropriate kit or robotic system.

ii) Primers and probes

EHV 1 Forward: GGG-GTT-CTT-AAT-TGC-ATT-CAG-ACC

EHV 1 Reverse: GTA-GGT-GCG-GTT-AGA-TCT-CAC-AAG

EHV 4 Forward: TAG-CAA-ACA-CCC-ACT-AAT-AAT-AGC-AAG

EHV 4 Reverse: GCT-CAA-ATC-TCT-TTA-TTT-TAT-GTC-ATA-TGC

EHV1gB/probe: {FAM}TCT-CCA-ACG-AAC-TCG-CCA-GGC-TGT-ACC{BHQ1}

EHV4ORF17/probe: {JOE}CGG-AAC-AGG-AAC-TCA-CTT-CAG-AGC-CAG-C{BHQ1}

iii) Real-time PCR standards

A DNA standard curve should be used to quantify the levels of viral DNA, comprising at least four standards containing EHV-1 and EHV-4 target DNA at known concentrations. All standards should be diluted in 1 ng/ml Polyinosinic–polycytidylic acid (Polyl/C) to stabilise the DNA in solution. These should be stored at –20°C and not subjected to multiple rounds of freeze–thaw.

iv) Test procedure

Due to the extreme sensitivity of PCR based tests, it is vital to eliminate all possible sources of nucleic acid contamination. All equipment and reagents must be of molecular biology/PCR grade and be guaranteed free from contaminating nucleic acids, nucleases, or other interfering enzymes.

Reactions should be prepared with appropriate PCR master mix kits. Reactions and collection of data are carried out in a real-time thermocycler using conditions that are optimised for that machine. The amount of viral DNA in each sample can be quantified against known DNA standards, however suitable positive and negative controls should also be included on each run: water as a non-template control, buffer that has been subjected to the sample extraction method (negative extraction control) and EHV-1 and EHV-4 virus as positive extraction controls. To ensure the ongoing quality of the assay, the cycle threshold (Ct) of a known low copy standard (e.g. 100 copies) should be recorded for each run and monitored regularly.

1.3. Virus isolation

For efficient primary isolation of EHV-4 from horses with respiratory disease, equine-derived cell cultures must be used. Both EHV-1 and EHV-4 may be isolated from nasopharyngeal samples using primary equine fetal kidney cells or equine fibroblasts derived from dermal (E-Derm) or lung tissue. EHV-1 can be isolated on other cell types, as will be discussed later. The nasopharyngeal swab and its accompanying transport medium are transferred into the barrel of a sterile 10 ml syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile tube. A portion of the expressed fluid can be filtered through a sterile, 0.45 μm membrane syringe filter unit into a second sterile tube if heavy bacterial contamination is expected, but this may also lower virus titre. Recently prepared cell monolayers in tissue culture flasks are inoculated with the filtered, as well as the unfiltered, nasopharyngeal swab extract. Cell monolayers in multiwell plates incubated in a 5% CO_2 environment

may also be used. Virus is allowed to attach by incubating the inoculated monolayers at 37°C. Monolayers of uninoculated control cells should be incubated in parallel.

At the end of the attachment period, the inocula are removed and the monolayers are rinsed twice with phosphate buffered saline (PBS) to remove virus-neutralising antibody that may be present in the nasopharyngeal secretions. After addition of supplemented maintenance medium (MEM containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics/antifungals [penicillin, streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C. The use of positive control virus samples to validate the isolation procedure carries the risk that this may lead to eventual contamination of diagnostic specimens. This risk can be minimised by using routine precautions and good laboratory technique, including the use of biosafety cabinets, inoculating positive controls after the diagnostic specimens, decontaminating the surfaces in the hood while the inoculum is adsorbing and using a positive control of relatively low titre. Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of cells). Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be blind-passaged into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum. Further blind passage is usually not productive.

Tissue samples: A number of cell types may be used for isolation of EHV-1 (e.g. rabbit kidney [RK-13 (AATC–CCL37)], baby hamster kidney [BHK-21], Madin–Darby bovine kidney [MDBK], pig kidney [PK-15], etc.). It can be useful to inoculate samples into both non-equine and equine cells in parallel to distinguish between EHV-1 and EHV-4, since EHV-4 can cause sporadic cases of abortion. Around 10% (w/v) pooled tissue homogenates of liver, lung, thymus, and spleen (from aborted fetuses) or of central nervous system tissue (from cases of neurological disease) are used for virus isolation. These are prepared by first mincing small samples of tissue into 1 mm cubes in a sterile Petri dish with dissecting scissors, followed by macerating the tissue cubes further in serum-free culture medium with antibiotics using a homogeniser or mechanical tissue grinder. After centrifugation at 1200 ***g*** for 10 minutes, the supernatant is removed and 0.5 ml is inoculated into duplicate cell monolayers in tissue culture flasks. Following incubation of the inoculated cells at 37°C for 1.5–2 hours, the inocula are removed and the monolayers are rinsed twice with PBS or maintenance medium. After addition of 5 ml of supplemented maintenance medium, the flasks are incubated at 37°C for up to 1 week or until viral CPE is observed. Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second time into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum.

Blood samples: EHV-1 and, infrequently, EHV-4 can be isolated from PBMC. Buffy coats may be prepared from unclotted blood by centrifugation at 600 ***g*** for 15 minutes, and the buffy coat is taken after the plasma has been carefully removed. The buffy coat is then layered onto a PBMC separating solution (Ficoll; density 1077 g/ml, commercially available) and centrifuged at 400 ***g*** for 20 minutes. The PBMC interface (without most granulocytes) is washed twice in PBS (300 ***g*** for 10 minutes) and resuspended in 1 ml of MEM containing 2% FCS. As a quicker alternative method, PBMC may be collected by centrifugation directly from plasma. An aliquot of the rinsed cell suspension is added to each of the duplicate monolayers of equine fibroblast, equine fetal or RK-13 cell monolayers in 25 cm^2 flasks containing 8–10 ml freshly added maintenance medium. The flasks are incubated at 37°C for 7 days; either with or without removal of the inoculum. If PBMCs are not removed prior to incubation, CPE may be difficult to detect in the presence of the massive inoculum of leukocytes: each flask of cells is freeze–thawed after 7 days of incubation and the contents centrifuged at 300 ***g*** for 10 minutes. Finally, 0.5 ml of the cell-free, culture medium supernatant is transferred to freshly made cell monolayers that are just subconfluent. These are incubated and observed for viral CPE for at least 5–6 days. Again, samples exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second time before discarding as negative.

Virus identity may be confirmed by PCR or by immunofluorescence with specific antisera. Virus isolates from positive cultures should be submitted to a WOAH Reference Laboratory to maintain a geographically diverse archive. Further strain characterisation for surveillance purposes or detection of the neurological marker can be provided at some laboratories.

1.4. Virus detection by direct immunofluorescence

Direct immunofluorescent detection of EHV antigens in samples of post-mortem tissues collected from aborted equine fetuses and the placenta provides a rapid preliminary diagnosis of herpesvirus abortion (Gunn, 1992). The diagnostic reliability of this technique approaches that of virus isolation attempts from the same tissues.

In the United States of America (USA), potent polyclonal antiserum to EHV-1, prepared in swine and conjugated with FITC, is available to veterinary diagnostic laboratories for this purpose from the National Veterinary Services Laboratories of the United States Department of Agriculture (USDA). The antiserum cross-reacts with EHV-4 and hence is not useful for serotyping, however virus typing can be conducted on any virus positive specimens by PCR.

Freshly dissected samples (5 × 5 mm pieces) of fetal tissue (lung, liver, thymus, and spleen) are frozen, sectioned on a cryostat at –20°C, mounted on to microscope slides, and fixed with 100% acetone. After air-drying, the sections are incubated at 37°C in a humid atmosphere for 30 minutes with an appropriate dilution of the conjugated swine antibody to EHV-1. Unreacted antibody is removed by two washes in PBS, and the tissue sections are then covered with aqueous mounting medium and a cover-slip, and examined for fluorescent cells indicating the presence of EHV antigen. Each test should include a positive and negative control consisting of sections from known EHV-1 infected and uninfected fetal tissue.

1.5. Virus detection by immunoperoxidase staining

Immunohistochemical (IH) staining methods, such as immunoperoxidase, have been developed for detecting EHV-1 antigen in fixed tissues of aborted equine fetuses, placental tissues or neurologically affected horses (Schultheiss *et al*., 1993; Whitwell *et al*., 1992). Such techniques can be used as an alternative to immunofluorescence described above and can also be readily applied to archival tissue samples. Immunohistochemical staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of the virus. Immunoperoxidase staining for EHV-1/4 may also be carried out on infected cell monolayers (van Maanen *et al*., 2000). Adequate controls must be included with each immunoperoxidase test run for evaluation of both the method specificity and antibody specificity. In one WOAH Reference Laboratory, this method is used routinely for frozen or fixed tissue, using rabbit polyclonal sera raised against EHV-1. This staining method is not type-specific and therefore needs to be combined with virus isolation or PCR to discriminate between EHV-1 and EHV-4, however it provides a useful method for rapid diagnosis of EHV-induced abortion.

1.6. Histopathology

Histopathological examination of sections of fixed placenta and lung, liver, spleen, adrenal and thymus from aborted fetuses and brain and spinal cord from neurologically affected horses should be carried out. In aborted fetuses, eosinophilic intranuclear inclusion bodies present within bronchiolar epithelium or in cells at the periphery of areas of hepatic necrosis are consistent with a diagnosis of herpesvirus infection. The characteristic microscopic lesion associated with EHV-1 neuropathy is a degenerative thrombotic vasculitis of small blood vessels in the brain or spinal cord (perivascular cuffing and infiltration by inflammatory cells, endothelial proliferation and necrosis, and thrombus formation).

2. Serological tests

EHV-1 and EHV-4 are endemic in most parts of the world and seroprevalence is high, however serological testing of paired sera can be useful for diagnosis of ER in horses. A positive diagnosis is based on the demonstration of significant increases (four-fold or greater) in antibody titres in paired sera taken during the acute and convalescent stages of the disease. The results of tests performed on sera from a single collection date are, in most cases, impossible to interpret with any degree of confidence. The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical signs, and the second (convalescent phase) serum sample should be taken 2–4 weeks later.

'Acute phase' sera from mares after abortion or from horses with EHV-1 neurological disease may already contain maximal titres of EHV-1 antibody, with no increase in titres detectable in sera collected at later dates. In such

cases, serological testing of paired serum samples from clinically unaffected cohort members of the herd may prove useful for retrospective diagnosis of ER within the herd.

Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids of equine fetuses can be of diagnostic value in cases of abortion especially when the fetus is virologically negative. The EHV 1/4 nucleic acid may be identified from these tissues by PCR.

Serum antibody levels to EHV-1/4 may be determined by virus neutralisation (VN) (Thomson *et al*., 1976), complement fixation (CF) tests (Thomson *et al*., 1976) or ELISA (Crabb *et al*., 1995). There are no internationally recognised reagents or standardised techniques for performing any of the serological tests for detection of EHV-1/4 antibody; titre determinations on the same serum may differ from one laboratory to another. Furthermore, the CF and VN tests detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the demonstration of a four-fold or greater rise in antibody titre to EHV-1 or EHV-4 during the course of a clinical illness provides serological confirmation of recent infection with one of the viruses.

The microneutralisation test is a widely used and sensitive serological assay for detecting EHV-1/4 antibody and will thus be described here.

2.1. Virus neutralisation test

This test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture grade) using a constant dose of virus and doubling dilutions of equine test sera. At least two replicate wells for each serum dilution are required. Serum-free MEM is used throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 $TCID_{50}$ (50% tissue culture infective dose) in 25 µl. Monolayers of E-Derm or RK-13 cells are monodispersed with EDTA/trypsin and resuspended at a concentration of 5×10^5/ml. Note that RK-13 cells can be used with EHV-1 but do not show CPE with EHV-4. Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the reciprocal of the highest serum dilution that protects 100% of the cell monolayer from virus destruction in both of the replicate wells.

Serum toxicity may be encountered in samples from horses repeatedly vaccinated with a commercial vaccine prepared from EHV-1 grown up in RK-13 cells. This can give rise to difficulties in interpretation of test reactions at lower serum dilutions. The problem can be overcome using E-Derm or other non-rabbit kidney derived cell line.

2.1.1. Test procedure

A suitable test procedure is as follows:

i) Inactivate test and control sera for 30 minutes in a water bath at 56°C.

ii) Add 25 µl of serum-free MEM to all wells of the microtitre assay plates.

iii) Pipette 25 µl of each test serum into duplicate wells of both rows A and B of the plate. The first row serves as the serum toxicity control and the second row as the first dilution of the test. Make doubling dilutions of each serum starting with row B and proceeding to the bottom of the plate by sequential mixing and transfer of 25 µl to each subsequent row of wells. Six sera can be assayed in each plate.

iv) Add 25 µl of the appropriately diluted EHV-1 or EHV-4 virus stock to each well (100 $TCID_{50}$/well) except those of row A, which are the serum control wells for monitoring serum toxicity for the indicator cells. Note that the final serum dilutions, after addition of virus, run from 1/4 to1/256.

v) A separate control plate should include titration of both a negative and positive horse serum of known titre, cell control (no virus), virus control (no serum), and a virus titration to calculate the actual amount of virus used in the test.

vi) Incubate the plates for 1 hour at 37°C in 5% CO_2 atmosphere.

vii) Add 50 µl of the prepared E-Derm or RK-13 cell suspension (5×10^5 cells/ml) in MEM/10% FCS to each well.

viii) Incubate the plates for 4–5 days at 37°C in an atmosphere of 5% CO_2 in air.

ix) Examine the plates microscopically for CPE and record the results on a worksheet. Alternatively, the cell monolayers can be scored for CPE after fixing and staining as follows: after removal of the culture fluid, immerse the plates for 15 minutes in a solution containing 2 mg/ml crystal violet, 10% formalin, 45% methanol, and 45% water. Then, rinse the plates vigorously under a stream of running tap water.

x) Wells containing intact cell monolayers stain blue, while monolayers destroyed by virus do not stain. Verify that the cell control, positive serum control, and serum cytotoxicity control wells stain blue, that the virus control and negative serum control wells are not stained, and that the actual amount of virus added to each well is between $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$. Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer remains intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in both duplicate wells is the end-point titre for that serum.

xi) Calculate the neutralisation titre for each test serum, and compare acute and convalescent phase serum titres from each animal for a four-fold or greater increase.

C. REQUIREMENTS FOR VACCINES

1. Background

Both live attenuated and inactivated vaccines are available for use in horses as licensed, commercially prepared products for use in reducing the impact of disease in horses caused by EHV-1/4 infection. The products contain different permutations of EHV-1 and EHV-4 and some also include equine influenza virus.

Clinical experience has demonstrated that vaccination can be useful for reducing clinical signs of respiratory disease and incidence of abortion, however none of the vaccines protects against neurological disease. Multiple doses repeated annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers. Vaccination schedules vary with a particular vaccine.

The indications stated on the product label for use of several available vaccines for ER are either as a preventative of herpesvirus-associated respiratory disease, or as an aid in the prevention of abortion, or both. Only four vaccine products have met the regulatory requirements for claiming efficacy in providing protection from herpesvirus abortion as a result of successful vaccination and challenge experiments in pregnant mares. None of the vaccine products has been demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4 that have been positively and unequivocally identified by both serological and genetic tests. Seed virus must be propagated in a cell line approved for equine vaccine production by the appropriate regulatory agency. A complete record of original source (including isolate number, location, year of isolation), passage history, medium used for propagation, etc., shall be kept for the master seed preparations of both the virus(es) and cell stock(s) intended for use in vaccine production.

2.1.1. Biological characteristics of the master seed

Permanently stored stocks of both MSV and master cell stock (MCS) used for vaccine production must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic.

Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest allowed for vaccine production. Results of all quality control tests on master seeds must be recorded and made a part of the licensee's permanent records.

2.1.2. Quality criteria

Tests for master seed purity include prescribed procedures that demonstrate the virus and cell seed stocks to be free from bacteria, fungi, mycoplasmas, and extraneous viruses. Special tests must be performed to confirm the absence of equine arteritis virus, equine infectious anaemia virus, equine influenza virus, equine herpesvirus-2, -3, and -5, equine rhinitis A and B viruses, the alphaviruses of equine encephalomyelitis, bovine viral diarrhoea virus (BVDV – common contaminant of bovine serum), and porcine parvovirus (PPV – potential contaminant of porcine trypsin). The purity check should also include the exclusion of the presence of EHV-1 from EHV-4 MSV and *vice versa*.

2.1.3. Validation as a vaccine strain

Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an experimental test vaccine prepared from the highest passage level of the MSV allowed for use in vaccine production. The test for MSV immunogenicity consists of vaccination of horses with low antibody titres to EHV-1/4, with doses of the test vaccine that will be recommended on the final product label. Second serum samples should be obtained and tested for significant increases in neutralising antibody titre against the virus, 21 days after the final dose.

Samples of each lot of MSV to be used for preparation of live attenuated ER vaccines must be tested for safety in horses determined to be susceptible to the virulent wild-type virus, including pregnant mares in the last 4 months of gestation. Vaccine safety must be demonstrated in a 'safety field trial' in horses of various ages from three different geographical areas. The safety trial should be conducted by independent veterinarians using a prelicensing batch of vaccine. EHV-1 vaccines making a claim for efficacy in controlling abortion must be tested for safety in a significant number of late gestation pregnant mares, using the vaccination schedule that will be recommended by the manufacturer for the final vaccine product.

2.2. Method of manufacture

2.2.1. Procedure

A detailed protocol of the methods of manufacture to be followed in the preparation of vaccines for ER must be compiled, approved, and filed as an Outline of Production with the appropriate licensing agency. Specifics of the methods of manufacture for ER vaccines will differ with the type (live or inactivated) and composition (EHV-1 only, EHV-1 and EHV-4, EHV-4 and equine influenza viruses, etc.) of each individual product, and also with the manufacturer.

2.2.2. Requirements for ingredients

Cells, virus, culture medium, and medium supplements of animal origin that are used for the preparation of production lots of vaccine must be derived from bulk stocks that have passed the prescribed tests for bacterial, fungal, and mycoplasma sterility; nontumorgenicity; and absence of extraneous viral agents.

2.2.3. Final product batch tests

i) Sterility

Samples taken from each batch of completed vaccine are tested for bacteria, fungi, and mycoplasma contamination. Procedures to establish that the vaccine is free from extraneous viruses are also required; such tests should include inoculation of cell cultures that allow detection of the common equine viruses, as well as techniques for the detection of BVDV and PPV in ingredients of animal origin used in the production of the batch of vaccine.

ii) Identity

Identity tests shall demonstrate that no other vaccine strain is present when several strains are propagated in a laboratory used in the production of multivalent vaccines.

iii) Safety

Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine in the host species by all vaccination route(s). Tests to assure safety of each production batch of ER vaccine must demonstrate complete inactivation of virus (for inactivated vaccines) as well as a level of residual virus-killing agent that does not exceed the maximal allowable limit (e.g. 0.2% for formaldehyde).

iv) Batch potency

Batch potency is examined on the final formulated product. Batch control of antigenic potency for EHV-1 vaccines only may be tested by measuring the ability of dilutions of the vaccine to protect hamsters from challenge with a lethal dose of hamster-adapted EHV-1 virus. Although potency testing on production batches of ER vaccine may also be performed by vaccination of susceptible horses followed by assay for seroconversion, the recent availability of virus type-specific MAbs has permitted development of less costly and more rapid *in-vitro* immunoassays for antigenic potency. The basis for such *in-vitro* assays for ER vaccine potency is the determination, by use of the specific MAb, of the presence of at least the minimal amount of viral antigen within each batch of vaccine that correlates with the required level of protection (or seroconversion rate) in a standard animal test for potency.

2.3. Requirements for authorisation/registration/licencing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2 Safety requirements

Vaccine safety should be evaluated in vaccinated animals using different assays (see Section 2.2.3.iii).

2.3.3 Efficacy requirements

Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to live pathogen challenge.

2.3.4 Duration of immunity

As part of the licensing or marketing authorisation procedure, the manufacturer may be required to demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test at the end of the claimed period of protection.

Tests to establish the duration of immunity to EHV-1/4 achieved by immunisation with each batch of vaccine are not required. The results of many reported observations indicate that vaccination-induced immunity to EHV-1/4 is not more than a few months in duration; these observations are reflected in the frequency of revaccination recommended on ER vaccine product labels.

2.3.5 Stability

As part of the licensing or marketing authorisation procedure, the manufacturer will be required to demonstrate the stability of all the vaccine's properties at the end of the claimed shelf-life period. Storage temperature shall be indicated and warnings should be given if product is damaged by freezing or ambient temperature.

At least three production batches of vaccine should be tested for shelf life before reaching a conclusion on the vaccine's stability. When stored at 4°C, inactivated vaccine products generally maintain their original antigenic potency for at least 1 year. Lyophilised preparations of the live virus vaccine are also stable during storage for 1 year at 4°C. Following reconstitution, live virus vaccine is unstable and cannot be stored without loss of potency.

Note: current vaccines are authorised for prevention of respiratory disease or as an aid in the prevention of abortion. Unless the vaccine's ability to prevent neurological disease is under investigation, the virus used in the challenge experiments should not be a strain with a history of inducing neurological disease.

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NB: There are WOAH Reference Laboratories for equine rhinopneumonitis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for equine rhinopneumonitis and to submit strains for further characterisation.

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 3.6.10.

EQUINE VIRAL ARTERITIS (INFECTION WITH EQUINE ARTERITIS VIRUS)

SUMMARY

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), an RNA virus classified in the genus, Arterivirus*, family* Arteriviridae*. Equine arteritis virus is found in horse populations in many countries world-wide. Although infrequently reported in the past, confirmed outbreaks of EVA appear to be on the increase.*

Description of the disease: *The majority of naturally acquired infections with EAV are subclinical. Where present, clinical signs of EVA can vary in range and severity. The disease is characterised principally by fever, depression, anorexia, dependent oedema, especially of the limbs, scrotum and prepuce in the stallion, conjunctivitis, an urticarial-type skin reaction, abortion and, rarely, a fulminating pneumonia, enteritis or pneumo-enteritis in young foals. Apart from mortality in young foals, the case-fatality rate in outbreaks of EVA is very low. Affected horses almost invariably make complete clinical recoveries. A long-term carrier state can occur in a variable percentage of infected stallions, but not in mares, geldings or sexually immature colts.*

Identification of the agent: *EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases. Diagnosis of EAV infection is laboratory dependent and based on virus isolation, detection of nucleic acid or viral antigen, or demonstration of a specific antibody response. Detection and identification of EAV nucleic acid in suspect cases of the disease can be attempted using various reverse-transcription polymerase chain reaction (RT-PCR) assays. The identity of isolates of EAV should be confirmed by RT-PCR assay, neutralisation test, or by immunocytochemical methods, namely indirect immunofluorescence or avidin–biotin–peroxidase techniques.*

Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries throughout the body. The characteristic vascular lesions present in the mature animal are not a notable feature in EVA-related abortions, diagnosis of which is based on virus isolation, viral nucleic acid detection by RT-PCR or demonstration of EAV antigens by immunohistochemical examination of placental and various fetal tissues.

Serological tests: *A variety of serological tests, including virus neutralisation (VN), complement fixation (CF), indirect fluorescent antibody, agar gel immunodiffusion, the enzyme-linked immunosorbent assay (ELISA), and the fluorescent microsphere immunoassay assay (MIA) have been used for the detection of antibody to EAV. The tests currently in widest use are the complement-enhanced VN test and the ELISA. The VN test is a very sensitive and highly specific assay of proven value in diagnosing acute infection and in seroprevalence studies. Several ELISAs have been developed. Although none have been as extensively validated as the VN test, some offer comparable specificity and close to equivalent sensitivity. The CF test is less sensitive than either VN test or ELISA, but it can be used for diagnosing recent infection.*

Requirements for vaccines: *Two commercial tissue culture derived vaccines are currently available against EVA. One is a modified live virus (MLV) vaccine prepared from virus that has been attenuated for horses by multiple serial transfers in primary equine and rabbit kidney cells and in an equine dermal cell line. It has been confirmed to be safe and protective for stallions and nonpregnant mares. Vaccination of foals less than 6 weeks of age and of pregnant mares in the final 2 months of gestation is not recommended. There is no evidence of back reversion of the vaccine virus to virulence or of recombination with naturally occurring strains of EAV following its use in the field. The second vaccine is an inactivated, adjuvanted product prepared from virus grown in equine cell culture that*

can be used in nonbreeding and breeding horses. In the absence of appropriate safety data, the vaccine is not currently recommended for use in pregnant mares.

A. INTRODUCTION

1. Description of disease and aetiology

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), a positive-sense, single-stranded RNA virus, and the prototype member of the genus *Arterivirus,* family *Arteriviridae*, order *Nidovirales* (Cavanagh, 1997). Only one major serotype of the virus has been identified so far. Epizootic lymphangitis pinkeye, fièvre typhoide and rotlaufseuche are some of the descriptive terms used in the past to refer to a disease of close clinical resemblance to EVA. The natural host range of EAV would appear to be restricted to equids, although very limited evidence would suggest it may also include new world camelids, *viz*. alpacas and llamas (Weber *et al*., 2006). The virus does not present a human health hazard (Timoney & McCollum, 1993).

While the majority of cases of acute infection with EAV are subclinical, certain strains of the virus can cause disease of varying severity (Timoney & McCollum, 1993). Typical cases of EVA can present with all or any combination of the following clinical signs: fever, depression, anorexia, leukopenia, dependent oedema, especially of the limbs, scrotum and prepuce of the stallion, conjunctivitis, ocular discharge, supra or periorbital oedema, rhinitis, nasal discharge, a local or generalised urticarial skin reaction, a period of temporary subfertility in acutely affected stallions, abortion, stillbirths and, rarely, a fulminating pneumonia, enteritis or pneumo-enteritis in young foals. Regardless of the severity of clinical signs, affected horses almost invariably make complete recoveries. The case-fatality rate in outbreaks of EVA is very low; mortality is usually only seen in very young foals, particularly those congenitally infected with the virus (Timoney & McCollum, 1993; Vaala *et al*., 1992), and very rarely in otherwise healthy adult horses.

A variable percentage of acutely infected stallions later become long-term carriers in the reproductive tract and constant semen shedders of the virus (Timoney & McCollum, 1993). The carrier state, which has been shown to be androgen dependant, has been found in the stallion, but not in the mare, gelding or sexually immature colt (Timoney & McCollum, 1993). While temporary down-regulation of circulating testosterone levels using a GnRH antagonist or by immunisation with GnRH would appear to have expedited clearance of the carrier state in some stallions, the efficacy of either treatment strategy has yet to be fully established. Concern has been expressed that such a therapeutic approach could be used to deliberately mask existence of the carrier state.

The gross and microscopic lesions described in fatal cases of EVA reflect the extensive vascular damage caused by the virus (Del Piero, 2000). EAV causes widespread vasculitis, primarily of the smaller arterioles and venules. This gives rise to oedema, congestion and haemorrhages, especially in the subcutis of the limbs and abdomen, and excess peritoneal, pleural and pericardial fluid (Jones *et al*., 1957). Pulmonary oedema, emphysema and interstitial pneumonia, enteritis and splenic infarcts have been described in fatal cases of EVA in young foals (Del Piero, 2000). Gross lesions are usually absent in cases of abortion and microscopic changes, and if present, are most often seen in the placenta, liver, spleen and lungs of the fetus.

Factors of considered importance in the epidemiology of EVA are phenotypic variation among virus strains, modes of transmission during acute and chronic phases of infection, carrier state in the stallion, nature and duration of acquired immunity and changing trends in the horse industry. EAV is present in the horse population of many countries world-wide (Timoney & McCollum, 1993). There has been an increase in the incidence of EVA in recent years that has been linked to the greater frequency of movement of horses and use of transported semen (Balasuriya *et al*., 1998). Transmission of EAV can occur by respiratory, venereal and congenital routes. Respiratory spread is most important during the acute phase of the infection. EAV can also be transmitted venereally by the acutely infected stallion or mare and by the carrier stallion.

Current EVA control programmes are aimed at preventing the dissemination of EAV in breeding populations to minimise the risk of abortion outbreaks, deaths in young foals and establishment of the carrier state in colts and stallions (Timoney & McCollum, 1993). Such programmes are based on observance of sound management practices in conjunction with a targeted vaccination program of breeding stallions and sexually immature colts against the disease.

2. Differential diagnosis

EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases, the most common of which are equine influenza, equine herpesvirus 1 and 4 infections, infection with equine rhinitis A and B viruses, equine adenoviruses and streptococcal infections, with particular reference to purpura haemorrhagica. The disease also has clinical similarities to equine infectious anaemia, equine encephalosis virus infection, African horse sickness fever, cases of Hendra virus infection, Getah virus infection and toxicosis caused by hoary alyssum (*Berteroa incana*) (Timoney & McCollum, 1993).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of equine viral arteritis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	–	+++	–	+++	–	–
PCR	–	+++	–	+++	–	–
Detection of immune response						
AGID	–	–	–	–	–	–
CFT	–	–	–	+++	–	–
ELISA	+	++	+	++	+++	+
VN	+	+++	+	+++	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; CFT = complement fixation test;
ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

Detection and identification of EAV from appropriate clinical and tissue samples can be accomplished by virus isolation in cell culture and by detection of viral nucleic acid using a range of reverse-transcription polymerase chain reaction (RT-PCR) assays. Both diagnostic approaches are appropriate for confirmation of clinical cases of EVA as well as establishing individual animal freedom from EAV infection. In the latter context, virus isolation and RT-PCR assays have been used in surveillance studies and in enabling animal movement to take place. Antigen detection through the use of various immunolabelling techniques also has diagnostic application when examining tissues from suspect cases of EVA abortion, death in young foals or older horses.

Isolation of EAV can be attempted in a limited number of cell lines of which the RK-13 rabbit kidney cell line (ATCC CCL37, or RK13-KY[1]) has proven to be optimal especially when testing stallion semen. Several comprehensive comparison studies have shown virus isolation to be of equivalent sensitivity to RT-PCR for the detection of EAV in

1 Available from the WOAH Reference Laboratory for EVA in the United States of America (USA) (please consult the web site for full contact details: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)

clinical and morbid material. Although many isolations of the virus are made in initial passage in cell culture, virus isolation is not a rapid diagnostic test in contrast to certain RT-PCR assays that can be completed in the same day.

A wide variety of RT-PCR assays (single step, nested, real-time) have been developed for EAV detection. Regrettably, very few have been adequately validated and compared with virus isolation for sensitivity and specificity. It is important to emphasise that the choice of reagent kits for both nucleic acid and extraction and amplification in the real-time RT-PCR assay can have a major influence on the overall diagnostic sensitivity and robustness of the assay (Miszczak *et al.,* 2011).

Immunohistochemical testing for EAV antigen in frozen or fixed tissue sections is best accomplished using a monospecific polyclonal serum against the virus or a monoclonal antibody (MAb) directed against the highly conserved nucleocapsid (N) viral protein.

Of the serological tests evaluated for the detection of antibodies to EAV, the complement-enhanced virus neutralisation (VN) test has been proven the most reliable for the diagnosis of acute EAV infection and for serosurveillance studies. Of the numerous enzyme-linked immunosorbent assays (ELISAs) that have been developed, a few offer comparable but not identical sensitivity and specificity to the VN test. A benefit of an EAV ELISA is that it can provide a same-day test result compared with the VN test, which is a 72-hour test. None of the available tests can reliably differentiate antibody titres resulting from natural infection from those due to vaccination.

1. Identification of the agent

1.1. *In-vitro* culture

In the event of a suspect outbreak of EVA, or when endeavouring to confirm a case of subclinical EAV infection, virus isolation should be attempted preferably from nasopharyngeal or deep nasal swabs, conjunctival swabs, unclotted blood samples, and semen from stallions considered putative carriers of the virus (Timoney & McCollum, 1993). To optimise the chances of virus isolation during an outbreak, relevant specimens should be obtained as soon as possible after the onset of fever in affected horses. In attempting virus isolation from peripheral mononuclear cells (PBMCs), blood should be collected in citrate on ethylenediaminetetraacetic acid (EDTA) anticoagulant. As heparin can inhibit the growth of EAV in rabbit kidney cells (RK-13 cell line), its use as an anticoagulant is contra-indicated as it may interfere with isolation of the virus from whole blood. Where EVA is suspected in cases of mortality in young foals or older animals, virus isolation can be attempted from a variety of tissues, especially the lymphatic glands associated with the alimentary tract and related organs, and also the lungs, liver and spleen (McCollum *et al*., 1971). In outbreaks of EVA-related abortion and/or cases of stillborn foals, placental and fetal fluids and a wide range of placental, lymphoreticular and other fetal tissues (especially lung) can be productive sources of virus (Timoney & McCollum, 1993).

Swabs for attempted isolation should be immersed in a suitable viral transport medium and these, together with any fluids or tissues collected for virus isolation and/or RT-PCR testing should be shipped either refrigerated or frozen in an insulated container to the laboratory, ideally within 24 hours. If swabs are intended for direct examination by RT-PCR, the swab shaft should not be made of wood, which might contain substances such as preservatives that could interfere with the PCR reaction. Unclotted blood samples must be transported refrigerated but not frozen. Where possible, specimens should be submitted to a laboratory with established competency in testing for this infection.

Nasopharyngeal swabs in transport medium are processed by transferring each into the barrel of a 10 ml syringe, the syringe plunger inserted and whatever fluid can be extracted is collected into a sterile tube. An aliquot of the fluid is passed through a prefilter and then filtered through a 0.45 µm membrane syringe fitter and collected aseptically for subsequent inoculation into cell culture.

Buffy coats can be harvested from unclotted blood by centrifugation at 600 ***g*** for 15 minutes, and the buffy coat taken off after the plasma has been carefully removed. The buffy coat is then layered onto a PBMC separating solution, Ficoll 1.077, and centrifuged at 400 ***g*** for 20 minutes. The PBMC interface (without most granulocytes) is washed twice in phosphate buffered saline (300 ***g*** for 10 minutes) and resuspended in 1 ml of Eagle's minimal essential medium (MEM) containing 2% FCS. A 0.5 ml volume of the rinsed cell suspension is added to monolayers of RK-13 cells in 25 cm^2 flasks or multiwall plates to which maintenance medium is added.

Although reportedly not always successful in natural cases of EAV infection (Timoney & McCollum, 1993), virus isolation should be attempted from clinical specimens or necropsy tissues using rabbit, equine or monkey kidney cell culture (Timoney *et al*., 2004; Timoney & McCollum, 1993). Selected cell lines, e.g. RK-13 (ATCC CCL-37), LLC-MK_2 (ATCC CCL-7), and primary horse or rabbit kidney cell culture can be used, with RK-13 cells being the cell system of choice (Timoney *et al*., 2004). Experience over the years has shown that primary isolation of EAV from semen can present more difficulty than from other clinical specimens or from infected tissues unless an appropriate cell culture system is used. Several factors have been shown to influence primary isolation of EAV from semen in RK-13 cells. Higher isolation rates have been obtained using 3- to 5-day-old confluent monolayers, a large inoculum size in relation to the cell surface area in the inoculated flasks or multiwell plates, and most importantly, the incorporation of carboxymethyl cellulose (medium viscosity, 400–800 cps) in the overlay medium. It should be noted that most RK-13 cells, including ATCC CCL-37, are contaminated with bovine viral diarrhoea virus, the presence of which appears to enhance sensitivity of this cell system for the primary isolation of EAV, especially from semen. In the case of specimens of low viral infectivity, isolation rates of EAV may be increased by using RK-13 cells of high passage history[2] (Timoney *et al*., 2004).

Inoculated cultures are examined daily for the appearance of viral cytopathic effect (CPE), which is usually evident within 2–6 days. In the absence of visible CPE, culture supernatants should be subinoculated on to confluent cell monolayers after 4–7 days. While the vast majority of isolations of EAV are made on the first passage in cell culture, a small minority only become evident on the second or subsequent passages *in vitro* (Timoney & McCollum, 1993). The identity of isolates of EAV can be confirmed by standard or real-time RT-PCR assays (Balasuriya *et al*., 1998), in a one-way neutralisation test, or by an immunocytochemical method (Little *et al*., 1995), indirect immunofluorescence (Crawford & Henson, 1973) or avidin–biotin–peroxidase (ABC) technique (Little *et al*., 1995). A polyclonal rabbit antiserum has been used to identify EAV in infected cell cultures. Mouse monoclonal antibodies (MAbs) to the nucleocapsid protein (N) and major envelope glycoprotein (GP5) of EAV as well as a monospecific rabbit antiserum to the unglycosylated envelope protein (M) (Balasuriya *et al*., 1998) have also been developed and these can detect various strains of the virus in RK-13 cells as early as 12–24 hours after infection (Balasuriya *et al*., 1998; Little *et al*., 1995).

1.2. Virus isolation from semen

There is considerable evidence that short- and long-term carrier stallions shed EAV constantly in the semen, but not in respiratory secretions or urine; nor has it been demonstrated in the buffy coat (peripheral blood mononuclear cells) of such animals (Timoney *et al*., 1987; Timoney & McCollum, 1993). Stallions should first be blood tested using the VN test or an appropriately validated ELISA or other serological test procedure. Virus isolation should be attempted from the semen of stallions serologically positive for antibodies to EAV e.g.VN titre ≥1/4, that do not have a certified history of vaccination against EVA, also with confirmation that they were serologically negative (VN titre <1/4) at time of initial vaccination. Virus isolation is also indicated in the case of shipped semen where the serological status and possible vaccination history of the donor stallion is not available. It is recommended that virus isolation from semen be attempted from two samples, which can be collected on the same day, on consecutive days, or after an interval of several days or weeks. There is no evidence that the outcome of attempted virus isolation from particular stallions is influenced by the frequency of sampling, the interval between collections, or time of the year. Isolation of EAV should be carried out preferably on portion of an entire ejaculate collected using an artificial vagina or a condom and a teaser or phantom mare. When it is not possible to obtain semen by this means, a less preferable alternative is to collect a dismount sample at the time of breeding. Care should be taken to ensure that no antiseptics/disinfectants are used in the cleansing of the external genitalia of the stallion prior to collection. Samples should contain the sperm-rich fraction of the ejaculate with which EAV is associated as the virus is not present in the pre-sperm fraction of semen (Timoney *et al*., 1987; Timoney & McCollum, 1993). Immediately following collection, the semen should be refrigerated on crushed ice or on freezer packs for transport to the laboratory as soon as possible. Where there is likely to be a delay in submitting a specimen for testing, the semen can be frozen at or below –20°C for a short period before being dispatched to the laboratory. Freezing a sample does not appear to interfere with isolation of EAV from semen. In situations where it is not feasible to determine the carrier status of a stallion by virus isolation or RT-PCR assay, the stallion

2 Such a line (RK-13-KY) is available from the WOAH Reference Laboratory for EVA in the USA (see footnote 1).

can be test bred to two seronegative mares, which are checked for seroconversion to the virus up to 28 days after breeding (Timoney & McCollum, 1993).

It is not possible to reliably determine the carrier status of stallions treated with GnRH antagonist or immunised with GnRH to modify reproductive activity or behaviour, as this may also temporarily interrupt EAV shedding.

1.2.1. Test procedure

i) On receipt in the laboratory, it should be noted whether a semen sample is frozen, chilled or at ambient temperature. Every sample should be checked to ensure that it contains the sperm-rich fraction of the ejaculate. Some stallions can produce large volumes of seminal plasma prior to ejaculating the sperm-rich and gel fractions of semen. Frequently, this pre-sperm fraction contains very few sperm and can be EAV negative even though the stallion is a carrier of EAV (Timoney *et al*., 1987). To optimise detection of virus, 50 µl of each semen sample should be transferred onto a glass slide, covered with a cover-slip and examined microscopically at a magnification of 100× to assess its sperm content. Ejaculates with less than an average of five sperm per ten fields examined should be considered of questionable diagnostic value. It is worth noting however, that the occasional oligospermic stallion can be EAV positive even with a low sperm count. If virus negative on the other hand, the test report on such a stallion should include the qualifier that freedom from EAV cannot be guaranteed based on the low sperm numbers in the sample submitted. Additionally, specimens of ejaculate should be visually inspected and recorded for colour and presence of gross particulate contamination. If a semen specimen is contaminated with blood, which can result from trauma to the external genitalia of the stallion at time of collection, a repeat sample should be requested as testing such a specimen from a serologically positive stallion may compromise the reliability of the virus isolation result due to the EAV antibodies in the serum. Very infrequently, an ejaculate may have a yellowish tinge due to contamination with urine. Such samples may be positive for equine rhinitis A virus.

ii) Although no longer considered an essential step, pretreatment of semen before inoculation into cell culture by short-term sonication (three 15-second cycles); facilitates effective mixing and dispersion of a sample.

iii) After removal of culture medium, 3- to 5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm^2 tissue culture flasks or multiwell plates, are inoculated with serial decimal dilutions (from 10^{-1} to 10^{-3}) of seminal plasma in tissue culture maintenance medium containing 2% fetal bovine serum and antibiotics. An inoculum of 1 ml per 25 cm^2 flask is used and no fewer than two flasks per dilution of seminal plasma are inoculated. Inoculum size and number of wells inoculated per dilution of a specimen should be pro-rated where multiwell plates are used. Appropriate dilutions of a virus positive control semen sample or virus control of known titre diluted in culture medium should be included in each test.

iv) The flasks are closed, lids replaced on multiwell plates and inoculated cultures gently rotated to disperse the inoculum over the cell monolayers.

v) Inoculated cultures are then incubated for 1 hour at 37°C either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO_2 in air, depending on whether flasks or multiwell plates are used.

vi) Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium with antibiotics.

vii) The flasks or plates are reincubated at 37°C and checked microscopically for viral CPE, which is usually evident within 2–6 days.

viii) In the absence of visible CPE, culture supernatants are subinoculated onto 3–5 day-old confluent cell monolayer cultures of RK-13 cells after 5–7 days. After removal of the overlay medium, monolayers are stained with 0.1% formalin-buffered crystal violet solution.

The identity of any virus isolates should be confirmed by standard or real-time RT-PCR (Balasuriya *et al*., 1998; 2002; Gilbert *et al*., 1997; Lu *et al*., 2007; Miszczak *et al*., 2011; Westcott *et al*., 2003) by VN, immunofluorescence (Crawford & Henson, 1973) or ABC technique, using a monospecific antiserum to EAV or MAbs to the structural proteins, N or GP5 of the virus (Balasuriya *et al*., 1998; Del Piero, 2000; Little *et al*., 1995).

In the one-way neutralisation test, serial decimal dilutions of the virus isolate are tested against a neutralising MAb or monospecific antiserum prepared against the prototype Bucyrus strain of EAV (ATCC VR 796) and also a serum negative for neutralising antibodies to the virus. Corresponding titrations of the prototype Bucyrus virus with the same reference antibody reagents are included as test controls. The test is performed in either 25 cm^2 tissue culture flasks or multiwell plates. Appropriate quantities of the known EAV positive and negative antibody reagents are inactivated for 30 minutes in a water bath at 56°C and diluted 1/4 in phosphate buffered saline, pH 7.2; then 0.3 ml of diluted antibody reagent is dispensed into five tubes for each isolate to be tested. Serial decimal dilutions (from 10^{-1} to 10^{-5}) of each virus isolate are made in Eagle's MEM containing 10% fetal bovine serum, antibiotics and 10% freshly diluted guinea-pig complement. Then, 0.3 ml of each virus dilution is added to the tubes containing positive and negative antibody reagents. The tubes are shaken and the virus/antibody mixtures are incubated for 1 hour at 37°C. The mixtures are then inoculated onto 3- to 5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm^2 flasks or multiwell plates, using two flasks or wells per virus dilution. Each flask is inoculated with 0.25 ml of virus/antibody mixture; the inoculum size is pro-rated where multiwell plates are used. Inoculated flasks or plates are incubated for 2 hours at 37°C, gently rocking after 1 hour to disperse the inoculum over the cell monolayers. Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium and incubated for 4–5 days at 37°C, either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO_2 in air. After removal of the medium, monolayers are stained with 0.1% formalin-buffered crystal violet solution. Plaques are counted and the virus infectivity titre is determined both in the presence and absence of EAV antibodies using the Spearman–Kärber method. Confirmation of the identity of an isolate is based on a reduction in plaque count of at least 10^2 logs of virus in the presence of antibody positive serum against the Bucyrus strain of EAV.

The vast majority of EAV isolates from carrier stallions are made in the first passage in cell culture using the described test procedure (Timoney & McCollum, 1993). The occurrence of nonviral cytotoxicity or bacterial contamination of specimens is not a significant problem when attempting isolation of this virus from stallion semen. Nonviral cytotoxicity, if observed, usually affects monolayers inoculated with the 10^{-1} and, much less frequently, the 10^{-2} dilution of seminal plasma. Treatment of seminal plasma with polyethylene glycol (Mol. wt 6000) prior to inoculation has been used with some success in overcoming this problem (Fukunaga *et al.,* 2000). The method described involves the addition of polyethylene glycol to the 10^{-1} to 10^{-3} dilutions of seminal plasma to give a final concentration of 10% in each dilution. The mixtures are held overnight at 4°C with gentle stirring, after which they are centrifuged at 2000 ***g*** for 30 minutes and the supernatants are discarded. The precipitates are suspended in cell culture maintenance medium to one-tenth the volume of the original dilutions and the mixtures are homogenised. They are then centrifuged at 2000 ***g*** for 30 minutes and the supernatants are taken off and used for inoculation. There is no evidence to indicate that pretreatment of seminal plasma in this manner reduces sensitivity of the virus isolation procedure (Fukunaga *et al*., 2000). Where bacterial contamination of a sample is a problem, it is preferable to request a repeat semen collection from the individual stallion. If this is not possible, an attempt can be made to control the contamination by pre-treatment of the sample with antibiotic containing viral transport medium, holding overnight at 4°C followed by ultracentrifugation and resuspension of the pellet before diluting and inoculating the specimen into cell culture.

There have been two reports of failure to isolate EAV from individual stallions whose semen was positive for viral nucleic acid on RT-PCR assay. In one case at least, failure to detect infectious virus may well have been the result of a very high level of neutralising activity in the seminal plasma of the stallion, emphasising the value of RT-PCR as an adjunct to virus isolation for detection of EAV.

1.3. Antigen detection

Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries and venules throughout the body, particularly in the caecum, colon, spleen, associated lymphatic glands and adrenal cortex (Crawford & Henson, 1973; Del Piero, 2000; Jones *et al*., 1957). The presence of a disseminated necrotising arteritis involving endothelial and medial cells of affected vessels is considered a pathognomonic feature of EVA. The characteristic vascular lesions present in the mature animal are not, however, a prominent a feature in many cases of EAV-related abortion.

EAV antigen can be identified in various tissues of EVA-affected animals either in the presence or absence of lesions (Del Piero, 2000). Antigen has been demonstrated in lung, heart, liver and spleen and the placenta of aborted fetuses (Del Piero, 2000). Immunohistochemical examination of biopsied skin specimens has also been investigated as a means of confirming acute EAV infection. Though of some value, it is not entirely reliable for the diagnosis of the disease. Viral antigen can be detected within the cytoplasm of infected cells by immunofluorescence using conjugated equine polyclonal anti-EAV serum (Crawford & Henson, 1973), or by the ABC technique using mouse MAbs to the GP5 or N proteins of the virus (Del Piero, 2000).

1.4. Molecular methods

The standard two-step RT-PCR, single-step RT-PCR, RT-nested PCR, and real-time RT-PCR (rRT-PCR) assays have become widely accepted as an alternative or adjunct to virus isolation in cell culture for the detection of EAV in diagnostic materials. The RT-PCR-based assays provide a means of identifying virus-specific RNA in clinical specimens, namely nasopharyngeal or nasal swab filtrates, buffy coats, raw and extended semen and urine, and in post-mortem tissue samples (Balasuriya *et al*., 2002; Gilbert *et al*., 1997; Lu *et al.,* 2007; Miszczak *et al*., 2011; Westcott *et al*., 2003). Standard, single-step RT-PCR, RT-nested PCR (RT-nPCR), and one tube TaqMan® rRT-PCR assays have been developed and evaluated for the detection of various strains of the virus in tissue culture fluid, semen and nasal secretions (Balasuriya *et al*., 2002; Gilbert *et al*., 1997; Lu *et al*., 2007; Miszczak *et al*., 2011; Westcott *et al*., 2003). These assays targeted six different open reading frames (ORFs) in the EAV genome (ORFs 1b, 3–7). However, there is considerable variation in the sensitivity and specificity among RT-PCR assays incorporating different primer pairs targeting various ORFs. Results comparable to virus isolation have been obtained with some but not all standard single-step RT-PCR, two-step RT-PCR, RT-nPCR or one tube TaqMan® rRT-PCR assays (Balasuriya *et al*., 2002; Gilbert *et al*., 1997; Lu *et al*., 2007; Miszczak *et al*., 2011). Compared with traditional virus isolation, these RT-PCR-based assays are frequently more sensitive and considerably more rapid to perform, the majority taking less than 24 hours to complete. In addition, RT-PCR assays have the advantage of not requiring viable virus for performance of the test. The one-tube rRT-PCR assay for EAV provides a simple, rapid and reliable method for the detection and identification of viral nucleic acid in equine semen and tissue culture fluid (Balasuriya *et al*., 2002; Lu *et al*., 2007; Miszczak *et al*., 2011). However, there is evidence to indicate that the choice of commercial kit used for nucleic acid extraction and also for amplification can have a major influence on the overall diagnostic sensitivity and robustness of the assay (Miszczak *et al*., 2011). This was demonstrated using a magnetic-bead-based nucleic acid extraction method in combination with specific commercial RT-PCR kits. The one tube rRT-PCR has the following important advantages over the standard two-step RT-PCR: 1) eliminating the possibility of cross contamination between samples with previously amplified products as the sample tube is never opened; and 2) reducing the chance of false-positive reactions where the rRT-PCR product is detected with a sequence-specific probe. Because of the high sensitivity of the RT-PCR assay, however, and in the absence of appropriate safeguards in the laboratory, there is the potential for cross-contamination between samples, giving rise to false-positive results. For example, the RT-nPCR assay, while it provides enhanced sensitivity for the detection of EAV, it also increases the likelihood of false-positive results. The risk of cross-contamination is greater using the RT-nPCR assay because of the second PCR amplification step involving the product from the first RT-PCR reaction. To minimise the risk of cross-contamination, considerable care needs to be taken, especially during the steps of RNA extraction and reaction setup. Relevant EAV positive and negative template controls and, where appropriate, nucleic acid extracted from the tissue culture fluid of uninfected cells, need to be included in each RT-PCR assay. Thus, in most circumstances, use of the single-step RT-PCR or one tube rRT-PCR assay will largely circumvent the problems associated with cross contamination.

Primer selection is critical to the sensitivity of the RT-PCR assay with primers (and probe in the case of the rRT-PCR assay) preferably designed from the most conserved region(s) of the EAV genome. Comparative nucleotide sequence analysis has shown that ORF 1b (encodes the viral polymerase), ORF 6 (M protein) and 7 (N protein) are more conserved than the other ORFs among EAV strains so far analysed from North America and Europe (Balasuriya *et al*., 2002; Lu *et al*., 2007; Miszczak *et al*., 2011; Westcott *et al*., 2003). The most conserved gene among different strains of EAV is ORF7 and primers specific for ORF7 (and probe for rRT-PCR) have detected a diversity of strains of the virus of European and North American origin (Balasuriya *et al*., 2002; Lu *et al*., 2007). Furthermore, the use of multiple primer pairs specific for different ORFs 1b ([forward: 5'-GAT-GTC-TAT-GCT-CCA-TCA-TT-3' and reverse: 5'-GGC-GTA-GGC-TCC-AAT-TGA-A-3']) and/or [forward: 5'-CCT-GAG-ACA-CTG-AGT-CGC-GT-3' and reverse 5'-CCT-GAT-GCC-ACA-TGG-AAT-GA-3']) (Gilbert *et al*., 1997), ORF 6 ([forward: 5'-

CTG-AGG-TAT-GGG-AGC-CAT-AG-3' and reverse: 5'-GCA-GCC-AAA-AGC-ACA-AAA-GC-3']) and ORF 7 ([forward 5'-ATG-GCG-TCA-AGA-CGA-TCA-CG-3' and reverse 5'-AGA-ATA-TCC-ACG-TCT-TAC-GGC-3']) markedly increases the likelihood of detecting North American and European strains of EAV in the RT-PCR assay. The two primer pairs specific for ORF 1b are suitable for use in a rRT-PCR assay (Gilbert *et al*., 1997). While the RT-PCR has been found to be highly sensitive for viral nucleic acid detection in raw semen, there is evidence that it is not of equivalent reliability when testing cryopreserved semen of very low virus infectivity (Zhang *et al*., 2004).

In addition to the foregoing RT-PCR assays, 2 TaqMan® fluorogenic probe-based one-tube rRT-PCR assays have been described for the detection of EAV nucleic acid (Balasuriya *et al*., 2002); primers ([forward: 5'-GGC-GAC-AGC-CTA-CAA-GCT-ACA-3', reverse: 5'-CGG-CAT-CTG-CAG-TGA-GTG-A-3'] and probe [5'FAM-TTG-CGG-ACC-CGC-ATC-TGA-CCA-A-TAMRA-3'] and (Westcott *et al*., 2003); primers [forward: 5'-GTA-CAC-CGC-AGT-TGG-TAA-CA-3', reverse: 5'-ACT-TCA-ACA-TGA-CGC-CAC-AC-3'] and probe [5'FAM-TGG-TTC-ACT-CAC-TGC-AGA-TGC-CGG-TAMRA-3']). It should be noted, however, that genomic variation among field isolates of EAV could reduce the sensitivity of both RT-PCR and rRT-PCR assays, even when the primers and probe are based on the most conserved region of the EAV genome (ORF 7 [Lu *et al*., 2007]). Phylogenetic studies of strains of EAV from certain regions/countries have confirmed the existence of clusters of isolates more closely related to one another than to virus strains of disparate geographic backgrounds (Mankoc *et al*., 2007). Under such circumstances, validated primers besides those already recommended may be more suitable for detection of these genomically distinct strains of EAV.

In the absence of widespread agreement on a universal primer set for EAV, and as no RT-PCR assay can determine the actual infectivity of a sample, there is a value to performing virus isolation in conjunction with RT-PCR or rRT-PCR for the identification of virus in clinical or post-mortem specimens and where indicated, genomic and phenotypic analysis of viral isolates.

Strains of EAV isolated from different regions of the world have been classified into different phylogenetic groups by sequence analysis of the GP3, GP5 and M envelope protein genes (ORFs 3, 5 and 6 respectively) and the nucleocapsid (N) protein gene (ORF 7 [Balasuriya *et al*., 1998; Zhang *et al*., 2010]). The GP5 gene has been found to be most useful and reliable for this purpose. The relationships between strains demonstrated by nucleotide sequencing are a useful molecular epidemiological tool for tracing the origin of outbreaks of EVA (Balasuriya *et al*., 1998; Zhang *et al*., 2010).

2. Serological tests

A variety of serological tests have been investigated for their ability to detect antibodies to EAV. These include the neutralisation (microneutralisation [Senne *et al*., 1985] and plaque reduction [McCollum, 1970]), the complement fixation (CF) test (Fukunaga & McCollum, 1977), the indirect fluorescent antibody test (Crawford & Henson, 1973), the agar gel immunodiffusion (Crawford & Henson, 1973), the ELISA (Cho *et al*., 2000; Hedges *et al*., 1998; Kondo *et al*., 1998; Nugent *et al*., 2000) and the fluorescent microsphere immunoassay (MIA) (Go *et al*., 2008).

Interestingly, only one major serotype of EAV represented by the prototype Bucyrus strain (ATCC VR 796) has been recognised so far (McCollum, 1970; Timoney & McCollum, 1993). Antiserum to unpurified EAV has been prepared in horses and in rabbits using conventional immunisation protocols. Mouse MAbs and monospecific rabbit antibodies have also been developed to the nucleocapsid protein (N) major envelope glycoprotein (GP5), and unglycosylated envelope protein (M) of EAV (Balasuriya *et al*., 1997).

2.1. Complement-enhanced microneutralisation test

The complement-enhanced microneutralisation is currently the test in widest international use to diagnose EAV infection, carry out seroprevalence studies, and test horses for movement. It has also been used to screen fetal heart blood as a means of retrospectively diagnosing cases of EVA-related abortion. Neutralising antibodies to EAV persist for several years after natural infection or vaccination with the modified live vaccine against EVA (Timoney & McCollum, 1993).

2.2. Virus neutralisation test

The VN test is used for diagnostic purposes to confirm infection in suspect cases/outbreaks of EVA and to screen horses e.g. stallions, for evidence of EAV infection. The test procedure currently in widest use

is that developed by the National Veterinary Service Laboratories of the United States Department of Agriculture (Senne *et al*., 1985). It is important to obtain a sterile blood sample as bacterial contamination of serum can interfere with the test result. It is recommended that the test be carried out in RK-13 cells using the approved CVL-Bucyrus (Weybridge) strain of EAV as reference virus[3] (Edwards *et al*., 1999). Although originally derived from the prototype Bucyrus virus, the passage history of the CVL (Weybridge) strain is not fully documented. Stocks of the reference virus are grown in the RK-13 cell line, clarified of cellular debris by low-speed centrifugation and stored in aliquots at –70°C. Several frozen aliquots are thawed and the infectivity of the stock virus is determined by titration in RK-13 cells. The sensitivity of the VN test for detection of antibodies to EAV can be significantly influenced by several factors, especially the source and passage history of the strain of virus used (Edwards *et al*., 1999). The CVL-Bucyrus (Weybridge) strain and the highly attenuated MLV vaccine strain of EAV are of comparable sensitivity for detecting low-titred positive sera, especially from EVA-vaccinated horses. Efforts are continuing to bring about greater uniformity in the testing protocol and serological results among laboratories providing the VN or other comparable serological assays for this infection. WOAH Approved Standard Sera for EAV are available[4] and these can facilitate international standardisation of the microneutralisation test and ELISA.

2.2.1. Test procedure

i) Sera are inactivated for 30 minutes in a water bath at 56°C (control sera, only once).

ii) Serial twofold dilutions of the inactivated test sera in serum-free cell culture medium (25 µl volumes) are made in a 96-well, flat-bottomed, cell-culture grade microtitre plate starting at a 1/2 serum dilution and using duplicate rows of wells for each serum to be tested. Most sera are screened initially at a 1/4 and 1/8 serum dilution (i.e. final serum dilution after addition of an equal volume of the appropriate dilution of stock virus to each well). Positive samples at the 1/8 dilution can, if desired, be retested and titrated out for end-point determination. Individual serum controls, together with negative and known low- and high-titred positive control sera must also be included in each test.

iii) A dilution of stock virus made up to contain from 100 to 300 $TCID_{50}$ (50% tissue culture infective dose) per 25 µl is prepared using as diluent, serum-free cell culture medium containing antibiotics and fresh guinea-pig or rabbit complement at a final concentration of 10%.

iv) 25 µl of the appropriate dilution of stock virus is added to every well containing 25 µl of each serum dilution, except the test serum toxicity control wells and cell control wells on each plate.

v) A virus back titration of the working dilution of stock virus is included, using four wells per tenfold dilution, to confirm the validity of the test results.

vi) The plates are covered and shaken gently to facilitate mixing of the serum/virus mixtures.

vii) The plates are incubated for 1 hour at 37°C in a humid atmosphere of 5% CO_2 in air.

viii) A suspension of cells from 3- to 5-day-old cultures of RK-13 cells are prepared using a concentration that will ensure confluent monolayers in the microtitre plate wells within 18–24 hours after seeding.

ix) 100 µl of cell suspension is added to every well, the plates covered with plate lids or sealed with tape and shaken gently.

x) The plates are incubated at 37°C in a humid atmosphere of 5% CO_2 in air.

xi) The plates are read microscopically for nonviral CPE after 12–18 hours and again for viral CPE after 48–72 hours' incubation. The validity of the test is confirmed by establishing that the working dilution of stock virus contained 30–300 $TCID_{50}$ virus and that the positive serum controls are within 0.3 log10 units of their predetermined titres.

A serum dilution is considered to be positive if there is an estimated 75% or greater reduction in the amount of viral CPE in the serum test wells compared with that present in the wells of the lowest virus

3 Available from the WOAH Reference Laboratory for EVA in the United Kingdom (see footnote 1).
4 Available from the WOAH Reference Laboratory for Equine viral arteritis in the USA (see footnote 1).

control dilution. End-points are then calculated using the Spearman–Kärber method. A titre of 1/4 or greater is considered to be positive. A negative serum should only have a trace (less than 25%) or no virus neutralisation at the lowest dilution tested. Antibody titres may, on occasion, be difficult to define as partial neutralisation may be observed over a range of several serum dilutions. Not infrequently, sera will be encountered that give rise to toxic changes in the lower dilutions tested. In such cases it may not be possible to establish whether the sample is negative or a low-titred positive. The problem may be overcome by retesting the toxic sample using microtitre plates with confluent monolayers of RK-13 cells that had been seeded the previous day. Also, the toxicity in serum samples can be reduced or eliminated if the sample is adsorbed with a packed suspension of RK-13 cells prior to testing or by substituting rabbit in place of guinea-pig complement in the virus diluent. It would appear that there is more than one type of cytotoxicity in sera. Vaccination status for equine herpesviruses should be considered when evaluating sera causing non-viral cytotoxicity. One of the equine herpesvirus vaccines currently available in Europe has been shown to stimulate antibodies to rabbit kidney cells used in the vaccine production. These, in turn, can give rise to cytotoxicity, usually in the 1/4 and/or 1/8 serum but sometimes at higher dilutions, and cause difficulties in interpretation of the test results (Newton *et al*., 2004).

2.3. Enzyme-linked immunosorbent assay

A number of direct or indirect ELISAs have been developed for the detection of antibodies to EAV (Cho *et al*., 2000; Hedges *et al*., 1998; Kondo *et al*., 1998; Nugent *et al*., 2000). These have been based on the use of purified virus or recombinant-derived viral antigens. The usefulness of earlier assays was compromised by the frequency of false-positive reactions. The latter were associated with the presence of antibodies to various tissue culture antigens in the sera of horses that had been vaccinated with tissue-culture-derived antigens. Identification of the importance of the viral GP5 protein in stimulation of the humoral antibody response to EAV led to the development of several ELISAs that employ a portion of, or the entire recombinant protein produced in a bacterial or baculovirus expression system (Cho *et al*., 2000; Hedges *et al*., 1998). Most recently, an ovalbumin-conjugated synthetic peptide representing amino acids 81–106 of the GP5 protein has been used (Nugent *et al*., 2000). Some of these assays appear to offer nearly comparable sensitivity and specificity to the VN test and may detect EAV-specific antibodies prior to a positive reaction being obtainable in the VN test. False-negative reactions can occur, however, with some of these assays. Screening a random peptide-phage library with polyclonal sera from EAV-infected horses led to the identification of ligands, which were purified and used as antigen in an ELISA for EAV. No correlation was found, however, between absorbency values obtained with this assay and neutralising antibody titres, indicating that the antibodies being detected were largely against nonsurface epitopes of the virus. An ELISA based on the use of a combination of the GP5, M or N structural proteins of EAV expressed from recombinant baculoviruses successfully detected viral antibody in naturally or experimentally infected horses but not in EVA-vaccinated animals (Hedges *et al*., 1998). Of major importance with respect to any GP5 protein-based ELISA for EAV is the fact that test sensitivity will vary depending on the ectodomain sequence(s) of this viral protein used in the assay. Considerable amino acid sequence variation within this domain has been found between isolates of EAV. To maximise sensitivity of a GP5-based ELISA, it may be necessary to include multiple ectodomain sequences representative of known phenotypically different isolates of EAV rather than depend on a single ectodomain sequence. Two more recently described ELISAs appear to offer most promise as reliable serodiagnostic tests for EAV infection (Cho *et al*., 2000; Nugent *et al*., 2000). A blocking ELISA involving MAbs produced against the GP5 protein was reported to have a sensitivity of 99.4% and a specificity of 97.7% compared with the VN test (Cho *et al*., 2000). Another assay, a GP5 ovalbumin-conjugated synthetic peptide ELISA was shown to have a sensitivity and specificity of 96.75% and 95.6%, respectively, using a panel of 400 VN positive sera and 400 VN negative samples (Nugent *et al*., 2000). Of the number of ELISAs that have been developed (Cho *et al*., 2000; Hedges *et al*., 1998; Kondo *et al*., 1998; Nugent *et al*., 2000), few, if any, have been as extensively validated as the VN test, though some would appear to offer nearly comparable sensitivity and specificity (Cho *et al*., 2000; Hedges *et al*., 1998; Nugent *et al*., 2000). It should be noted that unlike the VN test, a positive reaction in the ELISA is not necessarily reflective of the protective immune status of an individual horse to EAV as both non-neutralising and neutralising antibodies are involved.

2.4. Complement fixation test

The CF test has been used in the past for diagnosing recent infection with EAV based on the fact that complement-fixing antibodies are relatively short-lived in duration (Fukunaga & McCollum, 1977). The

test has been very largely superseded by the VN test and different ELISAs for carrying out serosurveillance studies and testing horses for movement.

2.5. Fluorescent-microsphere immunoassay

A fluorescent-MIA has been developed to detect equine antibodies to the major structural proteins of EAV (Go *et al*., 2008). It was based on cloning and expressing full-length individual major proteins, (GP5, M, N), as well as partial sequences of each structural protein and including these in separate assays. The different immunassays were analysed with a Luminex instrument. A partial GP5 protein based assay provided the best results, with sensitivity and specificity values of 92.6% and 93.9% respectively, compared with the VN test.

C. REQUIREMENTS FOR VACCINES

1. Background

A number of experimental and commercial vaccines have been developed against EVA. Currently, there are two commercially available vaccines, both tissue-culture derived. The first is a modified live virus (MLV) vaccine and the second an inactivated adjuvanated vaccine. The MLV vaccine is commercially available in the USA and Canada. It has also been used under ministerial control in Argentina and in New Zealand. The inactivated vaccine is licensed for commercial use in certain European countries, including Denmark, France, Germany, Hungary, Ireland, Sweden and the United Kingdom. Indications for use of these vaccines are to prevent outbreaks of EVA, including abortion in pregnant mares and establishment of the carrier state in the stallion. Since the carrier stallion is considered the principal reservoir of EAV, reduction in the carrier population would over time result in greater control over EVA and ultimately could contribute to eradication of the disease in certain countries. The MLV vaccine is prepared from virus that has been attenuated for horses by multiple serial transfers in primary equine and rabbit cells and in an equine dermal cell line (Doll *et al*., 1968; McCollum, 1970). This vaccine is licensed for use in stallions, nonpregnant mares and in nonbreeding horses. Whereas nonbreeding horses can be vaccinated at any time, stallions and mares should be vaccinated not less than 3 weeks prior to breeding. The vaccine is not recommended for use in pregnant mares, especially in the last 2 months of gestation, nor in foals under 6 weeks of age unless in the face of significant risk of exposure to natural infection.

The second commercially available vaccine against EVA is an inactivated product prepared from virus grown in equine cell culture, which is filtered, chemically inactivated and then combined with a metabolisable adjuvant. This vaccine is licensed for use in nonbreeding and breeding horses. In the absence of appropriate safety data, the vaccine is currently not recommended for use in pregnant mares.

An additional inactivated vaccine against EVA has been developed in Japan and is kept in storage for distribution should an outbreak of EVA occur in that country. It is an aqueous formalin-inactivated vaccine that has been shown to be safe and effective for use in nonbreeding and breeding horses. For optimal immunisation with this vaccine, horses require a primary course of two injections given at an interval of 4 weeks, with a booster dose administered every 6–12 months. As the vaccine is not commercially available, no details can be provided on its production.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

Both MLV and inactivated commercial vaccines are derived from the prototype Bucyrus strain of EAV (ATCC VR 796), an experimentally derived variant of a foetal lung isolate recovered during an extensive outbreak of respiratory disease and abortion near Bucyrus, Ohio, USA, in 1953 (Doll *et al*., 1957). Available evidence points to the existence of only one major serotype of the virus, and strain variation is not considered to be of significance in relation to vaccine efficacy (McCollum, 1970; Timoney & McCollum, 1993).

2.1.1. Biological characteristics of the master seed

In the case of the MLV vaccine, the prototype virus (ATCC VR 796) was attenuated by serial passage in primary cultures of horse kidney (HK-131), rabbit kidney (RK-111), and a diploid equine dermal cell line, ATCC CCL57 (ECID-24) (Doll *et al*., 1968; McCollum, 1970). The indications from

use of this vaccine are that the virus is safe and immunogenic between its 80th and 111th passage in primary rabbit kidney cells (Doll *et al*., 1968; McCollum, 1970).

The inactivated adjuvanted vaccine is prepared from the unattenuated prototype Bucyrus strain of EAV (ATCC VR 796) that has been plaque purified and in its fourth serial passage in the diploid equine dermal cell line (ECID-4). After growth in cell culture, the virus is then purified by filtration before being chemically inactivated and adjuvanted.

The virus for both MLV and inactivated vaccines should be grown in a stable cell culture system, such as equine dermal cells, using an appropriate medium supplemented with sterile bovine serum or bovine serum albumin as replacement for bovine serum in the growth medium. Cell monolayers should be washed prior to virus inoculation to remove traces of bovine serum. Extensive virus growth as evidenced by the appearance of cytopathic changes in 80–100% of the cells should be obtained within 2–3 days. Lots of master seed virus for each vaccine are maintained in liquid nitrogen or its equivalent.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Tests for sterility, purity and freedom of vaccines from contamination with extraneous agents can be found in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

2.1.3. Validation as a vaccine strain

In the case of both MLV and inactivated vaccines, the respective virus strains should be grown in an appropriate cell culture system that has been officially approved for vaccine production and confirmed to be free from extraneous bacteria, fungi, mycoplasmas and viruses (Moore, 1986). The identity of the vaccine virus in the master seed should be confirmed by neutralisation with homologous anti-EAV serum. Incomplete neutralisation of EAV by homologous horse or rabbit antisera has been scientifically documented (Moore, 1986; Senne *et al*., 1985) and is a problem when screening master seed virus for extraneous viruses and when attempting to confirm the identity of the vaccine virus. The problem has been circumvented by reducing the infectivity titre of the master seed virus below that required for seed virus production before conducting a neutralisation test on the diluted virus. Virus/serum mixtures are tested for residual live virus by serial passage in cell culture. No evidence of cytopathic viruses, haemadsorbing viruses, or noncytopathic strains of bovine virus diarrhoea virus should be found, based on attempted virus isolation in cell culture. If cells of equine origin are used, they should be confirmed to be free from equine infectious anaemia virus. Conventional technologies such as PCR and antigen-capture ELISAs are now more commonly used than virus isolation in screening for adventitious agents.

2.2. Method of manufacture

2.2.1. Procedure

Both the MLV and inactivated vaccines are produced by cultivation of the respective seed viruses in an equine dermal cell system. Cell monolayers should be washed prior to inoculation with seed virus to remove traces of bovine serum in the growth medium. Inoculated cultures should be maintained on an appropriate maintenance medium. Harvesting of infected cultures should take place when almost the entire cell sheet shows the characteristic CPE. Supernatant fluid and cells are harvested and clarified of cellular debris and unwanted material by filtration. In the case of the inactivated vaccine, the purified virus is then chemically inactivated and adjuvanted with a metabolisable adjuvant. The preservatives added to the MLV and inactivated vaccines are neomycin, polymycin B and amphotericin B.

2.2.2. Requirements for ingredients

Refer to Chapter 1.1.8 *Principles of veterinary vaccine production,* the focus of which is on products of biological origin of negligible risk.

2.2.3. In-process controls

The MLV and inactivated vaccines should be produced in a stable cell line that has been tested for identity and confirmed to be free from contamination by bacteria, fungi, mycoplasmas or other adventitious agents. In addition to the preproduction testing of the master seed virus for each vaccine and the cell line for adventitious contaminants, the cell cultures infected with the respective vaccine viruses should be examined macroscopically for evidence of microbial growth or other extraneous contamination during the incubation period. If growth in a culture vessel cannot be reliably determined by visual examination, subculture, microscopic examination, or both should be carried out.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use can be found in chapter 1.1.9. In the case of both MLV and inactivated vaccines, each production lot of vaccine should be checked for extraneous bacterial, fungal and mycoplasmal contaminants.

ii) Safety

The vaccine should be safety tested by the intramuscular inoculation of at least two horses seronegative for neutralising antibodies to EAV with one vaccine dose of lyophilised virus each (Moore, 1986). None of the inoculated horses should develop any clinical signs of disease other than mild pyrexia during the ensuing 2-week observation period. Transient local reactions may be observed in less than 10% of horses inoculated with either vaccine. In addition, nasopharyngeal swabs should be collected daily from each horse for attempted virus isolation; white blood cell counts and body temperatures should also be determined on a daily basis. No significant febrile or haematological changes should supervene following vaccination (Timoney & McCollum, 1993). Limited shedding of vaccine virus by the respiratory route and in semen may be demonstrated in the occasional horse within the first 7 days after vaccination. There is no evidence of persistence of the vaccine virus in the reproductive tract of vaccinated stallions (Timoney & McCollum, 1993).

To ensure complete inactivation of the vaccine virus, each serial lot of the inactivated vaccine should be checked for viable virus by three serial passages in equine dermal cells and by direct fluorescent antibody staining with specific EAV conjugate before being combined with adjuvant. This should be followed by a safety test in guinea-pigs and mice.

iii) Batch potency

Potency of the vaccine in the final containers is determined by plaque infectivity assay in monolayer cultures of equine dermal cells and by a vaccination challenge test in horses (Moore, 1986). The vaccine must be tested in triplicate in cell culture, the mean infectivity titre calculated and the dose rate determined on the basis that each dose of vaccine shall contain not less than 3×10^4 plaque-forming units of attenuated EAV. The *in-vivo* potency of the MLV and inactivated vaccines is evaluated in a single vaccination challenge test using 17–20 vaccinated and 5–7 control horses or in two tests each comprising ten vaccinates and five controls. The viral antigen concentration in the inactivated vaccine is over one-thousand times the concentration of viral antigen present in the MLV vaccine.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For vaccine registration, all relevant details concerning manufacture of the vaccine and quality control section should be submitted to the authorities. This information shall be provided from three consecutive batches with a volume of not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

The manufacturer of the MLV recommends a single dose of vaccine administered intramuscularlyfor primary vaccination followed by annual revaccination. The recommended vaccination regimen for the inactivated vaccine, which should also be administered by the intramuscular route, is a primary course of two vaccinations 3–6 weeks apart, followed by revaccination every 6 months.

i) Target and non-target animal safety

The MLV vaccine is considered safe for stallions and nonpregnant mares. There is no evidence to indicate that the vaccine virus can establish the carrier state in the vaccinated stallion. The MLV vaccine is not recommended for use in pregnant mares or in foals less than 6 weeks of age. Although contra-indicated by the manufacturer, this vaccine has been used in pregnant mares in the first two trimesters without any adverse sequelae. There is the risk of abortion in mares vaccinated within the last two months of gestation. The inactivated vaccine is safe for use in non-breeding and breeding animals. In the absence of appropriate safety data, the vaccine is not currently recommended for use in pregnant mares.

ii) Reversion-to-virulence for attenuated live vaccines and environmental considerations

Both experimental and extensive field studies conducted since the MLV vaccine was first released commercially in 1985, have failed to provide any evidence of back reversion to virulence or of recombination with naturally occurring strains of EAV (Timoney & McCollum, 1993).

iii) Precautions

The manufacturer of both the MLV and inactivated vaccines provides adequate information in the respective vaccine inserts as to the recommended usage of each vaccine, including certain contra-indications in the case of the MLV vaccine. Neither vaccine is harmful to vaccinators.

2.3.3. Efficacy requirements

Both MLV and inactivated vaccines have been evaluated for efficacy in vaccination – challenge studies. This involved respiratory challenge of a group of first-time vaccinated horses 4 weeks after primary immunisation, with the virulent prototype Bucyrus strain of EAV. The level of protective immunity engendered by vaccination was assessed based on failure to produce clinical signs of EVA in the challenged horses or a significant reduction in the severity of disease compared to that observed in the nonvaccinated controls. The efficacy of vaccination in preventing establishment of the carrier state in vaccinated stallions was similarly evaluated.

2.3.4. Duration of immunity

Detectable neutralising antibody titres to EAV should develop in the majority of horses within 1–2 weeks of vaccination with the MLV vaccine (Timoney & McCollum, 1993). Reported responses to primary vaccination have been variable in a couple of studies. In one stallion vaccination study, there was a rapid fall in antibody titres with a significant number of animals reverting to seronegativity 1–3 months after vaccination. On the other hand, other studies have been characterised by an excellent durable response, with persistence of high VN levels for at least 1–2 years. Revaccination with this vaccine results in an excellent anamnestic response, with the development of high antibody titres that remain relatively undiminished for several years (Timoney & McCollum, 1993).

Experimental studies have shown that most horses vaccinated with the inactivated vaccine develop low to moderate neutralising antibody titres to EAV by day 14 after the second vaccination. There is no published information on the duration of immunity conferred by this vaccine.

2.3.5. Stability

The lyophilised MLV vaccine can be stored for at least 3–4 years at 2–7°C without loss in infectivity, provided it is kept in the dark. Infectivity is preserved for much longer periods if vaccine is frozen at –20°C or below. Once rehydrated, however, the vaccine should be used within 1 hour or else destroyed. The inactivated vaccine is stored as a liquid suspension at 2–8°C, with no loss of potency for at least 1 year, provided it is protected from light.

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NB: There are WOAH Reference Laboratories for equine viral arteritis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for equine viral arteritis

NB: FIRST ADOPTED IN IN 1990. MOST RECENT UPDATES ADOPTED IN 2013.

CHAPTER 3.6.11.

GLANDERS AND MELIOIDOSIS

SUMMARY

Description and importance of the disease: *Glanders is a contagious and fatal disease of horses, donkeys, and mules, caused by infection with the bacterium* Burkholderia mallei*. The pathogen causes nodules and ulcerations in the upper respiratory tract and lungs. The skin form is known as 'farcy'.*

Melioidosis is an infectious disease caused by Burkholderia pseudomallei*. Melioidosis is uncommon in humans and animals and sometimes resembles glanders in horses. This chapter focuses on the disease in horses.* Burkholderia mallei *has evolved from* B. pseudomallei *by reduction of genetic information and is phylogenetically considered as a clone, i.e. a pathovar of* B. pseudomallei.

Control of glanders and melioidosis requires testing of suspect clinical cases, screening of apparently normal equids, and elimination of reactors. Stable hygiene and manure management are imperative. As B. mallei *and* B. pseudomallei *can be transmitted to humans, all infected or contaminated (or potentially infected or contaminated) material must be handled in a laboratory with appropriate biosafety and biosecurity controls following a biorisk analysis.*

Detection of the agent: *Smears from fresh material containing* B. mallei *bacteria may reveal Gram-negative nonsporulating, nonencapsulated rods.* Burkholderia mallei *grows aerobically and prefers media that contain glycerol. Standard media for isolation of* B. pseudomallei *can be used and selective enrichment techniques have been developed. The presence of a capsule-like cover has been demonstrated by electron microscopy in both agents. Unlike the* Pseudomonas *species and the closely related bacterium* B. pseudomallei, B. mallei *is nonmotile. For identification, biochemical phenotyping can be used. Commercially available biochemical identification kits lack diagnostic sensitivity. MALDI-TOF spectra have been made available for both agents in the past years. Whole genome sequences have been published. Specific monoclonal antibodies and polymerase chain reaction (PCR), as well as real-time PCR assays are available.*

Serological tests: *Complement fixation is an accurate and reliable serological method for diagnostic use in glanders in equids. A rose bengal plate agglutination test for glanders has been developed. An immunoblot test based on a crude formalin preparation of* B. mallei *antigens from isolates of different geographical regions is also a sensitive and specific assay. These tests may also be positive in horses with melioidosis. Enzyme-linked immunosorbent assays show promise for specific diagnosis in equids once their validation is complete.*

Mallein test: *The mallein test is a hypersensitivity skin test against* B. mallei*. The test is not generally recommended because of animal welfare concerns, however it can be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. Mallein, a water soluble protein fraction of the organism, is injected intradermo-palpebrally. In infected animals, the eyelid swells markedly within 1–2 days. This test may also be positive in horses with melioidosis.*

Requirements for vaccines and diagnostic biologicals: *There are no vaccines. Mallein purified protein derivative is commercially available.*

A. INTRODUCTION

Glanders is a bacterial disease of perissodactyls or odd-toed ungulates. It is a zoonotic disease and has been known since ancient times. It is caused by the bacterium *Burkholderia mallei* (Yabuuchi *et al.*, 1992) and has been variously classified in the past as *Pseudomonas, Pfeifferella*, *Loefflerella, Malleomyces* or *Actinobacillus*. It is a serious

contagious disease in equids and outbreaks may also occur in felids living in the wild or in zoological gardens. Susceptibility to glanders has been proven in camels, bears, wolves and dogs. Carnivores may become infected by eating infected meat, but cattle, and pigs are resistant. Small ruminants may be infected if kept in close contact with glanderous horses (Wittig *et al.*, 2006). Glanders generally takes an acute form in donkeys and mules with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia) and death occurs within a few days. In horses, glanders generally takes a more chronic course and horses may survive for several years. Chronic and subclinical 'occult' cases are potential sources of infection due to the permanent or intermittent shedding of bacteria (Wittig *et al.*, 2006). Khan *et al.* (2013) reviewed the disease, its epidemiology, diagnosis and control.

In horses, inflammatory pustules and ulcers develop in the nasal conchae and nasal septae, which give rise to a sticky yellow discharge, accompanied by enlarged firm submaxillary lymph nodes. Stellate scarring follows upon healing of the ulcers. The formation of reddish nodular abscesses with a central grey necrotic zone in the lungs is accompanied by progressive debility, febrile episodes, coughing and dyspnoea. Diarrhoea and polyuria can also occur. In the skin form ('farcy'), the lymphatics are enlarged and 0.5–2.5 cm sized nodular abscesses ('buds') develop, which ulcerate and discharge yellow oily pus. Dry ulcers may also develop. Pyogranulomatous nodules are sometimes found in the liver and spleen. Discharges from the respiratory tract and skin are infective, and transmission between animals, which is facilitated by close contact, inhalation, ingestion of contaminated material (e.g. from infected feed and water troughs), or by inoculation (e.g. via a harness) is common. The incubation period can range from a few days to many months (Wittig *et al.*, 2006).

Glanders is transmissible to humans by direct contact with diseased animals or with infected or contaminated material. In the untreated acute disease, the mortality rate can reach 95% within 3 weeks (Neubauer *et al.*, 1997). However, survival is possible if the infected person is treated early and aggressively with multiple systemic antibiotic therapies. A chronic form with abscessation can occur (Neubauer *et al.*, 1997).

Glanders has been eradicated from many countries by statutory testing, culling of infected animals, and import restrictions. It persists in numerous Asian, African and South American countries and can be considered a re-emerging disease. Glanders can be introduced into glanders-free areas by movement of equids (Neubauer *et al.*, 2005).

Burkholderia pseudomallei is the causative agent of melioidosis and originates from South-East Asia (areas within latitudes 20°N and 20°S) (Limmathurotsakul *et al.,* 2016). It is an important soil bacterium, denitrifying organic materials and is ubiquitous in those areas. Thus, *B. pseudomallei* is stable in the environment. It is also naturally resistant to various antibiotics and disinfectants (Currie, 2015; O'Connell *et al.,* 2009; Sprague & Neubauer, 2004). *Burkholderia pseudomallei* has an extremely broad host range including wildlife, farm animals and humans (Rush & Thomas, 2012). *Burkholderia pseudomallei* has regularly been isolated from abscesses in various organs (spleen, liver, lung, lymph nodes), from milk in cases with mastitis, or from faeces in diarrhoea cases. This chapter focuses on the disease in horses, which may closely resemble glanders, but more diverse presentations also occur. Melioidosis in horses has been reported as peracute cases with high fever, septicaemia, limb oedema, diarrhoea and death occurring within 24 hours, or acute cases with limb oedema, slight colic and intestinal hypermotility. More typically, melioidosis runs a subacute to chronic course from 3 weeks to 3 months with no loss of appetite. Further signs reported are emaciation, oedema and lymphangitis of the limbs, mild colic, diarrhoea, pneumonia, cough and nasal discharge. Skin involvement may initially resemble fungal eczema, later becoming papular without abscess formation. Acute meningoencephalitis and keratitis may be seen. If infection is acquired *per os*, intestinal signs may predominate. It is reported that the lungs are always affected and show signs of acute bronchopneumonia and numerous abscesses. Severe enteritis, multiple microabscessation in the kidneys, and necrotic foci in liver and spleen may be found at necropsy. Ulcers on the mucosa of the upper respiratory tract and cicatrical scar tissue on the septum nasi and epiglottis may be found. Ulcers and nodules in the skin and subcutis of the limbs may be confused with farcy. Conjunctivitis and keratitis have been reported, though rare. The incubation period for naturally occurring infection in animals is not known. Spread may be by infected animals or contaminated materials in the animals' environment. Infection is thought to occur by inoculation, ingestion or inhalation of environmental organisms. Inapparent infection may occur. (reviewed by Sprague & Neubauer, 2004). In humans the disease is frequently fatal despite timely institution of therapy (Currie, 2015; Kingsley *et al.,* 2016b). No approved antibiotic regimens for horses have been recommended.

Melioidosis can be considered an emerging disease and is present in many regions worldwide (Limmathurotsakul *et al.,* 2016). Control of melioidosis requires testing of suspect clinical cases, screening of apparently normal equids, and elimination of reactors. In areas with a temperate climate, special thought has to be given to stable hygiene (disinfection, removal of faeces several times a day, reduction of the use of water for cleaning, disinfection of hooves and lower limbs, movement control) and soil hygiene. Suitable methods to prevent spread by treatment of manure,

waste water or rodent control have not been investigated or reported (Dodin, 1992). Dodin (1992) stated that the enzootics in France declined and the infected soils were cleared only after all infected animals were removed. Consequently, Sprague & Neubauer (2004) proposed that equids with melioidosis should be culled immediately in non-endemic countries to prevent local establishment of environmental reservoirs that would pose an ongoing risk of infection for humans and animals.

When handling suspect or known infected animals or fomites, stringent precautions must be taken to prevent self-infection or transmission of the bacteria. Laboratory samples must be securely packaged, kept cool (not frozen) and shipped as outlined in Chapter 1.1.3 *Transport of biological materials*. All manipulations with potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of glanders and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
PCR	–	–	–	+	–	–
Culture	–	–	–	+	–	–
Detection of immune response						
Complement fixation	++	++[(a)]	+++	+	+++	–
ELISA	+	+	++	+	++	–
Mallein skin test	+	+	+	+	+	–
Western blotting	+	+	++	+	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[(a)]Horse samples only – care needed with interpretation of test on donkey samples.

For melioidosis, culture and polymerase chain reaction (PCR) methods may be used to detect and identify the organism for confirmation of clinical cases, as described in the text below, but serological methods are not yet available for this infection.

1. Interpretation of tests for the diagnosis of glanders

Confirmation of a diagnosis of glanders should be based on the isolation and identification of *Burkholderia mallei* in a sample from an equid or a product derived from that equid; or the identification in such samples of antigen or genetic material specific to *B. mallei.* Supporting evidence may be provided by positive serological test results such as a titre of 1/5 in the complement fixation test (CFT), confirmed by a second test with equal or higher sensitivity and higher specificity, e.g. *B. mallei*-specific lipopolysaccharide (LPS)-western blot, l-enzyme-linked

immunosorbent assay (ELISA) (indirect) (based on a recombinant protein from type VI secretion system) or C-ELISA (competitive ELISA) (based on *B. mallei*-specific monoclonal antibodies).

2. Detection of the agent

Cases for specific glanders investigation should be differentiated on clinical grounds from other chronic infections affecting the nasal mucous membranes, sinuses or the skin. Among these are strangles (*Streptococcus equi*), ulcerative lymphangitis (*Corynebacterium pseudotuberculosis*), pseudotuberculosis (*Yersinia pseudo-tuberculosis*) and sporotrichosis (*Sporotrichium* spp.). Glanders should be excluded from suspected cases of epizootic lymphangitis (*Histoplasma farciminosum*), with which it has many clinical similarities. In horses and humans in particular, glanders should be distinguished from melioidosis.

2.1. Morphology of *Burkholderia mallei*

Burkholderia mallei organisms are fairly numerous in smears from fresh lesions, but scarce in older lesions. Smears should be stained with methylene blue or Gram stain. The Gram-negative rods have rounded ends, are 2–5 μm long and 0.3–0.8 μm wide with granular inclusions of various size. The bacteria are generally located extracellularly and frequently stain irregularly and poorly when Gram stain is used. They do not have a readily visible capsule under the light microscope and do not form spores. The presence of a capsule-like cover has been verified by electron microscopy. This capsule is composed of neutral carbohydrates and serves to protect the cell from unfavourable environmental factors. Unlike other organisms in the *Pseudomonas* group and its close relative *B. pseudomallei*, *B. mallei* has no flagellae and is therefore nonmotile (Sprague & Neubauer, 2004). Nonmotility is the most important phenotypic characteristic diagnostically and must be demonstrated when pure culture is available. The organisms are difficult to detect in tissue sections, where they may have a beaded appearance. In culture media, they vary in appearance depending on the age of the culture and type of medium. In older cultures, there is much pleomorphism. Branching filaments form on the surface of broth cultures (Neubauer *et al.*, 2005).

2.2. Cultural characteristics

It is preferable to attempt isolation from unopened, uncontaminated lesions. *Burkholderia mallei* is aerobic and facultative anaerobic only in the presence of nitrate, growing optimally at 37°C. It grows well, but slowly, on culture media, including sheep blood agar. Incubation of cultures for 72 hours is recommended; glycerol enrichment is particularly useful. The tiny greyish shiny colonies of *B. mallei* on sheep blood agar can be easily overgrown by other bacteria; hence careful observation is needed not to overlook the bacteria after 72 hours of incubation. After a few days on glycerol agar, a confluent, smooth, moist and slightly viscous cream coloured growth can be observed. On continued incubation, the growth thickens and becomes dark brown and tough. *Burkholderia mallei* also grows well on glycerol potato agar and in glycerol broth, on which a slimy pellicle forms. On nutrient agar, the growth is much less effusive, and growth is poor on gelatine. Various commercially available *Burkholderia* selective agars enable the growth of *B. mallei* (Glass *et al.*, 2009). Even in fresh samples obtained under sterile conditions *B. mallei* is often overgrown by other bacteria, which makes isolation extremely difficult (Wernery, 2009).

Confirmation of the identity of suspected isolates is by biochemical reactions or by PCR. Growth characteristics may alter *in vitro*, so fresh isolates should be used for identification reactions. The positive biochemical reactions include reduction of nitrates, utilisation of arginine by arginine dihydrolase, assimilation of glucose, N-acetyl glucosamine and gluconate. Strain to strain variation is observed in the assimilation reactions of arabinose, fructose, mannose, mannitol, adipic acid, malate, trisodium citrate, phenyl acetic acid and VP reaction, which needs an incubation time of 48 hours. Indole is not produced, horse blood is not haemolysed and no diffusible pigments are produced in cultures. Various biochemical characteristics can be used for the differentiation of *B. mallei* from *B. pseudomallei*, as *B. mallei* has lost many fermentative activities during phylogeny (Neubauer *et al.,* 1997). Commercially available laboratory biochemical identification systems can be used for confirmation that an organism belongs to the *Pseudomonas* group. In general, however, commercially available systems are not suitable for unambiguous identification of members of the steadily growing number of species within the genus *Burkholderia* (Glass & Popovic, 2005, Hemarajata *et al*., 2016; Inglis *et al*., 2005; Kingsley *et al*., 2016a; Lau *et al*., 2015; Zong *et al*., 2012). Lack of motility is therefore of special relevance. Cell matrix-assisted laser desorption/ionisation mass spectrometric typing can be used (Cunningham & Patel, 2013; Karger *et al*. 2012). A bacteriophage specific for *B. mallei* is available. Reference laboratories or specialised

laboratories use *B. pseudomallei*-specific antibodies that are not commercially available and that have not been validated for use in equids (Sprague & Neubauer, 2004)

All prepared culture media should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are functioning correctly.

In contaminated samples, supplementation of media with substances that inhibit the growth of Gram-positive organisms (e.g. crystal violet, proflavine) has proven to be useful, as well as pre-treatment with penicillin (1000 units/ml for 3 hours at 37°C). A semi-selective medium (Xie *et al.*, 1980) composed of polymyxin E (1000 units), bacitracin (250 units), and actidione (0.25 mg) incorporated into nutrient agar (100 ml) containing glycerine (4%), donkey or horse serum (10%), and ovine haemoglobin or tryptone agar (0.1%) has been developed. Heavily contaminated samples should also be streaked onto stiff blood agar (3% agar) which inhibits the growth of *Proteus* spp., and onto Sabouraud dextrose agar which inhibits the growth of many Gram-positive and Gram-negative bacteria in glanders samples. These samples should also be streaked onto blood agar and incubated for 24 hours anaerobically to inhibit the growth of obligate aerobes. Isolation of *B. mallei* from the anaerobic plates needs a further 24 hours' incubation at 37°C. PCR methods may also prove useful for testing contaminated samples.

No validated procedures for the isolation of *B. pseudomallei* from horse samples exist. Cultivation may be successful from samples of ulcers, lesions or excretions. Standard media, i.e. blood or MacConkey agar, are used for isolation of *B. pseudomallei* and selective enrichment techniques e.g. Ashdown agar, Galimand's broth or *B. pseudomallei* selective agar (BPSA) have been developed (Limmathurotsakul *et al*., 2012; Peacock *et al*., 2005; Prakash *et al*., 2014; Roesnita *et al*., 2012; Sprague & Neubauer, 2004; Trung *et al*., 2011). Colony morphology varies from smooth to rough depending on the medium and strain, and often becomes wrinkled after a few days of incubation. A metallic sheen over the area of confluent growth on Columbia agar is an important feature for the presumptive identification of *B. pseudomallei* (Dance *et al*., 1989).

Outside the body, *B. mallei* shows little resistance to drying, heat, light or chemicals, so that survival beyond 2 weeks is unlikely (Neubauer *et al*., 1997). Under favourable conditions, however, it can probably survive a few months. *Burkholderia mallei* can remain viable in tap water for at least 1 month. For disinfection, benzalkonium chloride (1/2,000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol, and potassium permanganate have been shown to be highly effective. Phenolic disinfectants are less effective (St. Georgiev, 2008).

Burkholderia pseudomallei can survive in water up to 16 years (Pumpuang *et al.,* 2011), in muddy water for up to 7 months and in soil in the laboratory for up to 30 months. Chlorine has only a bacteriostatic effect on the agent as bacteria were recovered from water containing up to 1000 p.p.m. free chlorine (review: Sprague & Neubauer, 2004).

National regulations and guidelines for handling and application of disinfectants should be observed.

2.3. Identification of *Burkholderia mallei* by polymerase chain reaction (PCR) and real-time PCR

Several PCR and real-time PCR assays for the identification of *B. mallei* and differentiation of *B. pseudomallei* have been developed (review Lowe *et al*., 2014).

One conventional PCR and one real-time PCR assay were evaluated using samples from a natural outbreak of glanders in horses (Scholz *et al*., 2006; Tomaso *et al*., 2006). These two assays are therefore described in more detail, but inter-laboratory studies are needed to confirm the robustness of these assays. The guidelines and precautions outlined in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* should be observed.

2.3.1. DNA preparation

Single colonies are transferred from an agar plate to 200 µl deionised water. After heat inactivation (for example 99°C for 30 minutes), the DNA isolation can be performed using commercial DNA preparation kits for gram-negative bacteria (see Scholz *et al.,* 2006 and Tomaso

et al., 2006). Alternatively, heat-inactivated bacteria from pure cultures (eg 99°C, 10 minutes) can b used directly for PCR.

Tissue samples from horses (skin, lung, mucous membrane of the nasal conchae and septae, liver and spleen) that have been inactivated and preserved in formalin (48 hours, 10% v/v) are cut with a scalpel into pieces of 0.5 × 0.5 cm (approximately 500 mg). The specimens are washed twice in deionised water (10 ml), incubated overnight in sterile saline at 4°C, and minced by freezing in liquid nitrogen, followed by grinding with a mortar and pestle. Total DNA is prepared from 50 mg tissue using a commercial extraction kit according to the manufacturer's instructions. DNA is eluted with 80 µl dH_2O or as appropriate for the kit used.

For *B. pseudomallei,* kits have been evaluated for the extraction of DNA from tissue samples (Obersteller *et al*., 2016).

2.3.2. PCR assay (Scholz *et al.,* 2006)

The assay may have to be adapted to the PCR instrument used with minor modifications to the cycle conditions and the concentration of the reagents used.

The oligonucleotides used by Scholz *et al.,* (2006) are based on the differences between the *fliP* sequences from *B. mallei* ATCC 23344^T (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). Primers Bma-IS407-flip-f (5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3') and Bma- IS407-flip-r (5'-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3') are used to amplify a 989 bp fragment. The PCR uses 50 µl ready-to-go master mix, 15 pmol of each primer, and 4 µl of template DNA. Thermal cycling conditions are 94°C for 30 seconds and 35 cycles at 65°C for 30 seconds and 72°C for 60 seconds and succeeded by a final elongation step at 72°C for 7 minutes. Visualisation of the products takes place under UV light after agarose gel (1% w/v in TAE buffer) electrophoresis and staining with nucleic acid stain. No template controls containing PCR-grade water instead of template and positive controls containing *B. mallei* DNA have to be included in each run to detect contamination by amplicons of former runs or amplification failure. The lower detection limit of this assay is 10 fg or 2 genome equivalents.

2.3.3. Real-time PCR assay (Tomaso *et al.,* 2006)

The assay should be adapted to the real-time PCR instrument used, e.g. the cycling vials should be chosen according to the manufacturer's recommendations, the concentration of the oligonucleotides may have to be increased, or the labelling of the probes altered.

The oligonucleotides used by Tomaso *et al.* (2006) are based on differences in the *fliP* sequences of *B. mallei* ATCC 23344^T (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). The fluorogenic probe is synthesised with 6-carboxy-fluorescein (FAM) at the 5'-end and black hole quencher 1 (BHQ1) at the 3'-end. Oligonucleotides used were Bma-flip-f (5'-CCC-ATT-GGC-CCT-ATC-GAA-G-3'), Bma-flip-r (5'-GCC-CGA-CGA-GCA-CCT-GAT-T-3') and probe Bma-flip (5'-6FAM-CAG-GTC-AAC-GAG-CTT-CAC-GCG-GAT-C-BHQ1-3'). The 25 µl reaction mixture consists of 12.5 µl 2× master mix, 0.1 µl of each primer (10 pmol/µl), 0.1 µl of the probe (10 pmol/µl) and 4 µl template DNA. Thermal cycling conditions are 50°C for 2 minutes; 95°C for 10 minutes; 45 cycles at 95°C for 25 seconds and 63°C for 1 minute. Possible contaminations with amplification products from former reactions are inactivated by an initial incubation step using uracil *N*'-glycosilase. The authors suggest including an internal inhibition control based on a bacteriophage lambda gene target (Lambda-F [5'-ATG-CCA-CGT-AAG-CGA-AAC-A-3] Lambda-R [5'-GCA-TAA-ACG-AAG-CAG-TCG-AGT-3'], Lam-YAK [5'-YAK-ACC-TTA-CCG-AAA-TCG-GTA-CGG-ATA-CCG-C-DB-3']), which can be titrated to provide reproducible cycle threshold values. However, depending on the sample material a PCR targeting a housekeeping gene may be used additionally or as an alternative. No template controls containing 4 µl of PCR-grade water instead and positive controls containing DNA of *B. mallei* have to be included in each run to detect amplicon contamination or amplification failure.

The linear range of the assay was determined to cover concentrations from 240 pg to 70 fg bacterial DNA/reaction. The lower limit of detection defined as the lowest amount of DNA that was consistently detectable in three runs with eight measurements each is 60 fg DNA or four

genome equivalents (95% probability). The intra-assay variability of the *fliP* PCR assay for 35 pg DNA/reaction is 0.68% (based on Ct values) and for 875 fg 1.34%, respectively. The inter-assay variability for 35 pg DNA/reaction is 0.89% (based on Ct values) and for 875 fg DNA 2.76%, respectively.

To date, a positive result in real-time PCR confirms the diagnosis '*Burkholderia mallei*' for an isolate and the diagnosis 'glanders' in clinical cases. It has to be kept in mind, however, that future genetic evolution may well result in *B. mallei* clones that can no longer be detected by these standard PCRs.

The sensitivity of the PCR assays for clinical samples is unknown. A negative result therefore, is no proof of the absence of *B. mallei* in the sample and other diagnostic means must be applied for confirmation.

2.4. Other methods

Molecular typing techniques for *Burkholderia* isolates such as PCR-restriction fragment length polymorphism (Tanpiboonsak *et al.*, 2004), pulsed field gel electrophoresis (Chantratita *et al.*, 2006), ribotyping (Harvey & Minter, 2005), multilocus sequence typing (MLST) (Godoy *et al.*, 2003), or variable number tandem repeat analysis (Currie *et al.*, 2009) are only appropriate for use in specialised laboratories. Molecular typing and whole genome sequencing may be useful in the future (Gilling *et al.*, 2014; McRobb *et al.*, 2015; Price *et al.*, 2015).

3. Serological tests

3.1. Complement fixation test in horses, donkeys, and mules

The CFT is an accurate serological test that has been used for many years for diagnosing glanders. It will deliver positive results within 1 week post-infection and will also recognise sera from exacerbated chronic cases. Application of rigorous quality control in the formulation of CFT antigens, complement and haemolytic systems are crucial for the performance of this test as its specificity and sensitivity are critically dependent on the antigen used (Elschner *et al.*, 2011; Khan *et al.*, 2011). However, the specificity of CFT has been questioned (Neubauer *et al.*, 2005). The CFT is valid for horses, mules and camels; if used in donkeys particular care is needed to avoid misdiagnosis.

3.1.1. Antigen preparation

i) The stock culture strain of *B.mallei* stored at –80°C is revived by plating onto sheep blood agar and incubated at 37°C for 48 hours to get a confluent growth.

ii) From this 48 hours culture, a loopful (0.5 mm diameter) is inoculated to 5 ml of brain–heart infusion (BHI) broth with 3% glycerol and incubated at 37°C for 24 hours.

iii) 1 ml from the above culture broth is further inoculated to 100 ml BHI broth with 3% glycerol and incubated at 37°C for 48 hours with gentle agitation.

iv) The cultures are inactivated by exposing the flasks to flowing steam (100°C) for 60 minutes.

v) The clear supernatant is decanted and filtered. The filtrate is heated again by exposure to live steam for 1 hour, and clarified by centrifugation at 3000 rpm for 10 minutes.

vi) The clarified product is stored as concentrated antigen in brown glass bottles to shield from light and stored at 4°C. Antigen has been shown to be stable for at least 10 years in this concentrated state.

vii) Aliquots of antigen are prepared by diluting the concentrated antigen 1/20 with sterile physiological saline containing 0.5% phenol. The diluted antigen is dispensed into brown-glass vials and stored at 4°C. The final working dilution is determined by a block titration. The final working dilution for the CFT is prepared when performing the test.

The resulting antigen consists primarily of lipopolysaccharides (LPS). An alternative procedure is to use young cultures by growing the organism on glycerol–agar slopes for up to 48 hours and washing them off with normal saline. A suspension of the culture is heated for 1 hour at 70°C and

the heat-treated bacterial suspension is used as antigen. The disadvantage of this antigen preparation method is that the antigen contains all the bacterial cell components. The antigen should be safety tested by inoculating blood agar plates.

3.1.2. CFT procedure

i) Serum is diluted 1/5 in veronal (barbiturate) buffered saline containing 0.1% gelatine (VBSG) or CFD (complement fixation diluent – available as tablets) without gelatine or other commercially provided CFT buffers.

ii) Diluted serum is inactivated for 30 minutes at 58–60°C. Serum of equidae other than horses should be inactivated at 63°C for 30 minutes. Camel serum is inactivated for 30 minutes at 56°C.

iii) Twofold dilutions of the sera are prepared using veronal buffer or alternative commercially available CFT buffers in 96-well round-bottom microtitre plates.

iv) Guinea-pig complement is diluted in the chosen buffer and 4 or 5 complement haemolytic units-50% (CH_{50}) are used.

v) Sera, complement and antigen are mixed in the plates and incubated for 1 hour at 37°C. An alternative procedure is overnight incubation at 4°C.

vi) A 2-3% suspension of sensitised washed sheep red blood cells is added.

vii) Plates are incubated for 45 minutes at 37°C, and then centrifuged for 5 minutes at 600 ***g***.

When using commercially available CFT-antigens and ready-to-use CFT reagents, the manufacturers' instructions should be applied.

Recommended controls to verify test conditions:

i) Positive control: a control serum that gives a positive reaction;

ii) Negative control serum: a control serum that gives a negative reaction;

iii) Anti-complementary control (serum control): diluent + inactivated test serum + complement + haemolytic system;

iv) Antigen control: diluent + antigen + complement + haemolytic system;

v) Haemolytic system control: diluent + haemolytic system;

vi) Complement control: diluent + complement titration + antigen + haemolytic system.

3.1.3. Reading the results

The absence of anti-complementary activity must be checked for each serum; anti-complementary sera must be excluded from analyses. A sample that produces 100% haemolysis at the 1/5 dilution is negative, 25–75% haemolysis is suspicious, and no haemolysis (100% fixation) is positive. False-positive results can occur, and animals can remain positive for months. Moreover, *B. pseudomallei* and *B. mallei* cross react and cannot be differentiated by serology (Neubauer *et al.,* 1997). Healthy non-glanderous equids can show a false positive CFT reaction for a variable period of time following a mallein intradermal test.

3.2. Enzyme-linked immunosorbent assays

Both plate and membrane based ELISAs have been used for the serodiagnosis of glanders, but none of these procedures has been able to differentiate between *B. mallei* and *B. pseudomallei*. An avidin–biotin dot ELISA has been described, but has not yet been widely used or validated. The antigen used is a concentrated and purified heat-inactivated bacterial culture. A spot of this antigen is placed on a nitrocellulose dipstick. Using antigen-dotted, pre-blocked dipsticks, the test can be completed in approximately 1 hour. An I-ELISA was shown to be of limited value for the serological diagnosis of glanders (Sprague *et al.*, 2009). An I-ELISA based on recombinant *Burkholderia* intracellular motility A protein (rBimA) showed a promising sensitivity of 100% and a specificity of 98.88% (Kumar *et al.*, 2011). Pal *et al*. (2012) used also recombinant proteins to develop an ELISA.

A C-ELISA that makes use of an uncharacterised anti-LPS MAb has also been developed and found to be similar to the CFT in performance (Katz *et al.*, 2000). The C-ELISA was used again on a panel of horse sera originating mainly from Middle Eastern countries (Sprague *et al.*, 2009). A commercially available C-ELISA has recently been developed using anti-s *B. mallei* LPS MAb along with antigen prepared from a regional *B. mallei* isolate. This showed higher sensitivity than CFT in identifying field cases. The C-ELISA has been evaluated on donkey sera and reliable results obtained in an infection trial. Continuing development of monoclonal antibody reagents specific for *B. mallei* antigenic components will offer the possibility to develop more specific ELISAs that will help to resolve questionable test results of quarantined imported horses (Neubauer *et al.*, 1997).

For melioidosis, no serological techniques have been validated for use in veterinary medicine nor are any commercially available.

None of these tests has been fully validated to date.

3.3. Immunoblot assays

Immunoblot assays were developed for the serodiagnosis of glanders, but further validation was impossible because of the lack of a positive serum control panel (Katz *et al.*, 1999). Recently, the development of an immunoblot using *B. mallei* LPS antigen was reinitiated. The aim was to obtain a more sensitive test than the CFT in order to retest false positive CFT sera in non-endemic areas (Elschner *et al.*, 2011). The developed assay is based on crude antigen preparations of the *B. mallei* strains Bogor, Zagreb and Mukteswar, which are also the basis of most CFT antigen formulations. The antigens are separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subsequently transferred to nitrocellulose membranes. Anti-*B. mallei* LPS antibodies in a serum sample reacting to the antigen on the blot strip are visualised by animal species-specific (phosphatase) conjugate and the NBT-BCIP (Nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate) colour system. The immunoblot is scored positive if the banding pattern of the *B. mallei* LPS ladder within the 20–60 kDa region is clearly visible, suspicious if a weak colour reaction is detected and negative if no reaction is seen. 171 sera of glanderous horses and mules from Pakistan and Brazil and 305 sera of negative German horses were investigated and all glanders positive and negative animals were diagnosed correctly, however the test has not been fully validated to date. This test is not able to differentiate glanders from melioidosis infection and it has not yet been evaluated for use in donkeys because of the lack of a significant number of positive control sera.

For melioidosis, no serological techniques have yet been validated for use in veterinary medicine.

3.4. Other serological tests

The rose bengal plate agglutination test (RBT) has been described for the diagnosis of glanders in horses and other susceptible animals; and has been validated in Russia. In a study in Pakistan the RBT showed a sensitivity of 90% and a specificity of 100% (Naureen *et al.*, 2007). The antigen is a heat-inactivated bacterial suspension coloured with Rose Bengal, which is used in a plate agglutination test.

The accuracy of other agglutination and precipitin tests is unsatisfactory for control programmes. Horses with chronic glanders and those in a debilitated condition give negative or inconclusive results.

For melioidosis, no serological techniques have yet been validated for use in veterinary medicine.

4. Tests for cellular immunity

4.1. The mallein test

The mallein purified protein derivative (PPD), which is available commercially, is a solution of water-soluble protein fractions of heat-treated *B. mallei*. See section C below for details of its preparation and availability. The test is not generally recommended because of animal welfare concerns, however it can be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. It depends on infected horses being hypersensitive to mallein. Advanced clinical cases in horses and

acute cases in donkeys and mules may give inconclusive results requiring additional diagnostic methods.

The intradermo-palpebral test is the most sensitive, reliable and specific mallein test for detecting infected perissodactyls or odd-toed ungulates, and has largely displaced other methods. 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test is read at 24 and 48 hours. A positive reaction is characterised by marked oedematous swelling of the eyelid, and there may be a purulent discharge from the inner canthus or conjunctiva. This is usually accompanied by a rise in temperature. With a negative response, there is usually no reaction or only a little swelling of the lower lid.

No data are available for use of this PPD for equids with melioidosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available for glanders or melioidosis.

Mallein PPD is available commercially[1]. The following information outlines the requirements for the production of mallein PPD.

1. Seed management

Three strains of *Burkholderia mallei* are employed in the production of mallein PPD, namely Bogor strain (originating from Indonesia), Mukteswar strain (India) and Zagreb strain (Yugoslavia). The seed material is kept as a stock of freeze-dried cultures. The strains are subcultured on to glycerol agar at 37°C for 1–2 days. For maintaining virulence and antigenicity, the strains may be passaged in guinea-pigs.

2. Production

Dorset-Henley medium, enriched by the addition of trace elements, is used for the production of mallein PPD. The liquid medium is inoculated with a thick saline suspension of *B. mallei*, grown on glycerol agar. The production medium is incubated at 37°C for about 10 weeks. The bacteria are then killed by steaming for 3 hours in a Koch's steriliser. The fluid is then passed through a layer of cotton wool to remove coarse bacterial clumps. The resulting turbid fluid is cleared by membrane filtration, and one part trichloroacetic acid 40 % is immediately added to nine parts culture filtrate. The mixture is allowed to stand overnight during which the light brownish to greyish precipitate settles.

The supernatant is decanted and discarded. The precipitate is centrifuged for 15 minutes at 2500 ***g*** and the layer of precipitate is washed three or more times in a solution of 5% NaCl, pH 3, until the pH is 2.7. The washed precipitate is dissolved by stirring with a minimum of an alkaline solvent. The fluid is dark brown and has a pH of 6.7. This mallein concentrate is centrifuged again and the supernatant diluted with an equal amount of a glucose buffer solution. The protein content of this product is estimated by the Kjeldahl method and freeze-dried after it has been dispensed into ampoules.

3. In-process control

During the period of incubation, the flasks are inspected regularly for any signs of contamination, and suspicious flasks are discarded. A typical growth of the *B. mallei* cultures comprises turbidity, sedimentation, some surface growth with a tendency towards sinking, and the formation of a conspicuous slightly orange-coloured ring along the margin of the surface of the medium.

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4. Batch control

Each batch of mallein PPD is tested for sterility, safety, preservatives, protein content and potency.

Sterility testing is performed according to the European Pharmacopoeia guidelines.

The examination for safety is conducted on five to ten normal healthy horses by applying the intradermo-palpebral test. The resulting swelling should be, at most, barely detectable and transient, without any signs of conjunctival discharge.

Preparations containing phenol as a preservative should not contain more than 0.5% (w/v) phenol. The protein content should be no less than 0.95 mg/ml and not more than 1.05 mg/ml.

Potency testing is performed in guinea-pigs and horses. The animals are sensitised by subcutaneous inoculation with a concentrated suspension of heat-killed *B. mallei* in paraffin oil adjuvant. Cattle can also be used instead of horses. The production batch is bio-assayed against a standard mallein PPD by intradermal injection in 0.1 ml doses in such a way that complete randomisation is obtained.

In guinea-pigs, the different areas of erythema are measured after 24 hours, and in horses the increase in skin thickness is measured with callipers. The results are statistically evaluated, using standard statistical methods for parallel-line assays.

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NB: There are WOAH Reference Laboratories for glanders (please consult the WOAH web site: http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and diagnostic biologicals for glanders

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.

SECTION 3.7.

LAGOMORPHA

CHAPTER 3.7.1.

MYXOMATOSIS

SUMMARY

Description and importance of the disease: *Myxomatosis is a lethal, generalised viral disease of the European rabbit* (Oryctolagus cuniculus) *caused by the* Myxoma *virus* (*MYXV*)*, a member of the* Poxviridae *family. The original hosts were two lagomorphs:* Sylvilagus brasiliensis *in South America* (*South American strains*) *and* S. bachmani (*Californian strains*) *in California, USA. MYXV is now widely distributed, being endemic in wild rabbit populations, as well as in farmed, laboratory, and pet rabbits and occasionally in other lagomorphs. To date, myxomatosis is still the main infection threat for the rabbit industries.*

Myxomatosis is essentially a disease of European rabbits. Brown hares (Lepus europaeus) *are susceptible to MYXV infection, but rarely develop generalised disease. Large outbreaks of myxomatosis in Spanish hares* (*Lepus granatensis*) *were caused by a variant strain.* Sylvilagus *spp. are quite resistant and may act as healthy carriers. There is no health risk to humans. Two forms of the disease are observed: the nodular* (*classical*) *form, characterised by gross myxomatous skin lesions, and the amyxomatous* (*respiratory*) *form, in which signs are mainly respiratory, skin nodules being few and small. MYXV strains with different degrees of pathogenicity circulate in the field. The nodular form is caused by virulent MYXV strains, is naturally transmitted by biting insects during summertime, and is mainly observed in wild and pet rabbits and in small-scale rabbitries. During MYXV infection the copious viral immunomodulatory proteins progressively induce the collapse of the host immune system. This favours bacterial infections in the respiratory tract that contribute to the death of the animal. Mild and attenuated MYXV strains cause the amyxomatous* (*respiratory*) *form of the disease especially in farmed animals. Wild rabbits act as reservoirs while mosquitoes and fleas can transmit the virus to domestic rabbits, but in the case of close proximity between rabbits* (*i.e. in farmed animals*)*, virus may also be transmitted by direct contact. Introduced semen may also pose a risk. There is no age or sex predilection.*

Detection of the agent: *The diagnosis of myxomatosis, regardless of its clinical form, depends on the isolation and identification of the virus or the demonstration of its antigens or genome. When skin lesions are present, the viral antigen may be demonstrated by several rapid diagnostic methods such as agar gel immunodiffusion* (*AGID*)*, negative-staining electron microscopy* (*nsEM*)*, fluorescent antibody test* (*FAT*)*. Polymerase chain reaction* (*PCR*)*, and histopathology are also valuable assays in this circumstance. Monolayer cell cultures of rabbit kidney inoculated with lesion material will show the characteristic cytopathic effects of poxviruses. The presence of virus can be confirmed by immunoperoxidase monolayer assay, FAT, PCR and nsEM. To diagnose the disease* in vivo*, conjunctival swabs could be examined by PCR.*

Serological tests*: The presence of an overt humoral immune response: i*) *facilitates a retrospective diagnosis of the disease; ii*) *can provide an indication of the prevalence of infection in a rabbit population; and iii*) *contributes to evaluation of vaccination even if there is not a direct correlation between the anti-MYXV titres and the degree of protection of animals from the disease. Identification and titration of specific antibodies is mainly done by enzyme-linked immunosorbent assay. IFA and AGID may be used but are less sensitive.*

Requirements for vaccines: *Modified live virus vaccines prepared from fibroma virus or modified/deleted myxoma virus strains are available for immunisation of rabbits.*

A. INTRODUCTION

1 Description and impact of the disease

Myxomatosis is a major viral disease of wild and domestic European rabbits (*Oryctolagus cuniculus*). The natural hosts are two leporides: *Sylvilagus brasiliensis* in South America and *S. bachmani* in California, USA (Fenner, 1994) in which the viral strains produce only a benign fibroma. Following deliberate introductions into Australia and Europe as a biological control for wild European rabbits, *Myxoma* virus (MYXV) is now widely distributed, is endemic in wild rabbit populations, and can spill over into farmed, laboratory and pet rabbits (Fenner & Fantini, 1999). European brown hares (*Lepus europeus*) may rarely develop generalised disease (Fenner & Ratcliffe, 1965). However, a new cross-species jump of MYXV has been suggested in both Great Britain and Spain, where European brown hares (*Lepus europaeus*) and Iberian hares (*L. granatensis*), respectively, were found dead with lesions consistent with those observed in myxomatosis (Garcia-Bocanegra *et al*., 2019). A new genetic variant of MYXV, referred to as MYXV Toledo or ha-MYXV, was detected in the tissues of *L. granatensis* (Agueda-Pinto *et al.,* 2019; Dalton *et al.,* 2019).

Wild rabbits act as reservoirs, and insects (mainly mosquitoes and fleas but also midges and lice) can transmit the virus to domestic rabbits. Where there is close proximity between rabbits (i.e. in farmed animals), virus may also be transmitted by direct contact. MYXV is shed in ocular and nasal secretions or from skin lesions and it is also potentially present in semen and genital secretions. There is no age or sex predilection.

Two forms of the disease are observed: the nodular (classical) form and the amyxomatous (respiratory) form. Nodular myxomatosis is naturally transmitted by biting insects and mainly observed in wild and pet rabbits and in small-scale rabbitries. It is characterised by florid skin lesions and severe immune dysfunction, accompanied by supervening bacterial infections of the respiratory tract. After infection with a highly virulent strain, the first clinical sign is a lump at the site of infection, which increases in size, becomes protuberant and ulcerates. An acute blepharoconjunctivitis and an oedematous swelling of the genital area gradually develop. The secondary skin lesions appear on about the sixth or the seventh day (Fenner, 1994). Death usually occurs between the 8th and 15th day post-infection. After infection with milder or low virulent strains, the same clinical signs evolve more slowly and are less severe, and in surviving animals, the lesions progressively heal. The mortality rate fluctuates between 20 and 100% according to the grade of virulence of the viral strain. Secondary bacterial infections (in particular *Pasteurella* sp. and *Bordetella* sp.) are typical in rabbits that survive longer than 10–14 days after infection and may be the major cause of death in rabbits infected with subacute strains of MYXV. The clinical signs of amyxomatous myxomatosis are mainly respiratory and ocular (swollen eyelids, blepharoconjunctivitis and rhinitis) with fewer and smaller cutaneous lesions. Perineal oedema is also present. The amyxomatous form is regarded as more significant for farmed rabbits and it is considered an adaptation to contact transmission in the absence of vectors, presumably via respiratory and conjunctival secretions, as direct contact is needed for transmission. The clinical expression by amyxomatous viruses appears to depend on the presence of bacterial pathogens such as *Pasturella multocida* (Marlier *et al.,* 2000). So far, this form is commonly reported in countries with substantial rabbit meat production.

2. Nature and classification of the pathogen

MYXV is a poxvirus (family Poxviridae; subfamily Chordopoxvirinae; genus *Leporipoxvirus*) first isolated from laboratory rabbits in Uruguay in 1898. The MYXV DNA encodes about 170 genes, among which approximately 70 encode immunomodulatory and host interactive factors that are involved in subverting the host immune system and other anti-viral responses. Prototype strains of virus deriving from the Australian and European outbreaks have been designated that characterise the various virulence grades (from grade I to grade V) as determined in laboratory rabbits (Fenner & Ratcliffe, 1965). The MYXV Toledo/ha-MYXV isolate from hares in Spain is a recombinant strain, characterised by an insertion of four novel poxviral genes towards the 3' end of the negative strand of its genome (Agueda-Pinto *et al.,* 2019; Dalton *et al.,* 2019).

3. Zoonotic potential and biosafety and biosecurity requirements

There is no known risk of human infection with MYXV. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.*

4. Differential diagnosis

Clinical signs of classic myxomatosis are fairly clear-cut, although bacterial upper respiratory tract infections and bacterial conjunctivitis/keratoconjunctivitis can cause confusion and misdiagnosis. Rabbit fibroma virus (RFV, formerly Shope fibroma virus) produces a simple fibromatous local lesion that should be distinguished from MYXV.

B. DIAGNOSTIC TECHNIQUES

As the signs of the disease become less distinct with the attenuation of virus strains, the submission of samples for laboratory diagnosis becomes more important. Moreover, the expression of ectodermotropism is reduced for amyxomatous MYXV strains, so that the clinical diagnosis of the amyxomatous form is clearly more difficult than for the classical one. The different techniques available vary in their ability to detect MYXV in typical myxomatous lesions, oedema of the eyelids or genital oedema. Nevertheless, the diagnosis of attenuated typical myxomatosis or of atypical (amyxomatous) forms usually requires viral identification. In addition to classical methods i.e. the isolation of the virus by inoculation of sensitive cell lines such as the RK-13 (rabbit kidney) cell line and identification of the virus by immunological methods, nowadays, the MYXV is easily and quickly identified by demonstration of its nucleic acid. Indeed, molecular techniques can reveal subclinical infection (e.g. by testing conjunctival swabs) and permit the differentiation of vaccine from wild field strains (Cavadini *et al.,* 2010).

Table 1. Test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
nsEM	–	–	–	++	–	–
Histopathogy and immunostaining	–	–	–	++	–	–
Virus isolation (cell culture)	–	+	–	++	–	–
AGID	+	+	+	–	–	–
FAT	+	++	–	+	–	–
IPMA	+	+	–	++	–	–
PCR methods	++	+++	++	+++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
I-ELISA	++	+++	+++	++	+++	+++
C-ELISA	++	+++	+++	++	+++	+++
IFAT	+	+	+	–	++	++
AGID	+	+	+	–	+	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
nsEM = negative staining electron microscopy; AGID = Agar gel immunodiffusion; FAT = fluorescent antibody test;
IPMA = immunoperoxidase monlayer assay; PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay (I = indirect; C = competitive); IFAT = indirect fluorescent antibody test.
A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

In the case of the classical form of the disease, MYXV identification may be attempted from skin lesions (myxoma), eyelids, genital mucosa and internal organs (lungs, liver, spleen, kidney etc.). Myxomas are excised with scissors and separated from the epidermis and superficial dermis. Tissue samples (nodular and skin tissues, portion of organs and mucosal scrapings) are washed with phosphate-buffered saline (PBS) with antibiotics as defined below and ground or mechanically homogenised at a dilution rate of 1 g tissue/4.5–9.0 ml of PBS or sterile distilled water (dH_2O). Cells are disrupted by two freeze–thaw cycles, or by ultrasonication to liberate virions and viral antigens. This suspension is centrifuged for 5–10 minutes at 1500 ***g***. The supernatant fluid is used for the tests.

In the case of the amyxomatous respiratory form of the disease, nasal and conjunctival swabs and respiratory tissues (e.g. lungs) may be collected for viral identification. Swabs are put in a tube containing approximately 0.3 ml of sterile dH_2O and soaked for 10–15 minutes, scraping any remaining specimen off the cotton swab directly into the dH_2O with a wooden applicator stick.

1.1. Electron microscopy

Negative-staining electron microscopy (nsEM) can be applied to a portion of skin lesion (myxomas), eyelid, genital mucosa as well as on conjunctival and nasal swabs and lungs. The technique (drop method) is simple and rapid to perform, giving results in 1 hour. A drop of the tissue suspension is laid on a petri dish or watch glass, and a plastic/carbon-coated 200/400-mesh copper grid (plastic-side down) is placed on drop and let absorbed for 10 minutes. Any excess liquid is then removed with filter paper and the grid is transferred on a drop of stain for approximately 30 seconds. Either a 2% aqueous solution of ammonium molybdate, pH 7.0, or 2% phosphotungstic acid (PTA), pH 7.0, may be used for staining. The excess liquid is removed with filter paper and the grid is ready for the EM examination. In a positive case, particles showing typical poxvirus morphology can be seen, however MYXV cannot be distinguished from other poxviruses (e.g. RFV) using this method.

1.2. Histopathology

Histopathology of cutaneous lesions, fixed in 10% buffered formalin and embedded in paraffin, shows that the large lumps found in the skin are mainly due to an accumulation of mucinous material with destruction of the connective tissue architecture in the dermis rather than to an intense cellular proliferation (Marcato & Rosmini, 1986). The derma and epidermis are invaded by granulocytes and

enlarged, stellate, reticulo-endothelial cells with a large nucleus and abundant cytoplasm, called "myxoma cells". These cells destroy the endothelium of small vessels causing extravasation of red cells; they also replicate in the spleen and lymph nodes causing complete loss of lymphocytes from both B-cell and T-cell zones. After the viraemic phase, the virus spreads throughout the body and causes genital and visceral lesions, mainly congestive with vascular damage. In the lung the lesions are of variable intensity and the characteristic epidermal lesions are also observed in the bronchial epithelium (Joubert, 1973). Microscopic lesions may vary according to the virulence of strains and the type of animals i.e. in wild vs laboratory rabbits (Best *et al.,* 2000).

Fixed tissues can be also immunostained using an avidin–biotin complex (ABC) peroxidase method. The sections are first deparaffinised in xylene and alcohol, counter-stained with haematoxylin for 1 minute and rinsed in tap water. They are then put in a methanol bath containing 3% H_2O_2 and washed in PBS three times for 5 minutes each. To limit background interference caused by nonspecific antibody binding, the samples are incubated with normal rabbit serum for 1 hour at room temperature prior to the addition of biotin. The slides are incubated overnight in a humid chamber at room temperature with biotinylated anti-MYXV serum or monoclonal antibodies (MAbs), are washed as before and incubated again for 30 minutes at 37°C with an ABC peroxidase. The slides are then washed three times. Amino-ethyl-carbazole is used as substrate. Finally, the slides are rinsed in tap water and mounted.

1.3. *In-vitro* cell culture

Isolation of the virus in cell culture can be accomplished using primary cultures of rabbit kidney (RK) cells, or with established cell lines, such as RK-13 and SIRC (Statens Seruminstitut rabbit cornea) but also other mammalian cell lines such as Vero (African green monkey kidney) and BGMK (buffalo green monkey kidney), in minimal essential medium (MEM) containing 2% calf serum, 300 international units (IU)/ml penicillin; 300 μg/ml streptomycin; 100 μg/ml gentamycin; 50 IU/ml nystatin (mycostatin); and 5 μg/ml amphotericin (fungizone). The inoculum consists of the supernatant fluid from homogenised skin/mucosal lesions, internal organs (lungs, liver, spleen, kidney etc.) or oculo-respiratory discharge (including conjunctival swab prepared as described above) in MEM with 2% calf serum and antibiotics. This is removed from the cell layer after 2 hours. The cell layer is washed in a small volume of medium and then replenished with maintenance medium (MEM). The cultures are incubated at 37°C in a 5% CO_2 atmosphere.

A cytopathic effect (CPE) typical of poxviruses (Joubert, 1973) usually develops after 24–48 hours, but with some strains, depending on their virulence, it may take up to 7 days for CPE to be observed. According to the MYXV viral strain, groups of cells with confluent cytoplasm form syncytia that vary in size from 2 to 50 or even 100 nuclei together. The nuclei of some cells change, the chromatin forming basophilic aggregations that vary in size and number and give the culture a leopard-skin appearance. Eosinophilic intracytoplasmic inclusions remain discrete, if present at all. Affected cells round up, contract and become pyknotic. They then lyse and become detached from the glass or plastic support. Later, all cells become affected, and the cell monolayer detaches completely.

RFV at first produces well-defined voluminous masses of rounded cells, which proliferate and pile up (Joubert, 1973). At the edge, cells just becoming infected show discrete nuclear changes and acidophilic cytoplasmic inclusions that are numerous at an early stage. The cell layer is destroyed after several days.

In addition to the observation of CPE, different methods can be used to confirm the viral isolation on cell culture. Including nsEM (see Section B.1.1), FAT (see Section B.1.5.2), IPMA (see Section B.1.5.4) and PCR (see Section B.1.6).

1.4. *In-vivo* culture

1.4.1. Embryonated eggs

MYXV and RFV can be cultured on the chorioallantoic membrane of embryonated chicken eggs. Eleven-day-old eggs are inoculated chorioallantoically and incubated at 35°C for 3 days. In case of virus growth, specific pocks may be observed under the microscope after removing and washing the membrane.

1.4.2. Animal inoculation

Rabbit intradermal inoculation is not recommended as a diagnostic tool. Its use should be limited to when it is strictly necessary to characterise the pathogenicity (virulence grade, classical or amyxomatous forms) or to distinguish RFV from MYXV. Rabbits should be of a domestic breed, weighing approximately 2 kg, unvaccinated and previously tested for the absence of specific antibodies (Joubert, 1973).

The inoculum may be the supernatant fluid from a homogenised lesion (with antibiotics) or the product of a cell culture. Between 0.1 and 0.2 ml is administered intradermally behind the ear or into the dorso-lumbar region, which has previously been depilated. The inoculum may be assayed by injecting serial dilutions in saline buffer at one site for each dilution. A primary lesion will appear at the sites within 2–5 days, followed by conjunctivitis. Using five sites for each dilution allows a 50% infective dose (ID_{50}) to be obtained. If the animal survives, the disease can be confirmed serologically after 15 days.

1.5. Antigen detection – immunolabelling techniques

1.5.1. Agar gel immunodiffusion (AGID)

Agar gel immunodiffusion (AGID) (Sobey *et al.,* 1966) is a qualitative test that can be used to detect both antigen and antibodies. AGID tests are simple and rapid to perform – results can be obtained within 24 hours. Agar plates are prepared with Noble agar (0.6 g), ethylene diamine tetra-acetic acid (EDTA) (2.5 g), sodium chloride (4.5 g), and distilled water (500 ml) containing thiomersal (merthiolate) at 1/100,000 dilution. For antigen detection, standard antiserum and the test sample are placed in opposing wells that are 6 mm in diameter and 5 mm apart. Another technique is to deposit a small portion of the lesion directly into the agar, 5 mm away from a filter paper disk impregnated with the antiserum. A number of lines of precipitation, usually up to three, appear within 48 hours, indicating the presence of myxoma viral antigens. Only one line forms in the presence of heterologous reactions with RFV.

1.5.2. Fluorescent antibody test (FAT)

Tissue cryosections fixed in methanol can be directly immunostained by incubation for 1 hour with fluorescein-conjugated anti-MYXV serum or MAbs. Specific fluorescence can be detected in the cutaneous lesions, in eyelids, lungs, spleen, liver, kidney or genital mucosa. An *in-vivo* direct immunofluorescence (DIF) test on impressions of cornea, eyelid and conjunctival cells has been described (DIF-ET) (Cancellotti *et al.,* 1986). The DIF-ET is performed by a glass slide gently pressed on the surface of the eye. In this manner the cornea, eyelid and conjunctival cells adhere to the glass and can be stained and observed.

1.5.3. Indirect fluorescent antibody test (IFAT)

Indirect fluorescent antibody tests (IFAT) can be applied to cell cultures from 24 hours onwards. IFAT reveal intracytoplasmic multiplication of virus, but cannot distinguish MYXV from RFV. The inoculation of chicken embryo cells (trypsinised at day 11 of egg incubation) does not result in CPE, but it is useful for detecting the viral antigens by IFAT.

1.5.4. Immunoperoxidase monolayer assay (IPMA)

This test can be used to identify MYXV-infected cells. RK-13 cells are seeded on a six-well plate (see Section 2.1.1.ii). When cells are ready, the samples are inoculated one for each well, and the plate is incubated as described at Section B.2.3.1. The cells are:

i) Fixed using a solution of cold 80% acetone for 5–10 minutes and then air dried.

ii) Incubated with PBS with 0.3% H_2O_2 for 10 minutes at room temperature to quench the endogenous peroxide.

iii) Washed in PBS twice for 5 minutes each time.

iv) Incubated for 1 hour at 37°C with pre-titrated MYXV rabbit immune serum or specific MAbs in PBS containing 1% BSA.

v) Washed in PBS three times for 5 minutes each.

vi) Incubated for 1 hour at 37°C with pre-titrated IgG anti-IgG rabbit or mouse horse radish peroxide conjugate (depending on species antibody used in step iv).

vii) Washed in PBS four times for 5 minutes each.

viii) Stained with diaminobenzidine (DAB) solution (0.05% DAB, 50 mm Tris/HCl pH 7.4, 0.01% H_2O_2 (freshly prepared) for 10 minutes at room temperature.

ix) Washed with running tap water for 3 minutes.

x) Infected cells are clearly visible under an inverted microscope as brown stained cells.

1.6. Molecular methods – detection of nucleic acid

Polymerase chain reaction (PCR) (Cavadini *et al.,* 2010; Kwit *et al.,* 2019) or real-time PCR (Albini *et al.,* 2012; Belsham *et al.,* 2010; Duarte *et al*., 2014), can be used to amplify genome fragments of MYXV in diagnostic material including eyelid, ear and nasal myxomas, crusts and/or lung lesions, nasal and conjunctival swabs or semen. All these methods are also able to detect the recombinant "MYXV Toledo/ha-MYXV" strain found in the Iberian Hares (Garcia-Bocanegra *et al.,* 2019). A specific PCR method to detect this agent was set up by Dalton *et al.* (2019).

PCR and PCR-RFLPs (restriction fragment length polymorphism) can also be used to detect vaccine strains (Borghi strain and SG33 strains) (Camus-Bouclainville *et al.,* 2011; Cavadini *et al.,* 2010).

Purification of total DNA is a prerequisite for achieving optimal sensitivity. Various purification methods are commercially available and suitable for the assay. Special precautions should be taken during all steps to minimise the risk of contamination.

The procedures described here in detail are modified from those reported by Cavadini *et al.* (2010). The PCR assay consists of three successive procedures: (1) DNA extraction from the test or control sample followed by (2) PCR amplification and (3) detection of the PCR products by agarose gel electrophoresis.

1.6.1. Extraction of viral DNA

i) Homogenise ~1 g of sample (eyelid, ear and nasal myxomas, crusts and/or lung lesions) in 9 ml of PBS1X using manual or electric homogeniser, spin for 15 minutes at 4°C at low speed ~2000 ***g***.

ii) 100 µl supernatant is added to 100 µl lysis buffer (50 mM Tris/HCl, pH 8, 100 mM Na_2 EDTA, 100 mM NaCl, 0.5% sodium dodecyl sulphate [SDS]) plus 12 µl of proteinase K (stock 20 mg/ml, final concentration 1.2 mg/ml) and incubated for 2 hours at 45°C (modified by Stuart, 2004)

iii) To inactivate the proteinase K, the homogenate is denatured for 10 minutes at 94°C and centrifuged at 12,000 ***g*** for 1 minute.

iv) The resulting supernatant is transferred to a new tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) is added, the mixture is vortexed, centrifuged for 10 minutes at 12,000 ***g*** and the upper phase transferred to a new tube.

v) To precipitate the DNA, 0.1 volume of 3M sodium acetate (pH 5.2) and two volumes of absolute ethanol are added, and the mixture incubated at –20°C overnight or for shorter periods at –80°C (e.g. 20–30 minutes).

vi) Recover the precipitated DNA by centrifugation at 12,000 ***g*** for 5–15 minutes at 4°C.

vii) Remove the ethanol with care and wash the pellet with 1 ml of 70% ethanol (v/v).

viii) The dried DNA may be resuspended in 1 ml of TE buffer (10 mM Tris/Cl, pH 7.5, 1 mM EDTA) and stored at 4°C for further manipulation or at –20°C for long-term storage.

ix) For each session a negative control (100 µl of PBS) and a positive control (100 µl of homogenate from a positive rabbit) should be added.

1.6.2. PCR amplification and detection of *Myxoma* virus on agarose gel

i) Extracted DNA is amplified by PCR using primers (Cavadini *et al.,* 2010) (Table 2):

Table 2. PCR primers

Primer name	Primer sequence	PCR product[(a)]
M071-F	5'-ACC-CGC-CAA-GAA-CCA-CAG-TAG-T-3'	67.229 nt–67.250 nt
M071-R	5'-TAA-CGC-GAG-GAA-TAT-CCT-GTA-CCA-3'	67.700 nt–67.677 nt

[(a)]First and last nucleotides of PCR products obtained with the primers indicated. The nucleotide positions refer to myxomavirus strain "Lausanne" (GenBank Accession No.: AF170726)

ii) The PCR amplification is performed in 25 µl and the procedure and conditions are summarised in Tables 3 and 4.

iii) For each PCR reaction a negative control (water DNase free) and a positive control (5–10 ng of DNA previously extracted and tested from a positive rabbit) should be added.

Table 3. PCR reagents

Reagents	Final concentration	Volumes
DNA		5–10 ng
Buffer 5×	1×	5 µl
Primer-F (20 pmol/µl)	0.4 pmol/µl	0.5 µl
Primer-R (20 pmol/µl)	0.4 pmol/µl	0.5 µl
dNTPs (2.5 mM each)	0.2 mM	2 µl
BSA (1 mg/ml)	0.1 mg/ml	2.5 µl
Taq 5U/µl	0.04 U/µl	0.2 µl
H_2O DNase free		XX[1]
Final volume		25 µl

Table 4. PCR conditions

Steps	Temperature	Time	No. cycles
Denaturation	95°C	5 minutes	1
Denaturation	95°C	30 seconds	40
Annealing	60°C	30 seconds	
Extension	72°C	40 seconds	
Extension	72°C	7 minutes	1
	4°C	∞	1

iv) At the end of amplification 10 µl of the PCR reaction are analysed on 2% agarose gel and a positive result is indicated by the presence of a 471 bp band corresponding to a portion of M071L gene target in the positive control. If band appears in the negative control product, cross-contamination occurred during the PCR set-up and the test must be repeated.

1.6.3. PCR amplification and detection of Borghi and SG33 vaccine strains

i) To identify vaccine strains by PCR the following couple of primers are used (Cavadini *et al.,* 2010) (Table 5):

1 The volume of H_2O depends of the volume of DNA used in the reaction.

Table 5. PCR primers to identify vaccine strains

Primer names and position	Primer sequences	Amplicon (bp)	Specific for
VAX-F (138,997 nt–139,021 nt)	5'-ACA-AGA-ATA-TAC-TAA-AGA-ATA-CCA-CG-3'	405	Borghi vaccine strain
Borghi-R (139,398 nt–139,420 nt)	5'-TAG-CGC-GCA-TGG-CGA-CCC-TTG-GT-3'		
VAX-F (138,997 nt–139,021 nt)	5'-ACA-AGA-ATA-TAC-TAA-AGA-ATA-CCA-CG-3'	409	SG33 vaccine strain
SG33-R (139,398 nt–139,419 nt)	5'-GAC-GTG-CAT-GGC-GAC-CCT-TTT-TGC-GTG-T-3'		

ii) The PCR amplification and analysis are set up as previously described with the difference of the annealing temperature of 56°C.

1.6.4. Real-time PCR amplification method

The real-time PCR amplification method used at the WOAH Reference Laboratory is the following:

i) Extracted DNA is amplified by real-time PCR using the primers and probe indicated in table 6.

Table 6. Real-time PCR primers and probe

Primer names and position	Primer sequence
M071RT-F (67,528 nt – 67,555 nt)	**5'-TGTACATTTCATAACTCAACGTGGTAGA-3'**
M071RT-R (67,611 nt – 67,591 nt)	**5'-TTAAGATATCGGCCGCTACGA-3'**
Probe (67,558 nt – 67,587 nt)	**5'-FAM-TTCCTTTTGTTCTGATGATCTCCTCCACGA-BHQ1-3'**

ii) The real-time PCR amplification is performed in 20 µl and the procedure and conditions are summarised in Tables 7 and 8.

iii) For each run a negative control (water DNase free) and a positive control (5–10 ng of DNA previously extracted and tested from a positive rabbit) should be added.

Table 7. Real-time PCR reagents

Reagents	Final concentration	Volumes
DNA		5–10ng
2× MasterMix	1×	10 µl
MV_Probe (5 µM)	100 nM	0.4 µl
M071RT-F (20 µM)	500 nM	0.5 µl
M071RT-R (20 µM)	500 nM	0.5 µl
MilliQ water		6.6 µl
Final Volume	20 µl	18 µl

Table 8. Real-time PCR conditions

Steps	Temperature	Time	N° cycles
Initial denaturation	95°C	30 seconds	1
Denaturation	95°C	5 seconds	40
Annealing and extension	60°C	30 seconds	
	4°C	∞	1

iv) Expression of results: the sample is considered positive if the Ct≤36, negative if the Ct≥40. When Ct is between 36 and 40, the result is not conclusive, and the test should be repeated and/or a new sample taken.

2. Serological tests

Infection of rabbits with MYXV strains induces a strong adaptive immunological response with the production of specific antibodies of the IgM and IgG classes (Kerr, 1997). This occurs also in the cases of vaccination with live vaccine or infection with low pathogenic MYXV strains, although antibodies titres in these cases are lower than those induced by high virulent strains. IgM appears 5–6 days post-infection and usually persists for 30–40 days whereas IgG peaks at 20–30 days remaining positive in naturally infected rabbits for at least 2 years. In relation to the value of titre of the dose, IgG maternal antibodies to MYXV can be found in young rabbits up to approximately 2 months of age. As a consequence, MYXV serology is very useful for most of the purposes listed in Table 1. However, it must be considered that protection of rabbits from myxomatosis is dependent more on the cell-mediated immune response than the serum antibodies. As consequence anti-MYXV antibodies titres are not a direct indication of the level of disease protection. Finally, considering the very limited degree of genetic variation of the immuno-dominant MYXV proteins (i.e. immunodominant envelope protein – IMV – M071L) serology cannot be used for typing different field isolates of MYXV.

Numerous methods have been used to detect serum anti-MYXV antibodies, from traditional agar gel immunodiffusion to the more recent enzyme-linked immunosorbent assays (ELISAs). To date, ELISAs are preferred, for their simplicity, speed, low cost and high sensitivity and specificity. The complement fixation test (CFT) is no longer recommended because of its poor sensitivity (Gelfi *et al.,* 1999).

2.1. Indirect enzyme-linked immonosorbent assay (I-ELISA)

Two very similar indirect ELISAs (I-ELISA), with the antigen directly coated to the solid phase, were developed and used for MVXY serology. The results of the I-ELISA-1 described by Kerr (1997) have been compared with those obtained in neutralisation assay, whereas those obtained in the I-ELISA-2 developed by Gelfi *et al*. (1999) have been compared with indirect fluorescent antibody test (IFT) and CFT. Both ELISAs showed similar performances, consistently better than those of other serological methods.

2.1.1. I-ELISA-1 (Kerr, 1997)

i) Antigen preparation

a) The Lausanna (LU) strain, isolated in Brazil in 1949, is considered *de facto* as the international reference MYXV strain (ATCC code VR-115). Alternatively, considering the high degree of antigenic stability of the MYXV, a regional high virulent isolate can be used as MYXV laboratory reference. This virus should be adapted to grow *in vitro* to obtain stocks with high titres in 48–72 hours of incubation.

b) Virus stocks are cultured in RK-13 or SIRC cells grown in MEM supplemented with 10% calf serum, 200 units/ml penicillin and 100 μg/ml streptomycin. The foci-forming assay may be employed for the viral titration as described below. Virus stocks are aliquoted and stored frozen at –80°C.

c) Grow virions in RK-13 or SIRC cells in 180 cm^2 tissue-culture flasks using a multiplicity of infection (m.o.i.) of 0.02–0.05 (approximately 1 infectious virion each 20–50 cultured cells). Infect 6–12 flasks for each preparation leaving time to grow the virus until uniform CPE is observed.

d) Wash the cell monolayers twice with PBS, pH 7.2, scrape the cells from the flask and pellet by centrifugation (800 ***g*** for 10 minutes at 4°C).

e) Resuspend pellets in 5 ml cold PBS and sonicate to release the intracellular virus. Digest the suspension with DNase1 (25 μg/ml) and RNaseA (50 μg/ml) at 37°C for 30 minutes with frequent agitation.

f) Pellet the virions by centrifugation (250,000 ***g***, 20 minutes at 4°C) throughout a step gradient formed by overlaying 10% of dextran T10 with the same volume of 36% of sucrose, both solutions in 10 mM Tris/HCl, pH 8.0, and 1 mM EDTA.

g) Resuspend the pellet in cold PBS in the original volume and repeat the above pelleting step. Then, resuspend the pellet in 0.5–1.0 ml cold PBS, aliquot and store frozen at –20°C as antigen stock.

h) To establish the dilution at which to use the antigen in the ELISA, titrate the stock antigen against a positive reference serum to produce an optical density (OD) of 1.0 at a serum dilution of 1/100. Perform this step using the ELISA protocol given below.

ii) Quantification of MYXV by foci-forming assay (Smallwood *et al.,* 2010)

a) Seed a six-well tissue culture plate with 1/5 of a nearly confluent 75 cm^2 tissue culture flask (approximately 2–4 × 10^5 cells) of CV-1 cells (African green monkey kidney fibroblast) or alternatively RK-13 cells.

b) Mix the cells with complete MEM to a final volume of 12 ml for seeding each of the six wells. Pipette 2 ml into each well of the plate.

c) Incubate at 37°C in a CO_2 atmosphere. When cells appear to be 80–90% confluent (usually within 20–28 hours), proceed with the infection.

d) Before infection, check the viral preparation for the presence of clumps. If clumps are present or suspected, sonicate the solution with one single cycle of 10–15 seconds. During the sonication, keep the tube in ice.

e) Make serial ten-fold dilutions of virus in complete MEM starting from 10^{-2} to 10^{-8}. Eliminate the media from the six-well plate and add 0.5 ml of each dilution to a single well.

f) Incubate the plate at 37°C a CO_2 atmosphere to obtain the adsorption of the virus to the cells. If a platform rocker is not available inside the incubator, every 10 minutes manually and gently rock the plate to redistribute the liquid.

g) After 1 hour add 1.5 ml of complete MEM to each well. Incubate 2–5 days at 37°C a CO_2 atmosphere.

h) Aspirate media from wells. Add 0.4–0.5 ml of crystal violet solution (0.1% [w/v] crystal violet dissolved in 20% ethanol) to each well, applying gently along the side of the wells to avoid removing cells.

i) Rock gently and incubate for up 1 hour at room temperature. Aspirate the staining and invert plates to dry the wells.

j) Under an inverted microscope, count foci in wells that have <100 (this should be possible for at least two consecutive wells).

k) Calculate the titre (FFU [foci-forming units]/ml): titre = number foci × dilution × 2.

iii) Production of standard laboratory reference sera

Use adult rabbits (2–3 months old) not vaccinated against myxomatosis and obtained from a farm or rabbitry free from myxomatosis.

a) Standard positive-control sera: vaccinate for myxomatosis 3–5 rabbits. After 30 days inoculate rabbits with a virulent strain of myxomatosis (preferably use the same strain used for antigen preparation). If the virus titre is known, inoculate 100–200 plaque-forming units (PFU) in 0.1 ml using the intradermal route. On day 30 after challenge, kill the rabbits, collect the blood and produce the sera following the standard protocol.

b) Standard negative-control sera: collect the blood from 3–5 rabbits, and produce the sera following the standard protocol.

Store aliquoted sera at –20°C.

iv) ELISA protocol

Use high binding ELISA plates.

a) Coat the plate (50 µl in PBS, pH 7.2) with the antigen at the pre-determined dilution (see above) and incubate for 2 hours at 37°C.

b) Wash the plate with 5% (w/v) skim milk powder in PBS (milk/PBS).

c) Block the plate with milk/PBS overnight at 4°C.

d) Distribute the sera on the plate

- Identification of negative and positive sera: dilute 1/100 the standard and sample sera in milk PBS and add 50 µl per well. Leave two wells as blank (i.e. pipette 50 µl of milk/PBS only).
- Determination of the serum titre: all the sera are two-fold diluted starting from 1/100 dilution directly on the ELISA plate using a multichannel pipette. Leave two wells as blank (i.e. pipette 50 µl of milk/PBS only).

e) Incubate the plate for 2 hours at 37°C.

f) Wash three times with PBS and 0.05% Tween 20 (PBS/Tween). Add an anti-rabbit Ig horseradish-peroxidase conjugate at the dilution suggests by the supplier in PBS/milk. Incubate for 30 minutes at 37°C.

g) Wash the plate six times using PBS/tween. Add 100 µl of substrate (ABTS: 2,2-azino-bis 3-ethylbenzthiazoline sulphonate) at 1 mg/ml plus 0,06 % hydrogen peroxide, in 0.1 M citrate phosphate buffer at pH 4.0).

h) Incubate at room temperature for 20 minutes and read the absorbance at 405 nm using an ELISA microplate reader spectrophotometer.

v) Interpretation of the results

Before analysing the ELISA results, subtract the mean OD value of the two blank wells from the OD value of all the sera included in the plate. After this, the OD value of the negative control serum should be lower than 0.1 OD.

a) A serum is classified as negative when the corresponding OD value is equal to or lower than the OD value of the negative control sera plus 0.1 OD.

b) A serum is classified as positive when the corresponding OD value is higher than the OD value of the negative serum plus 0.25 OD.

c) A serum with OD value above the negative cut off but below the positive cut off is considered equivocal or doubtful.

In case of end point titration of positive sera, the titre corresponds to the last dilution with an OD value still positive, that is an OD equal to the OD of the negative control sera at the dilution 1/100 (always subtracted by the background value) plus 0.1 OD.

2.1.2. I-ELISA-2 (Gelfi *et al.,* 1999)

i) Antigen preparation

This step in very similar to that of I-ELISA-1 but with the following minor differences.

a) RK-13 are grown in MEM supplemented with 2% calf serum

b) Infected cells are scraped, pelleted by low-speed centrifugation and washed once in 20 mM Tris, 1 mM EDTA acid, 150 mM NaCl, pH 8.6 (TL20).

c) Cells are suspended in TL20, put on ice for 90 minutes (or overnight) and homogenised in a Dounce homogeniser.

d) After clarification of the homogenate at 1200 ***g*** for 10 minutes at 4°C, 7–9 ml of supernatant is layered on 2 ml of a 36% sucrose cushion in TL20 and ultracentrifuged at 200,000 ***g*** for 2 hours.

e) The pellet is resuspended in TL20 buffer at approximately 0.5 ml for each tube. Determinate protein concentration using a colorimetric method (i.e. Bradford or BCA method).

ii) Production of standard laboratory reference sera

This step is identical to that described for I-ELISA-1

iii) I-ELISA method

Use high-binding ELISA plate.

a) Coat the plate using 1 µg/ml of antigen in PBS pH 7.6 and incubate overnight at 37°C.

b) Wash the plate three times with PBS and block the plate by incubation in 15 mg/ml gelatin in PBS for 1 hour at 37°C.

c) Wash the plate three times in 0.1% PBS/Tween 20. Sera are serially two-fold diluted in PBS/Tween starting from the dilution 1/50. Include among the sera the negative and positive controls. Leave two wells with only PBS in place of the sera. Incubate the plate for 1 hour at 37°C.

d) Wash the plate three times in PBS/Tween. Add a goat anti-rabbit IgG conjugated to alkaline phosphatase diluted in PBS/Tween at the dilution indicated by the supplier. Incubate the plate for 1 hour at 37°C.

e) Wash the plate four times with PBS-Tween. Add disodium p-nitrophenyl phosphate at a concentration of 1 mg/ml in 10% diethanolamine. Keep the plate at room in the dark for 12 minutes and then stop the reaction adding 2 N NaOH. Read the absorbance values at 405 nm using an ELISA microplate reader spectrophotometer.

iv) Interpretation of the results

Use the OD value of the negative control sera as a reference in the interpretation of the results. This value should be equivalent to the average OD value of the wells containing PBS only. The results are expressed as negative or positive at a specific serum dilution. The serum sample titre is expressed as the inverse of the highest dilution for which the OD value is greater than three times the absorbance of the control negative serum. Serum samples are considered positive starting from 1/100.

2.1.3. Comments and suggestions on the use of I-ELISAs

Although considerable detail of the methods has been given above, each laboratory should standardise and validate them in relation to local conditions, as well as taking into account the disease epidemiology in the area. In addition, the anti-Ig rabbit enzyme conjugate is one of the most critical reagents in terms of specificity and sensitivity of the reaction. Considering that enzyme-Ig conjugate supplied by different companies or from different batches can show marked differences in technical performance, a new partial standardisation is necessary in each case. It may also be possible to improve antigen production (i.e. higher titres and degree of purification) using well described methods (Smallwood *et al*., 2010).

Although not specifically included in the above methods, laboratory procedures could also include the following steps:

i) After the coating step, to store the plates at –20°C in plastic bag for at least 3 weeks.

ii) To limit or better avoid antigen freezing and thawing, to add 50% glycerol to the antigen preparation that is then stored at –20°C at a liquid state.

2.2. Competitive ELISA (MYXV-C-ELISA)

At the WOAH Reference Laboratory for Myxomatosis, serological tests are routinely performed by using a competitive ELISA (C-ELISA), centred on the use of a MAb (1E5) that specifically recognises the MYXV immunodominant envelope protein (IMV – open reading frame M071L). As this protein is also expressed in the MYXV Toledo/ha-MYXV hare strain and as the method does not depend on secondary specific antispecies antibodies, it could also be used to detect antibodies in hares.

The main characteristics of this C-ELISA are:

i) the detection of all the anti MYXV immunoglobulin classes present in the serum;

ii) higher specificity than I-ELISA;

iii) the specificity is further increased by the use of MAb 1E5 anti-IMV protein.

The WOAH Reference Laboratory[2] can supply the main reagents for the MYXV C-ELISA in a kit format that includes detailed instruction of the methods including the result interpretation.

2.3. Preparation of standard reagents for AGID and IFAT

2.3.1. Preparation of antigen

Antigen is produced from cell cultures using the RK-13 cell line as described above (Section B.2.1.1.i). The monolayer is harvested about 48 hours after infection, when the cells clearly show CPE (80%), and is centrifuged (1000 ***g***). The supernatant fluid is retained. Infected cells are frozen and thawed three times to release additional virus and the viral suspension is clarified at 1000 ***g***. The newly harvested supernatant is added to the original supernatant. The final supernatant fluid is the antigen and is stored at –20°C or –70°C (for longer conservation). It is titrated in cell cultures before use.

2.3.2. Production of standard laboratory reference sera

This step is identical to that described for I-ELISAs (Section B.2.1)

2.4. Indirect fluorescent antibody test (IFAT)

IFAT (Gilbert *et al*., 1989) is carried out using RK-13 cell cultures in flat-bottomed wells of microtitre plates.

2.4.1. Test procedure

i) Cell suspensions, 4×10^4 cells diluted in medium, are distributed in all wells and a confluent cell sheet is formed within 24 hours.

ii) The medium is discarded and 100 µl of viral suspension (with a multiplicity of infection of 0.05) is added to each well.

iii) After 2 hours, 100 µl MEM containing 2% calf serum is added.

iv) After 48 hours of incubation, the plates are washed with PBS and fixed with acetone containing 50% ethanol for 30 minutes at –20°C, or paraformaldehyde (4% in PBS) at room temperature.

v) The plates are then dried at 37°C for 15 minutes. The plates can be stored at –30°C or –70°C for 3 months.

vi) Sera are tested by IFA using anti-rabbit IgG conjugated to fluorescein isothiocyanate.

vii) The test results may be qualitative with sera diluted 1/20, or quantitative with serial dilutions of serum.

2 List available at: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

2.5. Agar gel immunodiffusion test

Agar is prepared as described previously (Section B.1.5.1) using 6 ml per 10 cm Petri dish.

2.5.1. Test procedure

i) Strips of filter paper containing the standard antigen and antiserum, and discs containing test sera are arranged on the surface of the agar (discs between the strips).

ii) The plates are incubated in a humid atmosphere at 37°C and read after 24–48 hours.

iii) Three precipitation lines should appear. If the test sera contain MYXV-specific antibody, at least one of the three lines is bent towards the antigen band; otherwise it remains straight. If sera contain MYXV antigen, at least one of the lines is bent towards the standard serum strip. The test can also be carried out in a more conventional manner using liquid reagents in wells cut in the agar.

C. REQUIREMENTS FOR VACCINES

1. Background

Two types of live vaccine have been developed for vaccination against myxomatosis: heterologous vaccines prepared from RFV (Fenner & Woodroofe, 1954; Shope, 1932), and homologous vaccine prepared from attenuated strains of MYXV (Arguello Villares, 1986; Gorski & Mizak, 1985; Saurat *et al*., 1978; Tozzini & Mani, 1975; Von Der Ahe *et al*., 1981). They are both administered subcutaneously or intradermally. Each type of vaccine has its advantages and disadvantages. RFV-based vaccines may be considered less immunogenic. Their use has greatly diminished in recent years and they are not generally used in the meat rabbit industry. Live attenuated MYXV vaccines are more immunogenic and the duration of protection is longer, i.e. around 4–6 months. However, they may be immunosuppressive, particularly in young rabbits (Fenner & Woodroofe, 1954; McKercher & Saito, 1964).

A recombinant attenuated live MYXV strain expressing rabbit haemorrhagic disease virus (either RHDV or RHDV2) capsid protein and conferring double protection against myxomatosis and RHDV (Bertagnoli *et al*., 1996) has been developed and it is commercially available in Europe (Spibey *et al*., 2012). An attenuated field strain of MYXV from Spain (strain 6918) has been similarly engineered and tested in laboratory and field studies as a vaccine against both myxomatosis and RHDV for wild rabbits (Angulo & Barcena, 2007). A novel trivalent recombinant attenuated myxoma virus vectored RHDV1 and RHDV2 vaccine has been approved for use in the EU (Reemers *et al.,* 2020).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

A master seed virus (MSV) must be established and used according to a seed-lot system. A record must be kept of its origin, passage history and characteristics.

The viruses employed for vaccine are RFV or MYXV attenuated strains.

i) Rabbit fibroma virus heterologous vaccine

The strains of RFV are usually the original Shope's OA strain (1932), Boerlage's strain, IA variant or various closely related strains. Specific antigenic characteristics of the RFV strains are verified by AGID using monospecific sera against fibroma virus and MYXV.

RFV strains are maintained by passage in specific pathogen free (SPF) rabbits or in unvaccinated rabbits from a stock known to be free (serologically negative) from

myxomatosis. Skin on the backs of healthy adult rabbits is shaved, and multiple sites are inoculated with a 1% suspension of virulent material. Fibromas are fully developed within 8–10 days, at which time the rabbits are humanely killed and the tumours are removed aseptically and homogenised with distilled water. The suspension is stored at –30°C or –70°C in 50% buffered glycerol, or as a 5% dilution in a protein solution (bovine albumin). However, to avoid the use of animals, it is recommended to produce RFV *in vitro* by inoculation of rabbit dermal cell line.

ii) Live attenuated homologous myxomatosis vaccine

The strains of MYXV are field strains attenuated by serial passaging in embryonated chicken eggs, rabbit kidney cells at decreasing temperatures, or chicken embryo cells. The strains usually result from having been cloned several times. Various attenuated strains have been obtained for commercial vaccine preparation (MSD, SG33, Borghi, BT 84, MAV, Leon 162, Poxlap, Pisa, etc.).

The identity of MYXV is confirmed by neutralisation test, FAT or IPMA in RK-13 cells or other suitable cell lines, using a monospecific antiserum (produced by vaccination of rabbits with the specific vaccine viral strain). Identity of MYXV can also be achieved by using molecular methods (i.e. PCR using specific primers). In this way it is also possible to better characterise the genomic properties of the attenuated viral strain.

MYXV may be grown on chicken embryo cell culture obtained from flocks free from specified pathogens. MYXV can also be cultivated on suitable cell lines (rabbit dermal cell line) and on RK-13 cells.

iii) Recombinant attenuated live MYXV strain expressing RHDV

The vaccine is constructed from laboratory-attenuated strains of myxoma virus and the capsid protein gene of an RHDV isolate (either RHDV1 or RHDV2) (Bertagnoli *et al.,* 1996; Reemers *et al.,* 2020; Spibey *et al.,* 2012). Standard laboratory methods for MYXV live attenuated vaccine are used and the RHDVs capsid gene was inserted into the MGF/M11L locus of the myxoma virus genome. Vaccine material is prepared in rabbit kidney cells (RK-13).

2.1.2. Quality criteria (sterility, purity freedom from extraneous agents)

The master seed must be free from bacterial, fungal, mycoplasmal and viral contamination. Purity is determined by testing for a variety of contaminants, i.e. extraneous viruses, bacteria, mycoplasmas and fungi. Tests for sterility and freedom from contamination of biological materials should be performed according to chapter 1.1.4.

In particular, testing for contaminating viruses is done by inoculating a confluent monolayer of Vero cells. Vaccine, adjusted to the equivalent of 20 doses/ml, is neutralised with an equal volume of monospecific hyperimmune serum for 30 minutes at 37°C. The mixture is filtered through a 0.22 μm membrane filter, and 1 ml volumes are inoculated into five 25 ml bottles of cell cultures. These are kept under observation for 7 days. After harvesting, the cells are suspended in medium and subjected to several freeze–thaw cycles, followed by centrifugation and filtration, and the material is inoculated into fresh cultures and observed for 7 days. There should be no evidence of CPE. For excluding the presence of RHDV, haemagglutination of human O red blood cells, RT-PCR or ELISA can be performed.

2.1.3. Validation as a vaccine strain

The test is carried out for each route of administration indicated. Use at least five rabbits, of the minimum age recommended for vaccination and negative for myxomatosis (serologically negative). Administer by a recommended route a quantity of virus corresponding to not less than 10 times the maximum titre that may be expected in a dose of vaccine. Observe the rabbits for 28 days. Record the body temperature the day before vaccination, at vaccination, 4 hours after vaccination and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average temperature increase does not

exceed 1°C and no animal shows a rise greater than 2°C. A local reaction lasting less than 28 days may occur.

The innocuity of the viral strains (both RFV and MYXV), used for vaccine preparation, for pregnant females and for suckling rabbits should be assessed (see Section C.2.3.2). They should be safe also for other species (e.g. guinea-pigs, adult mice and hares).

2.2. Method of manufacture

2.2.1. Procedure

i) Rabbit fibroma virus heterologous vaccine

The original production of RFV was by multiple intradermal inoculations of seed virus into the skin on the back of a number of rabbits. The product of fibroma homogenate can be stored by freezing or used immediately. Alternative production in rabbit dermal cell line such as RK-13 (Jerabek, 1980) is recommended. After clarification by centrifugation, the supernatant fluid is mixed with a stabiliser containing antibiotics and is distributed into ampoules or bottles for lyophilisation. Kaolin may be added as an adjuvant (40 mg/ml) to strengthen intensity and duration of immunisation, in which case the vaccine is administered subcutaneously.

ii) Live attenuated homologous myxomatosis vaccine and recombinant attenuated live MYXV+RHDV

MYXV is propagated in a suitable cell line (e.g. RK-13). Virus is harvested after 2–6 days. The viral suspension may be stored at –70°C. The vaccine is prepared by diluting in specified proportions the viral preparation with a stabiliser for lyophilisation. After homogenisation, the product is distributed into bottles for lyophilisation, the bottles being sealed under vacuum or in sterile nitrogen.

2.2.2. In-process control

The batch potency is determined by measurement of virus content. Serial dilutions of the vaccine RFV or MYXV) are inoculated into suitable cell cultures. One dose of vaccine shall contain not less than the minimum titre previously established.

i) Rabbit fibroma virus heterologous vaccine

The RFV titre was originally measured *in vivo* by calculating the ID_{50} after intradermal inoculation of serial dilutions of the clarified supernatant fluid into several sites (e.g. five) on up to six rabbits. A dilution of a standard preparation of RFV was also inoculated into each rabbit to confirm the animal's correct response to inoculation. However, it is recommended to perform the titration *in vitro* in RK-13 cells ($TCID_{50}$).

ii) Live attenuated homologous myxomatosis vaccine and recombinant attenuated live MYXV+RHDV

Titration of MYXV can be done in RK-13 cells ($TCID_{50}$).

In each case the titre should correlate with the required potency as defined by the test for batch potency, see Section C.2.3.3.

2.2.3. Final product batch tests

i) Sterility

See Section C.2.1.2.

ii) Identity

The identity of RFV and MYXV are determined in RK-13 cells by one of the methods of identification of the agent (e.g. FAT or IPMA using monospecific serum or MAbs, or PCR).

iii) Safety

The use of target animal batch release safety tests or laboratory animal batch release safety tests should be avoided wherever possible and should always adhere to the '3 Rs' principles.

a) Rabbit fibroma virus heterologous vaccine

The pathogenicity of the RFV strains is tested by inoculating rabbits with serial dilutions of supernatant fluids obtained by centrifugation of tumour preparations. Macroscopic and histopathological features and the course of development of fibromas are tested in SPF rabbits periodically. Note that numerous serial passages in rabbits may induce mutation to the inflammatory IA strain, which produces severe lesions that are more inflammatory than neoplastic.

b) Live attenuated homologous myxomatosis vaccine and recombinant attenuated live MYXV+RHDV

The residual pathogenicity of the MYXV strains is tested by intradermal inoculation into SPF rabbits or unvaccinated rabbits free from myxomatosis (serologically negative). These rabbits should not develop more than a local reaction with possibly small secondary lesions on the head that disappear within a few days.

For all types of vaccines, the rectal temperature and the body weight should be recorded as additional parameters. Behavioural and feeding/drinking changes should be also monitored. Rabbits should be put under observation for 28 days.

iv) Batch potency

See Section C.2.2.2.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For regulatory approval of vaccine, all relevant details concerning manufacture of the vaccine (see Section C.2.2) and quality control testing (see Section C.2.1.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume. In-process controls are part of the manufacturing process.

2.3.2. Safety requirements

Several tests are performed to demonstrate different aspects of safety. The safety of ten times the normal dose must be demonstrated. Also, it is necessary to examine the organ dissemination of vaccine virus within the vaccinated animal, the ability of vaccine virus to spread from the vaccinated animal to in-contact animals and to test whether there is reversion-to-virulence of the vaccine virus.

i) To test the use of ten times the normal dose (overdose)

After rehydration, ten doses of the lyophilised RFV vaccine are injected subcutaneously into each of three susceptible rabbits, which are then observed for 21 days. Local reactions should be slight with no generalisation and effect on general health. MYXV vaccine is tested using ten doses injected intradermally into the ears of three susceptible rabbits, which are then observed for 21 days. The primary myxoma lesion should remain mild and no abnormal local or systemic reaction should occur,

ii) To test the use of the vaccine in pregnant rabbits

Administer the virus to not less than five pregnant rabbits according to the schedule to be recommended on the label. Prolong the observation period until 1 day after parturition. The rabbits remain in good health and there is no abnormal local or systemic reaction. No adverse effects on the pregnancy or the offspring are noted.

iii) To test the potential increase in virulence (reversion-to-virulence)

Administer by a recommended route to each of two rabbits, 5–7 weeks old and that do not have antibodies against myxoma virus, a quantity of virus that will allow recovery of virus for the passages described below. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Humanely kill the rabbits 5–10 days after inoculation and remove from each rabbit organs (lungs and liver), or tissues with sufficient virus to allow passage; homogenise the organs and tissues in a suitable buffer solution, centrifuge the suspension and use the supernatant for further passages. Inoculate the supernatant into suitable cell cultures (e.g. RK13) to verify the presence of virus. Administer by an appropriate route, at a suitable rate, a suitable volume of the supernatant to each of two other rabbits of the same age and the same susceptibility. This operation is then repeated at least five times. If the virus has disappeared, a second series of passages *in vitro* is carried out. Inoculate virus from the highest recovered passage level to rabbits, observe for 28 days and compare any reactions that occur with those seen in the test for safety described above. There should be no indication of an increase in virulence as compared with the non-passaged virus. If virus is not recovered in either of two series of passages, the vaccine virus also complies with the test.

2.3.3. Efficacy requirements

Different trials must be undertaken from representative batches of final product containing the minimum titre or potency. The same protocol is adopted for both RFV and MYXV vaccines. The protective effect is demonstrated as follows:

A minimum of five adult rabbits is inoculated with a dose of vaccine, and three rabbits serve as unvaccinated controls. After not less than 21 days after vaccination, all rabbits are inoculated by a suitable route (e.g. intradermally into the eyelids or in two sites on the flank), with a pathogenic strain of MYXV (example: 0.1 ml inoculum of the Lausanne virus strain containing 10^3 ID_{50} [median infectious dose]). Observe the rabbits for a further 21 days. The test is not valid if less than 66 per cent of the control rabbits display typical signs of myxomatosis. A vaccine containing MYXV or RFV complies with the test if not less than 80 per cent of vaccinated rabbits show no signs of myxomatosis.

The manufacturer shall have established a minimum titre or potency taking into account loss in potency during the shelf life.

2.3.4. Duration of immunity

Several groups of five susceptible rabbits are vaccinated with either RFV or MYXV vaccine. One batch is tested by challenge infection (as in efficiency test, see C.2.3.3), at 1, 2, 3, 6, and 9 months for MYXV. The duration of immunity is deduced from the time during which not less than 80 per cent of vaccinated rabbits show no signs of myxomatosis.

2.3.5. Stability

Stability studies (based on an acceptable potency test) are required to establish the validity of the expiry date that appears on the product package. Some authorities allow the use of accelerated stability tests to determine a provisional expiry date for products, e.g. incubating at 37°C for 1 week for each year of dating. Such estimates must be confirmed by periodic real-time potency tests on at least three different batches/serials through the period of time indicated by the expiry date, and 3–6 months beyond. For attenuated live vaccines, testing should be done at release and at the approximate expiry date until a statistically valid record has been established.

Titrations of vaccine virus are carried out at intervals until 3 months beyond the requested shelf life on at least three batches of vaccine.

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NB: There is a WOAH Reference Laboratory for myxomatosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for myxomatosis

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.7.2.

RABBIT HAEMORRHAGIC DISEASE

SUMMARY

Description and importance of the disease: *Rabbit haemorrhagic disease* (*RHD*) *is a highly contagious and acute fatal hepatitis of Leporids*, *caused by a calicivirus* (*genus* Lagovirus). *Up to 2010, all RHD viruses* (*RHDV*) *isolated belonged to one of the previously identified six genotypes* (*G1–G6*), *among which the G6 is an antigenic subtype* (*RHDVa*). *In 2010, an additional RHDV, phylogenetically and antigenically distinct from RHDV, emerged in Europe and was called RHDV2. Another lagovirus, called the European brown hare syndrome virus* (*EBHSV*), *causes a similar disease in hares* (Lepus europaeus, L. timidus *and* L. corsicanus).

RHD is characterised by high morbidity and a mortality of up to *90%. Infection mainly occurs by the oral route. Transmission follows contact with infected rabbits or via indirect contact with mechanical vectors* (*including insects, birds and humans*) *or contaminated tools and equipment. The incubation period of RHD varies from 1 to 3 days, and death usually occurs 12–36 hours after the onset of fever. The main clinical manifestations of the acute infection are nervous and respiratory signs, dullness and anorexia. In rabbits younger than 4–6 weeks, the RHDV/RHDVa infection course is subclinical, but when the causative agent is RHDV2, clinical signs and mortality are observed even in young animals from 7 to 15 days of age onwards.*

Detection and identification of the agent: *The liver and spleen of rabbits that died of acute RHD contain a very high concentration of virus: consequently, several test methods can guarantee a reliable diagnosis. Considering that no sensitive cell substrates have been established* in-vitro, *the main laboratory tests used are RNA amplification* (*reverse-transcription polymerase chain reaction* [*RT-PCR*]) *and sandwich enzyme-linked immunosorbent assay* (*ELISA*) *based on the use of monoclonal antibodies* (*MAbs*). *Specific primers and MAbs should be selected and used to distinguish among different lagoviruses. As RHDVs haemagglutinate human Group O red blood cells, the haemagglutination* (*HA*) *test can also be used bearing in mind that HA-negative RHDV variants have also been identified. The detection of RHDV particles in liver homogenates by electron microscopy is also possible. The diagnosis of chronic RHD can be complicated by the presence of high anti-RHDV antibody titres in the samples, causing possible false-negative results in ELISA and especially in HA tests.*

Serological tests: *Humoral immunity is the main defence against RHD and even a low level of specific and homologous anti-RHDV antibodies confers protection from the disease. The best RHD serological methods are based on the competitive ELISA using specific MAbs. These methods also allow RHDV and RHDV2 infection or vaccination to be distinguished in previously uninfected rabbits. In addition, ELISA quantification of RHDV-specific isotype immunoglobulins* (*IgM, IgA and IgG*), *helps in distinguishing the first infection from re-infection or vaccination. Classical direct ELISA, which needs purified RHDVs or recombinant virus-like particles* (*VLPs*) *to adsorb to the solid phase, shows a high diagnostic sensitivity. However, the exposition of the internal common epitopes shared by different lagoviruses decreases the test's specificity.*

Requirements for vaccines: *Indirect control of the disease is easily achieved by vaccination. Although RHDV capsid proteins have been expressed as recombinant VLPs and are commercially available, most vaccines used are still prepared from the livers of infected rabbits and are inactivated and adjuvanted. Vaccinated animals produce solid protective immunity against RHDV infection and experimental data indicate that protection lasts for a long period* (*over 1 year*). *As RHDV and RHDV2 have different antigenic profiles, combined vaccination with both serotypes, or the use of a vaccine homologous to the RHDV or RHDV2 strain identified during the epidemics or the outbreak, is highly advisable.*

A. INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious and fatal hepatitis of leporids. RHD is caused by a calicivirus (genus *Lagovirus*, family Caliciviriade), a non-enveloped small round RNA virus with only one major capsid protein (VP60) (Ohlinger *et al.*, 1990). The genus *Lagovirus* also includes the European brown hare syndrome virus (EBHSV), the causative agent of a disease of brown hare (*Lepus europaeus*) termed EBHS. Despite their high genetic relationship (VP60 nucleotide similarity of 70%), RHDV and EBHSV are two distinct viral species (Capucci *et al.,* 1991; Green *et al.,* 2000; Wirblich *et al.*, 1994).

A new classification of the genus *Lagovirus* has been proposed based on the VP60 coding sequences (Le Pendu *et al.,* 2017). In the proposal, not yet approved by the International Committee on the Taxonomy of Viruses (ICTV), all lagoviruses are grouped in a unique virus species (*Lagovirus europaeus)*. This could be divided in two genogroups (GI and GII) that can be subdivided into six genotypes (GI.1, GI.2, GI.3, GI.4, GII.1 and GII.2), and further into variants (four for GI.1 and three for GII.1). According to the new genome classification, the genomes of "classical RHDV", including previous genogroups G1–G5, first reported in China (People's Rep. of) in 1984 (Liu *et al*., 1984), and the subtype RHDVa previously corresponding to genogroup G6, identified in Europe in 1996 (Capucci *et al.*, 1998), are now classified as GI.1 (a–d). The new RHDV-related virus called RHDV2 (or RHDVb), which emerged in France in 2010 (Le Gall-Recule *et al*., 2013), is now classified as GI.2. The EBHSV genome is classified as GII.1. However, as the official ICTV taxonomy for Caliciviridae family is still that described by Green *et al.* (2000), the current *Lagovirus* classification is used in this chapter.

The European rabbit (*Oryctolagus cuniculus*) is the only susceptible host species for RHDV and RHDVa. Other lagomorph species, including cottontails (*Sylvilagus* spp.), black-tailed jackrabbits (*Lepus californicus*) and volcano rabbits (*Romerolagus diazzi*) are not susceptible. The RHDV2 host spectrum is broader as it can also cause disease in the Sardinian cape hare (*L. capensis* var *mediterraneus*), the Italian hare (*L. corsicanus*), the brown hare (*L. europaeus*), the mountain hare (*L. timidus*) and various hare (*Lepus*) and cottontail (*Sylvilagus*) species in North America, even if with a different degree of susceptibility.

EBHSV commonly causes disease in *Lepus europaeus, L. timidus* and *L. corsicanus,* and occasionally in cottontails (*Sylvilagus floridanus*), but apparently not in *L. granatensis*, *L. castroviejoi* and *L. capensis.*

RHDV has never been reported in humans. There has been a single report in alpine musk deer (*Moschus sifanicus*) in China (People's Rep. of). Inoculation of a positive RHDV tissue suspension into 28 different vertebrate species other than rabbits failed to produce disease, and no replication of the virus was detected by reverse-transcription polymerase chain reaction (RT-PCR) (Gould *et al.,* 1997). On the contrary, typical lesions and death were observed by challenging laboratory rabbits with RHDV2 positive extracts from two small rodents, the Mediterranean pine vole (*Microtus duodecimcostatus*) and white-toothed shrews (*Crocidura russula*), that were found positive for RHDV2 by RT-PCR. RHDV2 infection has also been reported in badgers (*Meles meles*) in Portugal.

The emergence of RHDV2 has drastically modified the global epidemiological situation for RHD, mainly because it is a serotype distinct from RHDV and can cause disease in young rabbits and in hares. Nowadays RHDV2 is associated with almost all RHD cases detected worldwide. Since 2010, RHDV2 quickly spread to North Africa and northern Europe and has been reported in West Africa. It also became endemic in Australia where it is replacing RHDV. In addition, several RHD outbreaks caused by RHDV2 have been reported in European rabbits and wild *Lepus* and *Sylvilagus* species in North and Central America.

RHD is characterised by high morbidity but with a variable mortality rate depending on the type of virus and the age of the rabbit. For infection with RHDV/RHDVa, mortality is around 80–90% and 5–10% of rabbits show a subacute or chronic clinical course. Although rabbits of all ages can be infected, the infection is subclinical in animals younger than 6–8 weeks of age. The disease caused by RHDV2 could last slightly longer and the mortality rate is highly variable (50–80%) depending on the strain; the most recently detected strains (from 2014 to 2015) have proven progressively more virulent than those initially identified in 2010–2011 (Capucci *et al.*, 2017). Death may occur even in unweaned rabbits from 7–15 days of age onwards.

The RHD incubation period varies between 1 and 6 days. In acute cases, infected animals develop high fever (>40°C) and die suddenly within 12–36 hours of its onset. The only clinical signs may be terminal squeals followed rapidly by collapse and death. Subclinical chronic RHD is characterised by generalised jaundice, loss of weight and lethargy. Death may occur within 1–2 weeks, but some rabbits survive after seroconversion. A specific and relevant IgM response appears within 3 days, immediately followed by an IgA and IgG response 2–3 days later. Viral RNA is detected using PCR in the blood and faeces of convalescent rabbits up to 15 weeks after the infection, as well as in

rabbits infected with RHDV but already protected by specific antibodies previously acquired (i.e. vaccinated or survivors of infection) (Gall *et al.*, 2007). Whether this is a consequence of a slow viral clearance or indicative of a real and prolonged virus replication (persistence) is yet to be established.

Following RHD serology testing, several non-pathogenic RHDV-related lagoviruses (rabbit calicivirus – RCV) have been isolated and partially characterised in Europe (Capucci *et al.,* 1996; Le Gall-Recule *et al.,* 2015; Marchandeau *et al.,* 2005), and Oceania (Strive *et al.,* 2009). Non-pathogenic lagoviruses induce a serological response that may interfere with and complicate RHD serological diagnosis (Capucci *et al.,* 1991; Cooke *et al.,* 2000; Robinson *et al.,* 2002). Recently, non-pathogenic lagoviruses have been detected in hares in Europe and Australia (Cavadini *et al.,* 2016; Droillard *et al.,* 2018; Mahar *et al.,* 2019).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of rabbit haemorrhagic disease and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[(a)]						
ELISA	+	–	++	+++	+	–
EM	–	–	–	++	–	–
HA	–	–	–	+	–	–
RT-PCR	+	–	++	+++	+	–
Detection of immune response						
C-ELISA	+++	+++	+++	+	+++	+++
IsoELISA	++	+++	++	+	++	++
HI	++	++	++	–	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
ELISA = enzyme-linked immunosorbent assay; EM = electron microscopy; HA = haemagglutination test;
RT-PCR = reverse-transcription polymerase chain reaction; C-ELISA = competitive ELISA;
isoELISA = isotype ELISA; HI = haemagglutination inhibition test.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

The liver of RHD-affected rabbits contains the highest viral titre (from 10^3 LD_{50} [50% lethal dose] to $10^{6.5}$ LD_{50}/ml of 10% homogenate and from 10^7 to 10^{10} copies of the genome for mg of tissue) and is the organ of choice for viral identification for both RHDV and EBHSV. The amount of virus present in other parts of the body is directly proportional to vascularisation; thus, spleen is suitable while serum may serve as alternative diagnostic material.

In the case of a subacute or chronic form of RHD, the antibody response triggers virus-clearance in the liver and spleen of rabbits, so that RHD virus-like particles (VLPs) are detected instead of RHDV, mainly in the spleen but also in the liver (Capucci *et al.,* 1991). This VLP is characterised by the lack of the outer shell on the viral capsid made up by the half C-terminal portion of the VP60 and consequently it is negative in the haemagglutination (HA) test as well as with anti-RHDV monoclonal antibodies (MAbs) directed to outer conformational epitopes (Capucci *et al.,* 1995).

The initial treatment of the diagnostic samples is almost identical irrespective of the diagnostic method to be applied, except for immunostaining techniques. An organ fragment is mechanically homogenised in 5–20% (w/v) phosphate buffered saline solution (PBS), pH 7.2–7.4, and clarified by centrifugation at 5000 ***g*** for 10–15 minutes. At this stage, the supernatant can be directly examined by the HA test or enzyme-linked immunosorbent assay (ELISA). If the sample is to be observed by electron microscopy (EM), it is advisable to perform a second centrifugation at 12,000 ***g*** for 15 minutes, before the final ultracentrifugation. For detection by PCR, viral RNA from the samples may also be directly extracted from tissues. Considering the high viral load of RHDV-positive samples and the high analytical sensitivity of PCRs methods, careful precautions must be adopted in the pre-analytical phase of sample preparation in order to avoid problems of cross contamination between samples.

1.1. Enzyme-linked immunosorbent assay

Virus detection by ELISA relies on a 'sandwich' technique and several variations of this have been described. 10% liver homogenates from RHD-affected rabbits tested positive in dilutions from 1/100 to 1/10000 with these ELISAs. Therefore, in spite of the limited sensitivity of ELISA in comparison with PCR techniques, ELISA is the best method for diagnosing acute RHD. One procedure uses the reagents, solutions, times and temperature that are used in the competitive ELISA (C-ELISA) for serology (see Section B.2.2). The microplate used should be of high adsorption capability. The liver homogenate is a 10% (w/v) suspension in standard PBS; 50 µl/well is the standard volume to use in each step. The ELISA buffer used for all steps is PBS with 1% yeast extract (or bovine serum albumin [BSA]), and 0.1% Tween 20, pH 7.4. All incubation steps are for 50–60 minutes at 37°C with gentle agitation. After all steps three washes of 3–5 minutes must be performed using PBS with 0.05% Tween 20. A positive and negative RHD rabbit liver homogenate must be used as controls. The horseradish peroxidase (HRPO) conjugate could be purified IgG from a specific polyclonal serum or MAbs (see Section B.2.2). Anti-RHDV MAbs have been produced in several laboratories and can be used instead of rabbit polyclonal sera. MAbs recognising specific epitopes expressed only by the RHDVa variant as well as by RHDV2 have also been produced (Le Gall-Recule *et al.,* 2013).

To type the RHDVs and relative variants present in the samples (RHDV, RHDVa or RHDV2) by sandwich ELISA, it is advisable to test each sample in at least four replicates, and then to use HRPO conjugates with different specificity, i.e. MAbs recognising antigenic determinants present on the virus surface and expressed alternatively by the classical strain, by the RHDVa or by RHDV2, and a pool of MAbs recognising internal epitopes that can detect smooth, degraded VLPs as well as EBHSV. Similar antigen-capture ELISAs have been described for the detection of either RHDV (Collins *et al.*, 1996) or RHDV2 (Dalton *et al.,* 2018).

1.1.1. Test procedure (example)

For steps that are not specifically indicated see the procedure of the C-ELISA for serology (Section B.2.2).

i) Coat the plate with anti-RHDV hyperimmune serum, with anti-RHDV2 hyperimmune serum and the negative RHDV serum. The latter serves as control for nonspecific reactions (false-positive samples). For each sample, eight wells must be sensitised with the positive sera and four wells with the negative one.

ii) Dilute the liver extract to 1/5 and 1/30 in ELISA buffer (see above), directly in the wells of the plate (e.g. add 45 µl of the buffer into all the wells of the plate, add 10 µl of the sample to the first two wells and then, after rocking, transfer 9 µl to the second wells). Treat the controls, both positive and negative, in the same way as the samples.

iii) After incubation and washing (see above), incubate with the specific HRPO conjugates.

iv) After a last series of washing, add the chromogenic substrate. Orthophenylene-diamine (OPD) can be used as peroxidase substrate for the final development of the reaction. Use 0.15 M citrate phosphate buffer, pH 5.0, with 0.5 mg/ml OPD and 0.02% H_2O_2. The reaction is stopped after 5 minutes by the addition of 50 µl of 1 M H_2SO_4.

v) Absorbance is read at 492 nm. Positive samples are those showing a difference in absorbance >0.3, between the wells coated with RHDV-positive serum and wells coated with the negative serum. Usually, at the dilution 1/30, positive samples taken from rabbits

with the classical acute form of RHD give an absorbance value >0.8, while the absorbance value of the negative sample, at the dilution 1/5, ranges from 0.1 to 0.25.

For diagnosis of EBHSV, it is possible to use this RHDV-specific sandwich ELISA, but, due to the high antigenic difference existing between these agents, there is a risk of obtaining false-negative results. Therefore, the adoption of an EBHSV-specific sandwich ELISA technique using either a high-titre positive anti-EBHSV hare serum, or cross-reacting RHDV MAbs (Capucci *et al.*, 1991; 1995), or specific EBHSV MAbs, instead of rabbit serum, is highly recommended (Capucci *et al.*, 1991).

1.2. Nucleic acid recognition methods

Owing to the low level of sequence variation among RHDV isolates and the high sensitivity of PCR, reverse transcription (RT)-PCR represents an ideal rapid diagnostic test for RHD as described by several authors (Gould *et al.*, 1997; Guittre *et al.*, 1995; Yang *et al.,* 2008). This method is carried out on organ specimens (optimally liver or spleen), urine, faeces and sera using different oligonucleotide primers derived from the capsid region of the RHDV genome. The WOAH Reference Laboratory for RHD uses a single-step RT-PCR, with the following primers specific for the VP60 gene: forward: 5'-CCT-GTT-ACC-ATC-ACC-ATG-CC-3'; reverse: 5'-CAA-GTT-CCA-RTG-SCT-GTT-GCA-3'; the primers are able to amplify all RHDV variants including RHDV2. For the amplification of RHDV2 only, specific primers should be used i.e. "14U1" (5'-GAA-TGT-GCT-TGA-GTT-YTG-GTA-3') and "RVP60-L1" (5'-CAA-GTC-CCA-GTC-CRA-TRA-A-3'), which amplify a 794 bp sequence located in the C-terminal of the gene encoding VP60 of RHDV2 (Le Gall-Recule *et al.,* 2013) or "Fra109-F" (5'-ACT-ACT-AGC-GTG-GTC-ACC-ACC-3') and "Fra567-R" (5'-TTG-TTA-TAA-ACG-CTC-AGG-ACC-AAC-3'), which amplify a 481 bp sequence located in the first part of the VP60 gene (Velarde *et al.,* 2017). Viral RNA can be directly amplified using a one-step standard RT-PCR or retrotranscribed firstly into cDNA and then amplified by PCR. To visualise the PCR product, the amplified DNA is subjected to electrophoresis on agarose gel. If needed, specificity of the PCR product can be determined by sequencing.

A similar RT-PCR method has been used to identify the non-pathogenic RCV (Capucci *et al.*, 1998) or HaCV (Cavadini *et al.*, 2016; Droillard *et al.,* 2018; Mahar *et al.,* 2019) using universal primers for lagoviruses (Strive *et al.,* 2009). RT-PCR represents an extremely sensitive method for the detection of RHDV and is at least 10^4-fold more sensitive than ELISA (Guittre *et al.*, 1995). It is not strictly necessary for routine diagnosis, but it is more sensitive, convenient and rapid than other tests. Similarly, RT-PCRs for the detection of EBHSV, using different primers pairs, have been applied to the detection and characterisation of EBHSV strains (Le Gall-Recule *et al.*, 2001; Velarde *et al.*, 2017).

An internally controlled multiplex real-time RT-PCR using fluorogenic probes and external standards for absolute RNA quantification has been developed as a further diagnostic tool for the detection of RHDV (Gall *et al.,* 2007). The oligonucleotides used in this method are: [VP60-7_forward: 5'-ACY-TCA-CTG-AAC TYA-TTG-ACG-3', vp60-8_reverse: 5' TCA-GAC-ATA-AGA-AAA-GCC-ATT-GG-3'] and probe [VP60-9_fam 5'-FAM-CCA-ARA-GCA-CRC-TCG-TGT-TCA-ACC-T-TAMRA-3'].

Real-time RT-PCRs specific for the detection of RHDV2 have been also developed. In the one described by Duarte *et al.*, 2015, the oligonucleotides used are: [RHDV2-F: 5'-TGG-AAC-TTG-GCT-TGA-GTG-TTG-A-3', RHDV2-R: 5'-ACA-AGC-GTG-CTT-GTG-GAC-GG-3'] and the probe [RHDV2: 5'-FAM-TGT-CAG-AAC-TTG-TTG-ACA-TCC-GCC-C-TAMRA-3'. Other real-time RT-PCR protocols have been used and are described in literature.

1.3. Electron microscopy (EM)

EM should preferably be performed after ultracentrifugation (at least 100,000 ***g*** for 30 minutes) of the sample (an organ suspension prepared as described in Section B.1) to concentrate the viral particles. The pellet obtained is resuspended in PBS or distilled water, put on to a grid for a few minutes, and then negatively stained with 2% sodium phosphotungstate (NaPT), pH 6.8, for 1.5 minutes. RHD virions are visible as uncoated particles, 32–35 nm in diameter, presenting an inner shell (25–27 nm in diameter), delineated by a rim from which radiate ten short regularly distributed peripheral projections. Smooth (s-RHDV) particles are identified by the complete loss of external portions, becoming perfectly hexagonal and smaller, with only the capsid rim visible (Capucci *et al.*, 1991).

Immuno-EM (IEM) which employs the same serological principles as ELISA in an EM technique has been reported for its usefulness in diagnosis and characterisation of various viruses, including RHDV. IEM, which works well with antisera or MAbs, can be more specific than traditional negative staining EM through the combination of morphological identification and antigen specificity (Lavazza *et al.,* 2015).

EBHSV can also be identified in diagnostic samples by EM examination. In addition, the IEM method using convalescent anti-EBHSV serum or specific anti-EBHS MAbs can be used to identify EBHSV. By using antisera that is specific for EBHSV and RHDV, it is possible to differentiate between the two viruses.

1.4. Haemagglutination test

HA was the first test to be used for routine laboratory diagnosis of RHD (Liu *et al.*, 1984). As RHDV2 showed an HA activity similar to RHDV/RHDVa (Le Gall-Recule *et al.,* 2013) this method could be used also for RHDV2 diagnosis. HA test should be performed with human Group O red blood cells (RBCs), freshly collected, stored overnight in Alsever's solution, and washed in 0.85% PBS at pH 6.5 (range 6–7.2). HA is less evident or non-existent when RBCs of other species are used. Washed RBCs are suspended at 0.75% in PBS. A twofold dilution of the clarified supernatant of a 10% tissue homogenate of liver or spleen is incubated with an equal volume of washed RBCs in a sealed round-bottom microtitre plate at, preferably, 4°C. After 1 hour (range from 20 minutes to 2 hours) of incubation, agglutination at an end-point dilution of >1/160 is considered to be positive. Lower titres should be regarded as suspicious and should be checked using other methods. Around 10% of samples found to be positive by ELISA or EM give negative results in HA (HA false-negative). Some RHD isolates may exhibit temperature-dependent differences in haemagglutinating characteristics and could show HA activity only when the test is performed at 4°C. Nevertheless, the HA false negativity is mainly detected in organs of rabbits showing a subacute/chronic form of the disease and it depends on the characteristics of the VLPs.

Hare organs rarely give a significant titre when the RHDV HA protocol is used. To demonstrate HA activity in organs from EBHSV-infected rabbits, a modified procedure should be adopted: all steps are carried out at 4°C, the organ suspension is treated with an equal volume of chloroform, and RBCs are used at a pH not higher than 6.5 (Capucci *et al.*, 1991). Even using this method, only about 50% of the samples give positive results. This is because the disease of hares is often subacute or chronic and therefore the virus has the antigenic and structural characteristics typical of the VLPs (Capucci *et al.*, 1991).

Due to the practical difficulty of obtaining and keeping human red cells and the risk from working with these cells, and because of the difficulty of obtaining consistent results, this test should be replaced by other virological methods such as antigen-detection ELISA or PCR.

1.5. Immunostaining

Tissue fixed in 10% buffered formalin and embedded in paraffin can be immunostained using standard methods by employing specific MAbs (Neimanis *et al.,* 2018).

Intense nuclear staining and diffuse cytoplasmic staining of necrotic hepatocytes in the liver, mainly in the periportal areas, are characteristic and specific. Positive staining of macrophages and Kupffer's cells is also observed, as well as hepatocellular reactions. Positive reactions can also be detected in the macrophages of the lungs, spleen and lymph nodes, in renal mesangial cells and in the bone marrow, which may exhibit a marked decrease in the proportion of myeloid to erythroid cells and an increase in the proportion of immature myeloid cells (Stoerckle-Berger *et al.*, 1992) (decreased myeloid-to -erythroid ratio) (Neimanis *et al.,* 2018).

Tissue cryosections fixed in methanol or acetone can be directly immunostained by incubation for 1 hour with fluorescein-conjugated rabbit anti-RHDV serum or MAbs. Specific fluorescence can be detected in the liver, spleen, and renal glomeruli.

1.6. Western blotting

When other tests such as HA or ELISA give doubtful results (low positivity) or the samples are suspected of containing s-RHDV particles, western blotting analysis can be used for determining the final diagnosis, while modern genome detection methods (real-time RT-PCR) are particularly useful for confirmation.

Homogenates are prepared as described previously, and virus particles are further concentrated (tenfold) by ultracentrifugation (100,000 ***g*** for 90 minutes) through a 20% (w/w) sucrose cushion.

Both the supernatant and the pellet can be examined to detect, respectively, the RHDV 6S subunits (Capucci *et al.*, 1995) and the denatured RHDV-VP60 structural protein or its proteolytic fragments, which can range in size from 50 to 28 kDa. A positive and negative control samples should be used on each occasion.

RHDV proteins could be detected with polyclonal antibodies or MAbs. If MAbs are used, they should recognise continuous epitopes. RHDV-specific MAbs recognising internal or buried epitopes could be used also to detect EBHSV. Rabbit anti-RHDV hyperimmune sera are less efficient than MAbs at recognising the same band patterns (Capucci *et al.*, 1995).

Western blot analysis can also be used to identify EBHSV. The pattern of protein bands, detected using either an anti-EBHSV polyclonal serum or cross-reacting anti-RHDV MAbs, is similar. However, the percentage of samples showing viral degradation is higher and therefore several fragments of lower molecular weight, originating from the VP60 structural protein, are often observed.

1.7. Rabbit inoculation

As no efficient *in-vitro* replication system has been established for RHDV and EBHSV, cell culture isolation cannot be included among the diagnostic methods. Rabbit inoculation therefore remains the only way of isolating, propagating and titrating the infectivity of the RHDV. However, **this method should be avoided on welfare grounds for routine diagnosis**. When a case can be made for this procedure, the rabbits involved must be fully susceptible to the virus, i.e. they should be over 2 months old and have no RHDV antibodies (see serological methods). RHD can be reproduced by using filtered and antibiotic-treated liver suspensions, inoculated either by the intramuscular, intravenous or oro-nasal route. When the disease is clinically evident, the signs and post-mortem lesions are similar to those described after natural infection. A rise in body temperature is registered between 18 and 24 hours post-infection (p.i.), followed by death in more than 80% of inoculated animals, depending on the type and virulence of the strain. A few individuals may survive until 6–8 days after infection. Animals that survive the disease show only a transient hyperthermia, depression and anorexia, but present a striking seroconversion that can be easily detected 3–4 days post-infection.

2. Serological tests

Infection with RHDV can be indirectly diagnosed in animals that have survived infection through detection of a specific antibody response. As the humoral response has great importance in protecting animals from RHD, determination of the specific antibody titre after vaccination or in convalescent animals is predictive of the ability of rabbits to resist RHDV infection. Considering the antigenic difference existing between RHDV/RHDVa and RHDV2, distinct specific antibody responses following infection or homologous vaccination are induced. As a consequence, serological diagnosis should be based on methods using RHDV- and RHDV2-specific immunological reagents. Therefore, especially when no or limited anamnestic or epidemiological information is available, tests for both RHDV and RHDV2 should be performed, and the results compared.

Three basic techniques are applied for the serological diagnosis of RHDV: haemagglutination inhibition (HI) (Liu *et al.*, 1984), indirect ELISA (I-ELISA) and C-ELISA (Capucci *et al.*, 1991). Each of these methods has advantages and disadvantages. With respect to the availability of reagents and the technical complexity of carrying out the test, HI is the most convenient method, followed by the I-ELISA and C-ELISA, respectively. On the other hand, both ELISAs are quicker and easier than HI, particularly when a large number of samples are tested. The specificity of the C-ELISA is markedly higher than those achieved with the other two methods (Capucci *et al.*, 1991). An alternative C-ELISA method has been described (Collins *et al.*, 1995). For improved serological interpretation and for correctly classifying the immunological status of rabbits, a combination of ELISA techniques that distinguish IgA, IgM and IgG antibody responses is also available (Cooke *et al.,* 2000).

Some other additional tests (Cooke *et al.*, 2000) could be used for particular investigations, and when a higher level of sensitivity is needed or to detect antibodies induced by cross-reacting non-pathogenic RCVs (see Section A. Introduction).

They are:

- *I-ELISA:* the antigen, an RHDV-positive liver homogenate, is linked to the solid phase by an MAb, the epitope of which is located on the outer shell of RHDV. The sera are then serially diluted starting from 1/40, and IgG bound to the antigen is detected using a reagent, preferably an MAb anti-rabbit IgG labelled HRPO. This ELISA has a higher sensitivity than C-ELISA, making possible measurement of highly cross-reactive antibodies and it can detect antibodies with low avidity.

- *Solid-phase ELISA* (*SP-ELISA*): the purified antigen is directly adsorbed to the solid phase and because of virus deformation, internal epitopes are exposed. Therefore, it detects a wider spectrum of RHDV antibodies and has high sensitivity and low specificity. For these reasons it can also be used for EBHSV serology. Together with I-ELISA, this test could be considered lagovirus specific, i.e. able to detect antibodies towards common lagovirus epitopes present in the NH2 half of VP60s.

- *Sandwich ELISA to detect IgM and IgG in liver or spleen samples already examined with the virological test:* such a test is particularly useful in those animals that die from the 'chronic' form of the disease, when detection of the virus may be difficult using HA or ELISA methods. In addition to the use of RT-PCR, a high level of RHDV-specific IgM and a low level, if any, of IgG are the unambiguous markers of positivity for RHD.

2.1. Haemagglutination inhibition

Antigen: The antigen is prepared using infected rabbit liver collected freshly at death. The liver is homogenised in 10% (w/v) PBS, pH 6.4, and clarified by two consecutive low speed centrifugations (500 ***g*** for 20 minutes and 6000 ***g*** for 30 minutes). The supernatant, drawn from the tube so as to avoid the superficial lipid layer, is filtered through a 0.22 μm pore size mesh, titrated by HA, and divided into aliquots, which are stored at –70°C.

Serum samples: Before testing, sera are inactivated by incubation at 56°C for 30 minutes. The sera are then treated with a 25% (w/v) kaolin suspension (serum final dilution: 1/10) at 25°C for 20 minutes and centrifuged. This is followed by a second kaolin treatment, also at 25°C for 20 minutes, this time with 1/10 volume of approximately 50% packed human Group O RBCs. These are freshly collected, stored overnight in Alsever's solution, and washed in 0.85% PBS, pH 6.5. The sera are clarified by centrifugation.

2.1.1. Test procedure

i) Dispense 50 μl of serum into the first well of a round-bottom microtitre plate and make double dilutions into wells 2–8 using PBS with 0.05% BSA.

ii) Add 25 μl of RHDV antigen containing 8 HA units to each well and incubate the plate at 25°C for 30–60 minutes.

iii) Add 25 μl of human Group O RBCs at 2–3% concentration to each well and allow to settle at 25°C for 30–60 minutes.

iv) Titrate the antigen with each test to ensure that 8 HA/25 μl were used and include positive and negative serum controls.

The serum titre is the end-point dilution showing inhibition of HA. The positive threshold of serum titres is correlated to the titre of the negative control sera; it usually is in the range 1/20–1/80.

As with the HA test (section B.1.4.) the difficulty of obtaining and working with human Group O blood cells has led to this test being superseded by the serological or antibody-detection ELISA.

2.2. Competitive enzyme-linked immunosorbent assay

Antigen: Due to the recent emergence of RHDV2, RHD serology should be based on the use of two antigens – classical RHDV and RHDV2.

The antigen can be prepared as described previously for HI (Section B.2.1), taking care to store it at –20°C in the presence of glycerol at 50% (v/v) to prevent freezing. If necessary, the virus can be inactivated before the addition of glycerol, using 1.0% binary ethylenimine (BEI) at 33°C for 24 hours. Antigen must be pretitrated in ELISA and then used as the limiting reagent: i.e. the dilution that corresponds to 60–70% of the plateau height (absorbance value at 492 nm in the range 1.1–1.3).

Anti-RHDV serum: specific polyclonal sera with high anti-RHDV or anti RHDV2 titre can be obtained in different ways. Two possible and currently used methods are as follows:

i) Rabbits older than 10 weeks of age are vaccinated with a vaccine homologous to the polyclonal serum that you want to produce (RHDV or RHDV2). To obtain sera containing a high level of anti-RHDV IgG, they are orally challenged 8 days later with 2 ml of a RHDV or RHDV2 positive 10% liver homogenate diluted 1/20 in PBS. Rabbits must be bled 35–45 days post-challenge to obtain the convalescent sera (titre in C-ELISA of around 1/10240). Alternatively, convalescent rabbits can be re-infected after 3–4 months and bled 10–15 days later to obtain RHDV hyperimmune sera. In the case of RHDV2, to obtain high titre immune sera it is advisable to use strains identified from 2015 onwards (i.e. high virulent isolates).

ii) The antigen (RHDV or RHDV2) is purified from the livers of naturally or experimentally infected rabbits that died from an acute form of the disease (between 28 and 40 hours post-Infection), using one of the methods that has been published (Capucci *et al.*, 1991; 1995; Ohlinger *et al.*, 1990). Then the purified RHDV antigen can be used to immunise sheep, goats or chickens according to classical protocols using oil adjuvants. The same procedure can also be used to inoculate rabbits, but of course the purified virus must be inactivated before inoculation.

Anti-RHDV MAbs may be used instead of rabbit polyclonal sera. Purification of rabbit IgG and conjugation to HRPO can be done following the standard protocols. The conjugated antibody is titrated in a sandwich ELISA in the presence and absence of RHDV antigen (negative rabbit liver). It is then used at the highest dilution showing maximum (plateau high) absorbance (if the serum had a good anti-RHDV titre, the value of the HRPO conjugate should range from 1/1000 to 1/3000).

Control sera: negative serum is taken from rabbits fully susceptible to RHDV infection. Positive serum is either a convalescent serum diluted 1/100 in a negative serum or a serum taken from a vaccinated animal.

2.2.1. Test procedure (example)

NB: This procedure is also valid for RHDV2 using the homologous reagents.

i) The rabbit anti-RHDV serum diluted to a predetermined titre, e.g. 1/5000 in 0.05 M carbonate/ bicarbonate buffer, pH 9.6, should be adsorbed to an ELISA microplate of high adsorption capability (e.g. Nunc Maxisorb Immunoplate) at 4°C overnight.

ii) Wash the plate three times for 3–5 minutes each time, in PBS, pH 7.4, with 0.05% Tween 20 (PBST). When the plates are not immediately used, they can be stored, closed in a plastic bag, for 1 month at –20°C.

iii) Distribute 25 µl/well PBST with 1% yeast extract (PBSTY) or 1% BSA (PBST-BSA) to all the wells needed on the plate (see below). Add 7 µl of the first serum sample to the first two wells (A1 and B1), 7 µl of the second serum to the second two wells (C1 and D1), and continue with the third (E1 and F1) and the fourth (G1 and H1) sera, thus completing the first column. If qualitative data (positive/negative) are needed, repeat the operation in the second column with sera samples from 5 to 8, and in the third column with sera samples from 9 to 12, and so on. If the titre of the serum needs to be determined, the serum must be diluted further. Agitate the plate and then use an eight-channel micropipette to transfer 7 µl from the wells in column 1 to the wells in column 2. This corresponds to a four-fold dilution of the sera. This last operation can be repeated once (titre 1/160), twice (titre 1/640), or four times (titre 1/10,240). Either in the case of testing sera for qualitative data (single dilution), or for getting the final titre (several dilutions), complete each plate leaving 12 wells free for the control sera. Add 7 µl of positive sera to wells G7 and H7, and 7 µl of negative sera to wells G10 and H10, then dilute them once and twice (1/40–1/160).

iv) Add 25 µl/well antigen suspended in PBSTY to all the wells on the plate, at a dilution that is double the calculated dilution, as described above in the antigen section (see the first part of this ELISA method description).

v) Incubate the plate at 37°C on a rocking platform for 50–60 minutes.

vi) Wash the plate as described in step ii.

vii) Add 50 µl/well rabbit IgG anti-RHDV conjugated with HRPO at the decided dilution, as described above in the 'anti-RHDV serum' section (see the first part of this ELISA test description).

viii) Incubate the plate at 37°C on a rocking platform for 50–60 minutes, and wash as described in step ii adding a fourth wash of 3 minutes duration.

ix) Use 50 µl/well OPD as hydrogen donor under the following conditions: 0.5 mg/ml OPD in 0.15 M phosphate/citrate buffer, pH 5, and 0.02% H_2O_2. Stop the reaction after 5 minutes by addition of 50 µl/well 1 M H_2SO_4.

x) Read the plate on a spectrophotometer using a 492 nm filter.

The serum is considered to be negative when the absorbance value of the first dilution (1/10) decreases by less than 15% of the reference value (dilution 1/10 of the negative control serum), while it is positive when the absorbance value decreases by 25% or more. When the absorbance value of the 1/10 dilution decreases by between 15% and 25% of the reference value, the sera is considered to be doubtful.

The serum titre corresponds to the dilution giving an absorbance value equal to 50% (±10) of the average value of the three negative serum dilutions. A wide range of titres will be found, depending on the origin of the sample. Positive sera range from 1/640 to 1/10,240 in convalescent rabbits, from 1/80 to 1/640 in vaccinated rabbits and from 1/10 to 1/160 in 'non-pathogenic' infection. Knowing the origin of the sample allows a choice to be made between testing one or more dilutions. Testing only the first dilution gives a positive or negative result. The titre is established by testing all dilutions, up to the sixth one.

The above criteria used to transform raw ELISA data into final serological results are the same for the RHDV and RHDV2 C-ELISAs. However, for a practical interpretation of the obtained results, some considerations must be borne in mind. The main one is that RHDV and RHDV2, although representing two distinct serotypes, share secondary antigenic determinants. These determinants induce a minor subset of cross-reactive antibodies that, although of limited importance in RHD protection, 'interfere' in ELISA reactions. This means that rabbits vaccinated (or infected) with RHDV will have medium or high titres in the homologous C-ELISA but will be positive to some extent in the heterologous C-ELISA (RHDV2 C-ELISA). The opposite is also true, when rabbits are vaccinated or infected with RHDV2 and tested with RHDV C-ELISA. However, to ascertain which vaccine was used (or which virus infected the rabbits) it may be possible to use the value obtained by the 'ratio' RHDV2 C-ELISA titre divided by the RHDV C-ELISA titre (RT2 value) (Velarde *et al.,* 2017). This value usually ranges from 4 to 64 in rabbits vaccinated or infected with RHDV2 and from 0.25 to 0.0156 in rabbits vaccinated or infected with RHDV. For an RT2 value from 2 to 0.5, it is not possible to assign the origin of the detected antibodies to one or the other viruses. This could occur when both titres are low (<1/80) or when rabbits are vaccinated with a bivalent vaccine (RHDV plus RHDV2), or two associated vaccines (RHDV and RHDV2).

Due to the significant antigenic differences existing between RHDV and EBHSV, the serological techniques described above, which use RHDV as antigen, are not recommended for the serological diagnosis of EBHS. However, a direct ELISA method could be employed for the detection of positive and negative EBHSV hare sera; in fact, the adsorption of RHDV on to the solid phase of an ELISA microplate exposes cross-reactive antigenic determinants. Alternatively, a specific C-ELISA for EBHSV can be arranged in a similar way, using specific reagent (antigen and antisera) prepared as described above for RHDV.

2.3. Isotype enzyme-linked immunosorbent assays (isoELISAs)

The isoELISAs enable the detection and titration of isotypes IgA, IgM and IgG. Isotype titres are critical for the interpretation of field serology in four main areas: cross-reactive antibodies, natural resistance of young rabbits, maternal antibodies, and antibodies in previously infected rabbits (Cooke *et al.*, 2000). In

fact, in the case of passive antibodies, only IgG are detected; in vaccinated animals, no IgA are usually detected and in recently infected rabbits, first IgM and then IgA and IgG are detected (Cooke *et al.*, 2000).

To detect RHDV-specific IgG, one RHDV-specific MAb is adsorbed to the plate at a concentration of 2 μg/ml by the method described above for the polyclonal serum in the C-ELISA (see above Section B.2.2, test procedure step i). Virus is added to the plates at a concentration double that used in the C-ELISA and after incubation and washing sera are added and serially diluted four-fold starting from 1/40. A MAb anti-rabbit IgG HRPO conjugate is used to detect IgG bound to the virus. The final step for the isoELISAs for IgG, IgM and IgA is the addition of OPD and H_2SO_4 as for the C-ELISA. To detect IgM and IgA isotypes the phases of the ELISA reaction are inverted in order to avoid competition with IgG, which is usually the predominant isotype. MAb anti-rabbit IgM or anti-rabbit IgA is adsorbed to the wells and then the sera are diluted as described above. Incubation with the antigen follows and then HRPO-conjugated MAb is used to detect the RHDV bound to the plate. Sera are considered to be positive if the OD_{492} (optical density) value at the 1/40 dilution is more than 0.2 OD units (two standard deviations) above the value of the negative serum used as a control. The titre of each serum is taken as the last dilution giving a positive value. Because isoELISA tests do not follow identical methodology, equivalent titres do not imply that isotypes are present in the same amounts. This method could be applied also for serology with RHDV2, obviously using the RHDV2 specific MAbs.

C. REQUIREMENTS FOR VACCINES

1. Background

In countries where RHD is endemic, indirect control of the disease in farmed animals and pet rabbits is achieved by vaccination. Most of the commercially available vaccines are based on inactivated infectious viruses. Due to the lack of a cell culture system for efficient virus propagation, such vaccines are prepared from clarified liver suspension of experimentally infected rabbits, and that is subsequently inactivated and adjuvanted. The methods of inactivation (formaldehyde, beta-propiolactone or other substances) and the adjuvants used (incomplete mineral oil, aluminium hydroxide or other emulsions), can vary according to the protocol used by the different manufacturers.

The level of cross protection induced by vaccination with RHDV/RHDVa vaccine against RHDV2 is poor and does not prevent infection and losses due to clinical disease. Therefore, combined vaccination with both antigenic types and/or vaccines homologous to the RHDV type identified during the epidemics or the outbreak should be used. Considering the current variability of RHDV2 strains, the use of vaccines based on strains showing a high homology with those circulating in an area/region/country is highly advisable.

Most vaccine manufacturers recommend a single basic vaccination, with yearly booster. Usually, a 1-ml dose is inoculated subcutaneously in the neck region, or intramuscularly. In those units with no history of disease, with negative serology for RHD, it is advisable to vaccinate only the breeding stock. Considering the high restocking rate in industrial rabbit farms, the usual vaccination programme is to administer the vaccine to all breeders, independently of their age, every 6 months. This should ensure that all animals get at least one vaccination per year. Booster vaccination is strongly recommended to ensure a good level of protection, although experimental data indicate that protection usually lasts for a long time (over 1 year).

Given the short life-cycle (approximately 80 days) of fattening rabbits and their natural resistance, up to the age of 6–8 weeks, to the disease caused by RHDV/RHDVa, but not by RHDV2, vaccinating these rabbits is not necessary if good biosecurity measures are applied on farm and there are no outbreaks of the disease in the area. Following an outbreak of RHD, and especially in the case of RHDV2, which could induce disease even in young animals, even if strict hygiene and sanitary measures are adopted, including cleaning and disinfection, safe disposal of carcasses and an interval before restocking, it is strongly recommended to vaccinate meat animals at the age of 30–40 days, because the incidence of re-infection is very high. Only after several (>3) production cycles it is advisable to stop vaccination of meat animals. To verify the persistence of infective RHD inside the unit, a variable number of rabbits, starting with a small sentinel group, should not be vaccinated.

Given that immunity starts after about 7–10 days, vaccination could also be considered a quite effective post-exposure treatment. In some situations, in particular, it may be included in the emergency strategies applied when RHD occurs on those farms having separate sheds and where good biosecurity measures are applied. Indeed, better results in limiting the spread of the disease and reducing economic losses could be obtained by using

serotherapy through the parenteral administration of anti-RHDV hyperimmune sera, which produces a rapid, but short-lived, protection against RHDV infection. In both situations (vaccination followed by post-exposure treatment and passive protection with hyperimmune sera), it is necessary to use vaccine and sera homologous to the causative RHDV strain. This is particularly true in the case of RHDV2 given the poor cross-protection induced by classical vaccines based on RHDV/RHDVa.

Vaccine should be stored at 2–8°C and it should not be frozen or exposed to bright light or high temperatures.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

At present, RHDV replication can only be produced by infection of susceptible animals. Therefore, the source of seed virus for the production of inactivated tissue vaccines is infected liver homogenates obtained by serial passages in rabbits that have been inoculated with a partially purified RHD viral suspension. The rabbits used for inoculation are selected from colonies shown to be healthy and susceptible to the disease by periodic serological testing. More variability could be encountered when obtaining livers for RHDV2 vaccine because of the different level of mortality registered in experimental infections depending on the virulence of the strain used.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The partially purified RHD viral suspension is obtained by centrifuging the 1/5 liver suspension (w/v) in PBS at 10,000 ***g*** for 20 minutes at 4°C. The resulting supernatant is treated with 8% (v/v) polyethylene glycol (PEG 6000) overnight at 4°C. The pellet is re-suspended at a dilution of 1/10 in PBS, and subsequently centrifuged at 10,000 ***g*** for 20 minutes at 4°C. The supernatant is ultracentrifuged at 80,000 ***g*** for 2 hours at 4°C through a 20% cushion of sucrose. The pellet is re-suspended in PBS (1/100 of the starting volume).

This viral suspension is then characterised by any of the following methods: negative-stain EM examination, determination of reactivity in ELISA with different specific MAbs, and HA activity at room temperature (HA titre against RBCs of human Group O higher than 1/1280).

The absence of viable bacteria, or fungi should be determined by using common laboratory bacteriological methods. PCR methods may be used for the detection of mycoplasma and of rabbit specific extraneous viruses (e.g. *Myxoma* virus).

Seed virus is controlled by direct inoculation into susceptible rabbits followed by evaluation of the clinical signs in the course of the experimental infection. Suitable seed virus should cause a variable death rate among animals according to the type of strains, i.e. 70–80% of the rabbits in the case of RHDV/RHDVa and up to 80% in the case of RHDV2 depending on the virulence of the strain, within 24–96 hours post-inoculation, with the internal organ lesions characteristic of RHD. To validate the test, gross and histopathological examination of all rabbits should be performed to exclude intercurrent diseases.

Seed virus is titrated before use and should contain at least 10^5 LD_{50}. It should be stored frozen (–70°C), better with the addition of 1:1 volume of glycerol or freeze-dried.

2.1.3. Validation as a vaccine strain

Due to the lack of effective cross protection when using heterologous vaccines (i.e. RHDV/RHDVa vs RHDV2), for vaccine preparation it is recommended to use those strains that are highly homologous with the dominant RHDV serotype causing outbreaks in an area.

It is also important to have current and homologous vaccine controls because of induced immunity with respect to the expressed antigenic pattern of the field strains.

2.1.4. Procedure for provisional acceptance of new master seed virus

Current epidemiological data clearly show that RHDV2 has almost replaced classical RHDVs, and thus there is an increasing demand for RHDV2 vaccines. As RHDV2 is a 'new' emerging virus and not just a genetic variant of RHDV/RHDVa, it has undergone a significant evolution since 2010 resulting in changes in virulence and in antigenic profile (Capucci *et al.,* 2017). As a consequence, it is recommended to choose as master seed for vaccine production one of those strains isolated from 2015 onwards, and to base the selection on their antigenic profile.

Nevertheless, it should be remembered that while wild and domestic rabbit populations are rapidly gaining a high herd immunity against RHDV2, they are simultaneously losing any protection against classical RHDV/RHDVa strains that have not completely disappeared. This fact should be considered in the design of RHD surveillance systems so that countries can be ready to react promptly to epizootics by updating vaccine composition.

2.2. Method of manufacture

2.2.1. Procedure

The vaccine manufacturing procedure for both antigenic types (RHDV and RHDV2) follows a similar protocol. Following inoculation of susceptible rabbits, the liver and spleen of those rabbits that die between 24 and 96 hours post-inoculation are collected. Rabbits that died later must be discarded. The organs are minced in 1/10 (w/v) sterile PBS, pH 7.2–7.4, and the mixture is homogenised for 10 minutes in a blender in a refrigerated environment. The mixture is then treated with 2% chloroform (18 hours at 4°C), followed by centrifugation at 6000 ***g*** for 1 hour at 4°C. The supernatant is collected by high pressure continuous pumping and is subsequently inactivated. The viral suspension is assayed by HA test and ELISA and, once the number of HA units from the initial titration is known, more sterile PBS is added in sufficient volume to provide, after inactivation and adsorption/addition of the adjuvant, a concentration of 640–1280 HA units/ml in the commercial product. Various agents have proved effective at abolishing viral infectivity. The most frequently used are formaldehyde and beta-propiolactone, which can be used at different concentrations and temperatures, for variable periods of time and also in combination. During inactivation, it is advisable to continuously agitate the fluid. Aluminium hydroxide, Freund's incomplete adjuvant or another oil emulsion is then incorporated into the vaccine as adjuvant. A preservative, thiomersal (merthiolate), is finally added at a dilution of 1/10,000 (v/v) before distribution into bottles.

2.2.2. Requirements for ingredients

As the virus cannot be grown *in vitro,* the only requirements are those concerning infected animals. Rabbits must be free from RHDV and myxomatosis virus and should not have anti-RHDV antibodies, including cross-reactive antibodies induced by the non-pathogenic RHDV-related rabbit calicivirus (RCV).

The animals (at least 4 months old) must be kept in strict quarantine on arrival, in a separate area and reared under satisfactory and biosecure health conditions (see Laboratory animal facilities in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Seed virus propagation and production of vaccine batches rely on the same protocol of experimental infection, involving intramuscular injection of a dose of at least 100 LD_{50}.

2.2.3. In-process controls

i) Antigen content

The RHDV titre is determined before inactivation by calculating the HA titre, which should be higher than 1/1280, and the ELISA reactivity. Both values are again determined after inactivation and adsorption/addition of the adjuvant. The identity of RHD could be also confirmed by negative-staining EM or real-time RT-PCR analyses.

ii) Sterility

The organs are tested for the presence of viable bacteria, viruses, fungi or mycoplasma according to the protocol used for testing master seed virus. PBS solution and aluminium hydroxide gel are sterilised by autoclaving; oil emulsion is sterilised by heating at 160°C for 1 hour.

iii) Inactivation

Before incorporation of the adjuvant, the inactivating agent and the inactivation process must be shown to inactivate the vaccine virus under the conditions of manufacture. Thus, a test is carried out on each batch of the bulk harvest as well as on the final product.

Thirty adult rabbits (>4 months of age) are used in three groups of 10. The first and second group are injected with concentrated antigen and kept under observation for 15 and 7 days, respectively. The second group is humanely killed after 7 days. The third group is injected with the liver of rabbits from the second group and kept under observation for 21 days. The dose of the inoculum, administered parenterally (intramuscular or subcutaneous), is 1 ml of concentrated antigen (PEG precipitation) corresponding to at least 10 doses (HA ≥20480). The observation period is: 10 rabbits for 7 days, 10 rabbits for 15 days and 10 rabbits for 21 days. All the rabbits kept under observation must survive without any clinical signs. The liver should give negative results using the HA test and sandwich ELISA. The rabbits inoculated with antigen should have a positive serological titre (e.g. >1/80 using the C-ELISA method specific for the homologous virus) and those injected with livers obtained after the first passage should be serologically negative.

2.2.4. Final product batch tests

Batch release safety tests are not required, except in the case of autogenous vaccines. Sterility, and potency tests should be carried out on each batch of final vaccine; tests for duration of immunity should be carried out once using a typical batch of vaccine, and stability tests should be carried out on three batches.

i) Sterility/purity

Each batch of vaccine must be tested for the presence of viable bacteria, viruses, fungi or mycoplasma according to the same protocol recommended for testing master seed virus.

iii) Safety

Where safety tests are required, the following procedure should be carried out:

a) The safety of the administration of one dose;

b) The safety of the administration of an overdose (at least two doses of inactivated vaccine);

c) The safety of the repeated administration of one dose.

The test is carried out for each approved route of administration. Use at least 10 adults (>4 months of age) that are RHDV antibody free. Observe these animals for 21 days by evaluating the following life parameters: general conditions and reactions, sensory condition, water and food consumption, characteristics of faeces, and local abnormal reactions at the inoculum point. Record the body temperature the day before vaccination, at vaccination, 4 hours after vaccination and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction should occur; the average body temperature increase should not exceed 1°C and no animal should have a temperature rise greater than 2°C. A local reaction lasting less than 21 days may occur. If the vaccine is intended for use in pregnant rabbits, administer the vaccine to at least 10 pregnant does according to the schedule to be recommended. Prolong the observation period until 1 day after parturition. The does should remain in good health and there should not be abnormal local or systemic reactions. No adverse effects on the pregnancy or on the offspring should be noted.

iii) Batch potency

Use susceptible adult rabbits (>4 months old), free from antibodies against RHDV and reared in suitable isolation conditions to ensure absence of contact with RHDV. Five rabbits are vaccinated with one full dose of vaccine given by the recommended route. Two other groups of five animals each are vaccinated with 1/4 and 1/16 of the full dose, respectively. A fourth group of five unvaccinated rabbits is maintained as controls. All animals are challenged not less than 21 days post-vaccination by intramuscular inoculation of a dose of RHDV containing at least 100 LD_{50} or presenting a HA titre higher than 1/2560. Observe the rabbits for a further 21 days. The test is not valid if: a) during the period between vaccination and challenge more than 10% of the vaccinated or more than 20% of control rabbits show abnormal clinical signs or die from causes not attributable to the vaccine; b) following challenge with RHDV/RHDVa, less than 60% of control rabbits died with typical signs of RHD; or c) following challenge with RHDV2, less than 20% of control rabbits die and less than 60% of them show high antibody titres (>1/1280 using the homologous C-ELISA). The vaccine complies with the test if: a) not less than 80% of vaccinated rabbits show no signs of RHD; b) the mean antibody level of vaccinated animals, is not significantly less than the level recorded in the protection test performed using as vaccine the inactivated seed virus.

2.3. Requirements for regulatory approval

The tests for safety, potency and sterility of the final product must be performed after bottling and packaging. Thus, it is important that these two last manufacturing steps be performed following standardised good manufacturing procedures. The tests are conducted by removing samples from a statistically determined number of randomly taken multi-dose containers (20, 50 or 100 doses) of vaccine.

2.3.1. Manufacturing process

For approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and Section C.2.2 *Method of manufacture*) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Rabbit is the sole species susceptible to RHDV (with the exception of some hare species susceptible to RHDV2) and in the interest of animal welfare, tests and trials must be held only on target animals. The safety requirements of the final product for rabbits should be verified in field studies on both fattening and breeder rabbits. At least 30 breeder rabbits, >4 months of age, and 70 rabbits 30–45 days of age should be used. Breeder rabbits are vaccinated subcutaneously at the back of the neck twice (at an interval of 3 weeks) with one dose. Fattening rabbit are vaccinated either at 30 or 45 days of age. Animals are observed for 4 months from the first vaccination. Unvaccinated animals are kept as controls.

The control of the safety of the vaccine in breeder rabbits is done by evaluating their reproductive performance. The following parameters are considered: local or general reactions; total number of rabbits born, and the number of live rabbits born; percentage of mortality at the time of weaning; average weight of young rabbits at the weaned period; daily consumption of food. The control of the safety of the vaccine in fattened rabbits is done by evaluating their daily health. The following parameters are considered: local or general reactions; individual weight increase from weaning (30 days) and every 15 days; daily consumption of feed; conversion index; mortality during the fattening period. Vaccinated rabbits should not show any changes in their general health or abnormal local or systemic reactions for the whole test duration.

ii) Reversion-to-virulence for attenuated/live vaccines

Not applicable.

iii) Precautions

The vaccine should not contain any ingredients that are likely to pose a risk for consumers of vaccinated rabbits. However, as the inactivated vaccine contains a mineral oil adjuvant, there is an associated risk that might arise from accidental self-injection. Accidental injection can cause intense swelling and severe consequences if expert medical advice is not sought promptly.

During the safety and efficacy field trials, interactions with other vaccines (e.g. vaccine against myxomatosis) or pharmaceutical products (medicated feeding-stuffs containing antibiotics against respiratory diseases and bacterial enteritis) should be checked and recorded. No interactions have been reported to date.

The inactivated vaccine does not spread in the environment and, in previous trials, there were no signs of ecotoxicity problems for the viral antigens. The risk of ecotoxicity caused by the use of vaccine is zero because of the nature of the vaccine (inactivated vaccine for parenteral use). The vaccine contains no ingredients likely to pose a risk to the environment. In addition, the vaccine is administered by injection so environmental contamination is unlikely. To achieve the highest standard of safety in accordance with good hygiene rules, the bottles must be dipped in an antiseptic solution after use.

2.3.3. Efficacy requirements

The efficacy should be tested in the laboratory with both challenge and serology tests. Twenty rabbits (10 vaccinated and 10 unvaccinated), at least 4 months of age, are challenged with virulent virus: at least 90% of the vaccinated animals must be protected, giving positive serological titres and a proportion of the control unvaccinated animals similar to that naturally recorded according to the type of strain (i.e. 70–90% for RHDV and 50–80% for RHDV2) must have died within the observation period.

The in-field efficacy of the vaccine may be determined by evaluating the seroconversion in blood samples taken from both fattening and breeder rabbits at different check-points from vaccination. Titres are measured by C-ELISA and anti-isotype IgM, IgA and IgG ELISAs, by using specific and homologous methods according to the type of virus (RHDV/RHDVa or RHDV2).

Before the first vaccination, the C-ELISAs should confirm, in all rabbits, the absence of anti-RHDV antibodies. Vaccinated animals develop an RHDV protective immunity in a short period of time: in the serum of infected animals, circulating antibodies are present just 3–4 days post-infection (IgM and IgA), whereas in rabbits vaccinated with the inactivated adjuvant vaccine, the first antibodies usually appear after 7–10 days (only IgM). IgG appear after approximately 15–20 days. After vaccination there is very low or no IgA production. As it is produced only during infection with the live virus following oro-nasal dissemination, IgA could be considered to be a marker of contact with the field virus. The mucosal immuno-system may also be involved in protection to the disease even if the vaccine is parenterally and not orally administered. This is suggested by oral challenge experiments in vaccinated rabbits when IgA but no IgM appear very quickly in the serum. This suggests that B memory cells able to produce IgA are already present at the mucosal level, which is usually the first site of replication of RHDV.

There is a definite correlation between the titres obtained by C-ELISAs and the state of protection from the disease induced by the different strains (see Section B.2.2.) taking into account the RT2 value found, i.e. rabbits with antibodies titres specifically induced by one strain (RHDV/RHDVa/RHDV2) did not show any sign of disease when challenged with the same virulent strain. In convalescent rabbits, serological titres could be as high as 1/20480, whereas in vaccinated rabbits they are usually between 1/40 and 1/640 according to the time elapsed since vaccination. Maternal antibodies (IgG only) usually disappear within 30 days of age in young rabbits born to vaccinated healthy does, but they last longer (until 45–55 days of age) when rabbits are born to convalescent does, as the passive titres of young are directly related to that of their mothers. This is true for young rabbits from industrial farms that are weaned quite early (25–35 days of age), whereas in young wild rabbits, maternal antibodies can last for 80 days. In young rabbits (<35–40 days old), a low level of antibody (1/80–1/320) could also be induced by an active infection with RHDV/RHDVa not leading to disease, as commonly occurs in animals of this age.

2.3.4 Duration of immunity

The data reported in the literature indicate the long-term duration of immunity induced by a single vaccination (up to 15 months). At 9–12 months post-vaccination, titres are 2–4 times lower than observed 2–3 weeks after vaccination. The booster effect, in the case of natural infection or re-vaccination, depends on the time elapsed since vaccination, i.e. it is lower 5–7 months post-vaccination and higher in animals vaccinated before that time.

To exactly determine the duration and efficacy of immunity, it is advisable to carry out the following test: 20 rabbits vaccinated once are divided into four groups and are serologically tested at monthly intervals over a period of 1 year. Each group is inoculated with virulent RHDV at 3, 6, 9 months or 1 year post-vaccination. Challenge infection should produce increasing seroconversion, which is directly related to the time that has elapsed since vaccination. The absence of clinical signs of disease and mortality supports the efficacy of the vaccine.

2.3.5 Stability

Evidence should be provided to show that the vaccine passes the batch potency test at 3 months beyond the suggested shelf life.

A suitable preservative is normally required for vaccine in multi-dose containers. Its persistence throughout the shelf life should be checked.

3. Vaccines based on biotechnology

Biotechnology-based approaches for vaccine production have been, and are being, developed. The aim will be to circumvent welfare concerns with conventional vaccine production, to avoid biosecurity risks, and to provide a broad spectrum protection against multiple strains of the virus.

Several studies have been carried out on the expression of RHDV/RHDVa/RHDV2 capsid protein in *Escherichia coli*, in vaccinia virus, and in attenuated *Myxoma* virus (MV). Moreover, it has been shown by various authors that a recombinant capsid protein, VP60, expressed in the baculovirus/Sf9 cell expression system, self-assembled into VLPs that are structurally and antigenically identical to RHD virions. While the fusion protein expressed in *E. coli* is highly insoluble and of low immunogenicity, active immunisation can be achieved with VLPs obtained in the baculovirus system or by using recombinant vaccinia, MV and canarypox, administered either intramuscularly or orally. In particular, rabbits vaccinated with recombinant MV expressing the RHDV and or RHDV2 capsid protein were protected against lethal RHDV and MV challenges.

The VP60 structural protein has also been expressed in transgenic plants, either with a new plum pox virus (PPV)-based vector (PPV-NK), or in transgenic potato plants under the control of a cauliflower mosaic virus 35S promoter or a modified 35S promoter. In both cases the immunisation of rabbits with extracts of *Nicotiana clevelandii* plants infected with the PPV-NK VP60 chimera and with leaf extracts from potatoes carrying this modified 35S promoter, respectively, induced an efficient immune response that protected animals against a lethal challenge with RHDV. However, at the present time, none of these vaccines has been registered and therefore, they are not commercially available.

A vaccine that is a combination of a traditional inactivated liver-derived RHD vaccine and a live attenuated Myxoma virus vaccine, and which can be administered by the intradermal route, has been developed in France and then marketed in some European countries.

Recently, two new biotechnology vaccines, one bivalent and the other trivalent have been developed and registered and are commercially available in several countries for administration by the parenteral route. In the first, the RHDV or RHDV2 VP60 gene has been inserted into the genome of a vaccine strain of myxomatosis virus, which, when inoculated into the rabbit, replicates, also producing RHDV VP60. The second vaccine is based on the same principle, contains two different myxoma virus strains each producing the VP60 of RHDV and RHDV2, respectively (Reemers *et al.,* 2020). These vaccines need approximately 3 weeks to induce detectable antibodies.

In addition, another bivalent vaccine against RHDV and RHDV2, in which the viral capsid is expressed in the form of virus-like particles from a baculovirus growing on the pupae of *Lepidoptera* (Dalton *et al.,* 2021), has been approved by the European Medicines Agency and should soon be available on the market. These recombinant-type vaccines do not need the inactivation step, and only the one based on the baculovirus system uses aluminium hydroxide as

adjuvant. Similarly to inactivated organ vaccines, this vaccine induces a quick immune response and antibodies are detectable at 7–10 days post-vaccination.

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NB: There is a WOAH Reference Laboratory for rabbit haemorrhagic disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for rabbit haemorrhagic disease

NB: FIRST ADOPTED IN 1991 AS VIRAL HAEMORRHAGIC DISEASE OF RABBITS.
MOST RECENT UPDATES ADOPTED IN 2023.

SECTION 3.8.

CAPRINAE

CHAPTER 3.8.1.

BORDER DISEASE

SUMMARY

Border disease (BD) is a viral disease of sheep and goats first reported in sheep in 1959 from the border region of England and Wales, and since recorded world-wide. Prevalence rates in sheep vary from 5% to 50% between countries and from region to region within countries. Clinical signs include barren ewes, abortions, stillbirths and the birth of small weak lambs. Affected lambs can show tremor, abnormal body conformation and hairy fleeces (so-called 'hairy-shaker' or 'fuzzy' lambs) and the disease has been referred to as 'hairy shaker disease'. Vertical transmission plays an important role in the epidemiology of the disease. Infection of fetuses can result in the birth of persistently infected (PI) lambs. These PI lambs are viraemic, antibody negative and constantly excrete virus. The virus spreads from sheep to sheep, with PI animals being the most potent source of infection. Infection in goats is less common with abortion being the main presenting sign.

BD is caused by the Pestivirus *border disease virus (BDV), but in some parts of the world, especially where there is close contact between sheep or goats and cattle, the same clinical signs may be caused by infection with bovine viral diarrhoea virus (BVDV). Therefore the genetic and antigenic differences between BDV and BVDV need to be taken into consideration when investigating disease outbreaks or certifying animals or germplasm for international movement, It is important to identify the viraemic PI animals so that they will not be used for breeding or trading purposes. Serological testing is insufficient. However, it is generally considered that serologically positive, nonviraemic sheep are 'safe', as latent infections are not known to occur in recovered animals.*

Identification of the agent: *BDV is a* Pestivirus *in the family* Flaviviridae *and is closely related to classical swine fever virus and BVDV. Nearly all isolates of BDV are noncytopathogenic in cell culture. There are no defined serotypes but virus isolates exhibit considerable antigenic diversity. A number of separate genotypes, have been identified.*

Apparently healthy PI sheep resulting from congenital infection can be identified by direct detection of virus or nucleic acid in blood or tissues or by virus isolation in cell culture followed by immunostaining to detect the noncytopathogenic virus.

Diagnostic methods: *The demonstration of virus by culture and antigen detection may be less reliable in lambs younger than 2 months that have received colostral antibody. Acute infection is usually subclinical and viraemia is transient and difficult to detect. The isolation of virus from tissues of aborted or stillborn lambs is often difficult but virus can be detected by sensitive polymerase chain reaction methods that are able to detect residual nucleic acid. However, tissues and blood from PI sheep more than a few months old contain high levels of virus, which can be easily identified by isolation and direct methods to detect antigens or nucleic acids. As sheep may be infected with BVDV, it is preferable to use diagnostic assays that are 'pan-pestivirus' reactive and will readily detect all strains of BDV and BVDV.*

Serological tests: *Acute infection with BDV is best confirmed by demonstrating seroconversion using paired or sequential samples from several animals in the group. The enzyme-linked immunosorbent assay and virus neutralisation test (VNT) are the most commonly used antibody detection methods. Due to the antigenic differences between BDV and BVDV, assays for the detection of antibodies to BDV, especially by VNT, should preferably be based on a strain of BDV.*

Requirements for vaccines: *There is no standard vaccine for BDV, but a commercial killed whole-virus vaccine has been produced. Ideally, such a vaccine should be suitable for administration to females before breeding for prevention of transplacental infection. The use of BVDV vaccines has been advocated, but the antigenic diversity of BD viruses must be considered.*

BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells or containing sheep serum. This potential hazard should be recognised by manufacturers of biological products.

A. INTRODUCTION

Border disease virus (BDV) is a *Pestivirus* of the family *Flaviviridae* and is closely related to classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV). There are four officially recognised species, namely – CSFV, BVDV types 1 and 2 and BDV (ICTV, 2016), but a number of other pestiviruses that are considered to be distinct species have been reported. While CSF viruses are predominantly restricted to pigs, examples of the other three species have all been recovered from sheep. While the majority of isolates have been identified as BD viruses in areas where sheep or goats are raised in isolation from other species (Vilcek *et al*., 1997), in regions where there is close contact between small ruminants and cattle, BVDV may be frequently identified (Carlsson, 1991). Nearly all virus isolates of BDV are noncytopathogenic, although occasional cytopathic viruses have been isolated (Vantsis *et al*., 1976). BDV spreads naturally among sheep by the oro-nasal route and by vertical transmission. It is principally a cause of congenital disease in sheep and goats, but can also cause acute and persistent infections. Infection is less common in goats, in which persistent infection is rare as abortion is the main presenting sign. Pigs may also be infected by pestiviruses other than CSFV and antibodies to BDV in pigs may interfere with tests for the diagnosis of CSF (Oguzoglu *et al*., 2001). Several genotypes of BD viruses from sheep, goats and Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) have been described. Phylogenetic analysis using computer-assisted nucleotide sequence analysis suggests that genetic variability among BD viruses is greater than within each of the other *Pestivirus* species. Four distinguishable genogroups of BDV have been described as well as putative novel *Pestivirus* genotypes from Tunisian sheep and a goat (Becher *et al*., 2003; Vilcek & Nettleton, 2006). The chamois BD virus is similar to isolates from sheep in the Iberian Peninsula (Valdazo-Gonzalez *et al*., 2007). This chapter describes BDV infection in sheep. Chapter 3.4.7 *Bovine viral diarrhoea* should also be consulted for related diagnostic methods.

1. Acute infections

Healthy newborn and adult sheep exposed to BDV usually experience only mild or inapparent disease. Slight fever and a mild leukopenia are associated with a short-lived viraemia detectable between days 4 and 11 post-infection, after which virus neutralising antibody appears in the serum (Thabti *et al*., 2002).

Acute infections are best diagnosed serologically using paired sera from a representative number of sheep. Occasional BDV isolates have been shown to produce high fever, profound and prolonged leukopenia, anorexia, conjunctivitis, nasal discharge, dyspnoea and diarrhoea, and 50% mortality in young lambs. One such isolate was recovered from a severe epidemic of BD among dairy sheep in 1984 (Chappuis *et al*., 1986). A second such isolate was a BDV contaminant of a live CSFV vaccine (Wensvoort & Terpstra, 1988).

2. Fetal infection

The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal infection is subclinical or mild, the consequences for the fetus are serious. Fetal death may occur at any stage of pregnancy, but is more common in fetuses infected early in gestation. Small dead fetuses may be resorbed or their abortion may pass unnoticed as the ewes continue to feed well and show no sign of discomfort. As lambing time approaches, the abortion of larger fetuses, stillbirths and the premature births of small, weak lambs will be seen. Confirmation that an abortion or stillbirth is due to BDV is often difficult to establish, but virus may be isolated from fetal tissues in some cases. The use of an appropriate real-time reverse-transcription polymerase chain

reaction (RT-PCR) assay may give a higher level of success because of the advantages of high sensitivity and the ability to detect genome from non-infectious virus. In aborted fetuses, it is also possible to detect virus by immunohistochemistry of brain, thyroid and other tissues (Thur *et al*., 1997). Samples of fetal fluids or serum should be tested for BDV antibody.

During lambing, an excessive number of barren ewes will become apparent, but it is the diseased live lambs that present the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very variable and depend on the breed of sheep, the virulence of the virus and the time at which infection was introduced into the flock. Affected lambs are usually small and weak, many being unable to stand. Nervous signs and fleece changes are often apparent. The nervous signs of BD are its most characteristic feature. The tremor can vary from violent rhythmic contractions of the muscles of the hindlegs and back, to barely detectable fine trembling of the head, ears, and tail. Fleece abnormalities are most obvious in smooth-coated breeds, which develop hairy fleeces, especially on the neck and back. Abnormal brown or black pigmentation of the fleece may also be seen in BD-affected lambs. Blood samples to be tested for the presence of BDV or antibody should be collected into anticoagulant from suspect lambs before they have received colostrum. Once lambs have ingested colostrum, it is difficult to isolate virus until they are 2 months old and maternal antibody levels have waned. However, during this period, it may be possible to detect viral antigen in skin biopsies, by immunohistochemistry, in washed leukocytes by enzyme-linked immunosorbent assay (ELISA) or by real-time RT-PCR. ELISAs directed at detection of the Erns antigen appear to be less prone to interference by maternal antibodies and can often be used to detect antigen in serum.

With careful nursing, a proportion of BD lambs can be reared, although deaths may occur at any age. The nervous signs gradually decline and can disappear by 3–6 months of age. Weakness, and swaying of the hind-quarters, together with fine trembling of the head, may reappear at times of stress. Affected lambs often grow slowly and under normal field conditions many will die before or around weaning time. In cases where losses at lambing time have been low and no lambs with obvious signs of BD have been born, this can be the first presenting sign of disease.

Some fetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia, hydranencephaly and porencephaly resulting from necrotising inflammation. The severe destructive lesions appear to be immune mediated, and lambs with such lesions frequently have high titres of serum antibody to BDV. Most lambs infected in late gestation are normal and healthy and are born free from virus but with BDV antibody. Some such lambs can be weak and may die in early life (Barlow & Patterson, 1982).

3. Persistent viraemia

When fetuses survive an infection that occurs before the onset of immune competence, they are born with a persistent viraemia. The ovine fetus can first respond to an antigenic stimulus between approximately 60 and 85 days of its 150-day gestation period. In fetuses infected before the onset of immune competence, viral replication is uncontrolled and 50% fetal death is common. In lambs surviving infection in early gestation, virus is widespread in all organs. Such lambs appear to be tolerant of the virus and have a persistent infection, usually for life. A precolostral blood sample will be virus positive and antibody negative. Typically, there is no inflammatory reaction and the most characteristic pathological changes are in the central nervous system (CNS) and skin. Throughout the CNS, there is a deficiency of myelin, and this causes the nervous signs. In the skin, primary wool follicles increase in size and the number of secondary wool follicles decreases, causing the hairy or coarse fleece.

Persistently viraemic sheep can be identified by the detection of viral antigens, nucleic acids or infectious virus in a blood sample. Viraemia is readily detectable by testing of serum at any time except within the first 2 months of life, when virus may be masked by colostral antibody and, possibly, in animals older than 4 years, some of which develop low levels of anti-BDV antibody (Nettleton *et al*., 1992). Methods other than virus isolation may be preferred to avoid interference from antibodies. When the presence of colostral antibodies is suspected, the virus may be detected in washed leukocytes and in skin by using sensitive ELISAs. Although virus detection in blood during an acute infection is difficult, persistent viraemia should be confirmed by retesting animals after an interval of at least 3 weeks. The use of real-time RT-PCR should be considered at all times and for any sample type due to its high analytical sensitivity and the lack of interference from antibodies in a sample.

Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams are always persistently viraemic. Persistently viraemic sheep are a continual source of infectious virus to other

animals and their identification is a major factor in any control programme. Sheep being traded should be screened for the absence of BDV viraemia.

Usually persistently infected (PI) rams have poor quality, highly infective semen and reduced fertility. All rams used for breeding should be screened for persistent BDV infection on a blood sample. Semen samples can also be screened for virus, but virus isolation is much less satisfactory than from blood because of the toxicity of semen for cell cultures. Real-time RT-PCR for detection of pestivirus nucleic acid would usually overcome toxicity problems, and thus this assay should be useful for testing semen from rams.

4. Late-onset disease in persistently viraemic sheep

Some PI sheep housed apart from other animals spontaneously develop intractable diarrhoea, wasting, excessive ocular and nasal discharges, sometimes with respiratory distress. At necropsy such sheep have gross thickening of the distal ileum, caecum and colon resulting from focal hyperplastic enteropathy. Cytopathic BDV can be recovered from the gut of these lambs. With no obvious outside source of cytopathic virus, it is most likely that such virus originates from the lamb's own virus pool, similar to what occurs with BVDV. Other PI sheep in the group do not develop the disease. This syndrome, which has been produced experimentally and recognised in occasional field outbreaks of BD, has several similarities with bovine mucosal disease (Nettleton *et al*., 1992).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of border disease and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	+	++	++	+++	–	–
Antigen detection by ELISA	+	++	+++	+++	–	–
NA detection by real-time RT-PCR	+++	+++	+++	+++	+++	–
NA detection by ISH	–	–	–	+	–	–
Detection of immune response						
ELISA	++	++	++	+	++	++
VN	+++	+++	++	+++	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry; NA = nucleic acid; RT-PCR = reverse-transcription polymerase chain reaction; ISH = *in-situ* hybridisation; VN = virus neutralisation.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

There is no designated WOAH Reference Laboratory for BDV, but the reference laboratories for BVDV or CSFV will be able to provide advice[1]. One of the most sensitive proven methods for identifying BDV remains virus isolation. However, a broadly reactive real-time RT-PCR assay (preferably pan-pestivirus reactive) will usually provide higher analytical sensitivity than virus isolation, can be used to test samples that are difficult to manage by virus isolation and can be performed in a few hours. Antigen-detection ELISA and immunohistochemical techniques on tissue sections are also valuable methods for identifying BDV-infected animals.

1.1. Virus isolation

It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free susceptible cells and bovine serum, or equivalent, that contain no anti-pestivirus activity and no contaminating virus. It is important that a laboratory quality assurance programme be in place. Chapter 3.4.7 provides detailed methods for virus isolation in either culture tubes or microplates for the isolation of pestiviruses from sheep or goat samples, including serum, whole blood, semen and tissues. The principles and precautions outlined in that chapter for the selection of cell cultures, medium components and reagents are equally relevant to this chapter. Provided proven pan-pestivirus reactive reagents (e.g. monoclonal antibodies [MAbs], primers and probes for real-time RT-PCR) are used for antigen or nucleic acid detection, the principal difference is the selection of appropriate cell cultures.

BD virus can be isolated in a number of primary or secondary ovine cell cultures (e.g. kidney, testes, lung). Ovine cell lines for BDV growth are rare. Semicontinuous cell lines derived from fetal lamb muscle (FLM), whole embryo (Thabti *et al*., 2002) or sheep choroid plexus can be useful, but different lines vary considerably in their susceptibility to the virus. Ovine cells have been used successfully for the isolation and growth of BD viruses and BVDV types 1 and 2 from sheep. In regions where sheep may become infected with BVD viruses from cattle, a virus isolation system using both ovine and bovine cells could be optimal. However, bovine cells have lower sensitivity for the primary isolation and growth of some BD viruses, so reliance on bovine cells alone is inadvisable. Details of suitable bovine cell cultures are provided chapter 3.4.7. The precautions outlined in that chapter for the establishment of cells and medium components that are free from contamination with either pestiviruses or antibodies, and measures to ensure that the cells are susceptible to a wide range of local field strains are equally relevant to systems for detection of BDV.

From live animals, serum is the most frequently used sample to be tested for the presence of infectious virus. However, for difficult cases, the most sensitive way to confirm pestivirus viraemia is to wash leukocytes repeatedly (at least three times) in culture medium before co-cultivating them with susceptible cells in either cell culture tubes or microplates. After culture for 5–7 days, the cultures should be frozen and thawed once and an aliquot of diluted culture fluid passaged onto further susceptible cells grown in microplates or on chamber slides to allow antigen detection by immunocytochemistry. Staining for noncytopathic pestiviruses will usually detect virus at the end of the primary passage, but to detect slow-growing viruses in poorly permissive cells two passages are desirable. It is recommended that the culture supernatant used as inoculum for the second passage is diluted approximately 1/100 in new culture medium because some high titred field isolates will replicate poorly if passaged undiluted (i.e. at high multiplicity of infection – moi).

Tissues should be collected from dead animals in virus transport medium. In the laboratory, the tissues are ground to give a 10–20% (w/v) suspension, centrifuged to remove debris, and the supernatant passed through 0.45 µm filters. Spleen, lung, thyroid, thymus, kidney, brain, lymph nodes and gut lesions are the best organs for virus isolation.

Semen can be examined for the presence of BDV, but raw semen is strongly cytotoxic and must be diluted, usually at least 1/10 in culture medium. As the major threat of BDV-infected semen is from PI rams, blood is a more reliable clinical sample than semen for identifying such animals. There are many

1 Please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

variations in virus isolation procedures. All should be optimised for maximum sensitivity using a standard reference virus preparation and, whenever possible, recent BDV field isolates. Most of the limitations of virus isolation for the detection of BDV in serum or blood, tissues or semen can be overcome by the use of a proven, sensitive pan-pestivirus reactive real-time RT-PCR. Some laboratories screen samples by real-time RT-PCR and undertake virus isolation on positive samples to collect BDV strains for future reference or research purposes.

For specific technical details of virus isolation procedures, including immunoperoxidase staining, refer to chapter 3.4.7.

1.2. Nucleic acid detection methods

The complete genomic sequences of three BD viruses have been determined and compared with those of other pestiviruses (Becher *et al*., 1998; Ridpath & Bolin, 1997). Phylogenetic analysis shows BD viruses to be more closely related to CSFV than to BVDV (Becher *et al*., 2003; Van Rijn *et al*., 1997; Vilcek & Nettleton, 2006; Vilcek *et al*., 1997). Real-time RT-PCR for diagnosing pestivirus infection is now used widely and a number of formats have been described. Real-time RT-PCR assays have the advantages of being able to detect both infectious virus and residual nucleic acid, the latter being of value for investigating abortions and lamb deaths. Furthermore, the presence of virus-specific antibodies in a sample will have no adverse effect on the sensitivity of the real-time RT-PCR assay. These assays are also useful for screening semen and, when recommended nucleic acid extraction protocols are followed, are less affected by components of the semen compared with virus isolation. Because of the potential for small ruminants to be infected with genetically different strains of BDV or with strains of BVDV, a proven pan-pestivirus reactive real-time RT-PCR should be used. Suitable protocols for both nucleic acid extraction as well as the real-time RT-PCR are described in chapter 3.4.7. All precautions to minimise laboratory contamination should be followed closely.

After testing samples in a pan-pestivirus reactive assay, samples giving positive results can be investigated further by the application of a BDV-specific real-time RT-PCR (Willoughby *et al*., 2006). It is important to note however that different genotypes of BDV may be circulating in some populations, especially wild ruminants such as chamois and deer, and may be transferred to sheep. An assay that is specific for the detection of BDV should be used with some caution as variants or previously unrecognised genotypes may not be detected, hence the value of initially screening samples with a pan-pestivirus reactive real-time RT-PCR.

1.3. Enzyme-linked immunosorbent assay for antigen detection

ELISAs for the direct detection of pestivirus antigen in blood and tissues of infected animals have proven to be extremely useful for the detection of PI animals and the diagnosis of disease. The first ELISA for pestivirus antigen detection was described for detecting viraemic sheep and was later modified into a double MAb capture ELISA for use in sheep and cattle (Entrican *et al.,* 1994). The test is most commonly employed to identify PI viraemic sheep using washed, detergent-lysed blood leukocytes. The sensitivity is close to that of virus isolation and it is a practical method for screening large numbers of blood samples. As with virus isolation, high levels of colostral antibody can mask persistent viraemia. The ELISA is more effective than virus isolation in the presence of antibody, but may give false-negative results in viraemic lambs younger than 2 months old. The ELISA is usually not sensitive enough to detect acute BDV infections on blood samples. As well as for testing leukocytes, the antigen ELISA can also be used on tissue suspensions, especially spleen, from suspected PI sheep and, as an alternative to immunofluorescence and immunoperoxidase methods, on cell cultures. Several pestivirus ELISA methods have been published but there are at present no commercially available kits that have been fully validated for detecting BDV. Prior to use for regulatory purposes, these kits should be validated in the region where they are to be used to ensure that a wide range of field strains of BDV can be detected and that they are suitable for the sample types to be tested.

1.4. Immunohistochemistry

Viral antigen demonstration is possible in most of the tissues of PI animals (Braun *et al*., 2002; Thur *et al*., 1997) although this is not a method that is routinely used for diagnostic purposes. This should be done on acetone-fixed frozen tissue sections (cryostat sections) or paraffin wax embedded samples using appropriate antibodies. Pan-pestivirus reactive antibodies with NS2-3 specificity are suitable.

Tissues with a high amount of viral antigen are brain, thyroid gland, lung and oral mucosa. Skin biopsies have been shown to be useful for *in-vivo* diagnosis of persistent BDV infection.

2. Serological tests

Antibody to BDV is usually detected in sheep sera using VN or an ELISA. The less sensitive agar gel immunodiffusion test is not recommended. Control positive and negative reference sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. Single sera can be tested to determine the prevalence of BDV in a flock, region or country. For diagnosis, however, acute and convalescent sera are the best samples for confirming acute BDV infection. Repeat sera from one animal should always be tested alongside each other on the same plate to provide a reliable comparison of titres.

2.1. Virus neutralisation test

Due to antigenic diversity among pestiviruses the choice of test virus is difficult (Dekker *et al*., 1995; Nettleton *et al*., 1998). No single strain of BDV is ideal. A local strain that gives the highest antibody titre with a range of positive sheep sera should be used.

Because there are few cytopathogenic strains of BDV available, it is more usual to employ a representative local non-cytopathogenic strain and read the assay after immunoperoxidase staining of the cells. Proven highly sensitive, pestivirus-free sheep cells such as lamb testis or kidney cells are suitable and can be maintained as cryogenically frozen stocks for use over long periods of time. The precautions outlined for selection of pestivirus-free medium components are equally applicable to reagents to be used in VN tests. A recommended procedure follows.

2.1.1. Test procedure

i) The test sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, three or four wells are used at each dilution depending on the degree of precision required. Also, for each sample and at each serum dilution, one well is left without virus to monitor for evidence of sample toxicity that could mimic viral cytopathology or interfere with virus replication. Control positive and negative sera should also be included in each batch of tests.

iii) An equal volume (e.g. 50 µl) of a stock of BDV containing 100 $TCID_{50}$ (50% tissue culture infective dose) is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30–300 $TCID_{50}$).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. ovine testis or kidney cells) is trypsinised and the cell concentration is adjusted to 2×10^5/ml. 100 µl of the cell suspension is added to each well of the microtitre plate.

vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO_2 atmosphere or with the plate sealed.

vii) The wells are examined microscopically to ensure that there is no evidence of toxicity or cytopathic effect (CPE), then fixed and stained by immunoperoxidase staining using an appropriate MAb. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber or Reed Muench methods. A seronegative animal will show no neutralisation at the lowest dilution (i.e. 1/4), equivalent to a final dilution of 1/8. For accurate comparison of antibody titres, and particularly to demonstrate significant (more than fourfold) changes in titre, samples should be tested in parallel in the same test

viii) Occasionally there may be a need to determine whether antibody in a flock is against a virus belonging to a particular *Pestivirus* serogroup. A differential VN test can be used in which sera are titrated out against representative viruses from each of the four *Pestivirus* groups, i.e. BDV, BVDV types 1 and 2, and CSFV. Maximum titre will identify the infecting serotype and the spectrum of cross-reactivity with the other serotypes will also be revealed.

2.2. Enzyme-linked immunosorbent assay

An MAb-capture ELISA for measuring BDV antibodies has been described. Two pan-pestivirus MAbs that detect different epitopes on the immunodominant nonstructural protein NS 2/3 are used to capture detergent-lysed cell-culture grown antigen. The results correlate qualitatively with the VN test (Fenton *et al*., 1991).

2.2.1. Antigen preparation

Use eight 225 cm^2 flasks of newly confluent FLM cells; four flasks will be controls and four will be infected. Wash the flasks and infect four with a 0.01–0.1 m.o.i. of Moredun cytopathic BDV. Allow the virus to adsorb for 2 hours at 37°C. Add maintenance media containing 2% FBS (free from BDV antibody), and incubate cultures for 4–5 days until CPE is obvious. Pool four control flask supernatants and separately pool four infected flask supernatants. Centrifuge at 3000 ***g*** for 15 minutes to pellet cells. Discard the supernatants. Retain the cell pellets. Wash the flasks with 50 ml of PBS and repeat the centrifugation step as above. Pool all the control cell pellets in 8 ml PBS containing 1% Nonidet P40 and return 2 ml to each control flask to lyse the remaining attached cells. Repeat for infected cells. Keep the flasks at 4°C for at least 2 hours agitating the small volume of fluid on the cells vigorously every 30 minutes to ensure total cell detachment. Centrifuge the control and infected antigen at 12,000 ***g*** for 5 minutes to remove the cell debris. Supernatant antigens are stored at –70°C in small aliquots.

2.2.2. Test procedure

i) The two MAbs are diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. All wells of a suitable ELISA-grade microtitre plate (e.g. Nunc maxisorb, Greiner 129b) are coated overnight at 4°C.

ii) After washing three times in PBST, a blocking solution of PBST containing 10% horse serum (PBSTH) is added to all wells, which are incubated at 37°C for 1 hour.

iii) The antigen is diluted to a predetermined dilution in PBSTH and alternate rows of wells are coated with virus and control antigens for 1 hour at 37°C. The plates are then washed three times in PBST before addition of test sera.

iv) Test sera are diluted 1/50 in PBSTH and added to duplicate virus and duplicate control wells for 1 hour at 37°C. The plates are then washed three times in PBST.

v) Anti-ovine IgG peroxidase conjugate is diluted to a predetermined dilution in PBSTH and added to all wells for 1 hour at 37°C. The plates are washed three times in PBST.

vi) A suitable activated enzyme substrate/chromogen, such as ortho-phenylene diamine (OPD) or tetramethyl benzidine (TMB), is added). After colour development, the reaction is stopped with sulphuric acid and the absorbance read on an ELISA plate reader. The mean value of the two control wells is subtracted from the mean value of the two virus wells to give the corrected absorbance for each serum. Results are expressed as corrected absorbance with reference to the corrected absorbance of known positive and negative sera. Alternatively, ELISA titres can be extrapolated from a standard curve of a dilution series of a known positive reference serum.

If antigens of sufficient potency can be produced the MAb capture stage can be omitted. In this case alternate rows of wells are coated with virus and control antigen diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6, overnight at +4°C. The plates are washed and blocked as in step ii above. After washing, diluted test sera are added and the test proceeds from step iv as above.

C. REQUIREMENTS FOR VACCINES

1. Background

To be useful, a BDV vaccine should be effective when administered to female sheep before breeding to prevent transplacental infection. Experimental and commercial inactivated whole virus BDV vaccines have been produced in Europe (Brun *et al*., 1993; Vantsis *et al*., 1980). Unlike vaccines for BVDV, there is limited demand for vaccines

against BDV and those produced have only been inactivated products. No live attenuated or recombinant subunit vaccines for BDV have been produced commercially.

Pestivirus contaminants of modified live virus vaccines have been found to be a cause of serious disease following their use in pigs, cattle, sheep and goats. Contaminated vaccines have included those used for the control of Aujesky's disease, CSF, rotavirus, coronavirus, rinderpest, sheep pox and contagious pustular dermatitis. The insidious ability of pestiviruses to cross the placenta, and thus establish PI animals, gives them the potential to contaminate vaccines through cells, serum used as medium supplement, or seed stock virus. As nearly all isolates of pestiviruses are noncytopathic, they will remain undetected unless specific tests are carried out. Although such contamination should be less likely to be a problem with an inactivated vaccine, nevertheless steps should be taken to ensure that materials used in production are not contaminated.

1.1. Characteristics of a target product profile

Traditionally, pestivirus vaccines fall into two classes: modified live or inactivated virus vaccines. The essential requirement for both types is to afford a high level of fetal infection. Only inactivated vaccines have been produced for BDV. Properly formulated inactivated vaccines are very safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. Because of the propensity for antigenic variability, the vaccine should contain strains of BDV that are closely matched to viruses found in the area in which they are used. This may present particular challenges with BDV in regions where several antigenic types have been found. Due to the need to customise vaccines for the most commonly encountered strains within a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon globally

Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

An ideal vaccine should contain a strain or strains of virus that give protection against all sheep pestiviruses. This may be challenging however, because of the range of pestiviruses with which sheep can be infected. There is considerable antigenic variation across these viruses – both between viruses that have been classified in the BDV genogroup as well as between viruses in the BVDV1 and BVDV2 genotypes (Wensvoort *et al*., 1989; Becher *et al*., 2003; Vilcek & Nettleton, 2006). Infection of sheep with the putative BVDV-3 genotype has also been described (Decaro *et al.,* 2012). It is likely that the antigenic composition of a vaccine will vary from region to region to provide an adequate antigenic match with dominant virus strains. Cross-neutralisation studies are required to establish optimal combinations. Nevertheless it would appear that any BDV vaccine should contain at least a representative of the BDV and BVDV (type 1) groups. Characterisation of the biologically cloned vaccine viruses should include typing with MAbs and genotyping (Paton *et al*., 1995).

2.1.1. Quality criteria (sterility, purity, freedom from extraneous agents)

It is crucial to ensure that all materials used in the preparation of the bulk antigens have been extensively screened to ensure freedom from extraneous agents. This should include master and working seeds, the cell cultures and all medium supplements such as bovine serum. Some bovine viruses and particularly BVDV can readily infect small ruminants such as sheep. Therefore, it is particularly important to ensure that any serum used that is of bovine origin is free of both adventitious BVDV and antibodies against BVDV strains because low levels of either virus or antibody can mask the presence of the other. Materials and vaccine seeds should be tested for sterility and freedom from contamination with other agents, especially viruses as described in the chapter 1.1.8 and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the capacity to prevent transplacental transmission. Effective challenge of vaccinated pregnant ewes at 50–60 days gestation has been achieved by intranasal installation of virus or by mixing

with PI sheep (Brun *et al.,* 1993). Usually this reliably produces persistently viraemic offspring in non-immune ewes. In regions where multiple genotypes of BDV viruses are commonly encountered, efficacy in protecting against multiple strains should be measured.

2.2. Method of manufacture

2.2.1. Procedure

Inactivated vaccines have been prepared using conventional laboratory techniques with stationary or rolled cell cultures. Inactivants have included formalin and beta-propriolactone. Adjuvants have included aluminium hydroxide and oil (Brun *et al*., 1993; Vantsis *et al*., 1980). Optimal yields depend on the cell type and isolate used. A commercial BDV vaccine containing two strains of virus has been prepared on ovine cell lines (Brun *et al*., 1993). Cells must be produced according to a seed-lot system from a master cell seed (MCS) that has been shown to be free from all contaminating microorganisms. Vaccine should only be produced in cells fewer than 20 passages from the MCS. Control cells from every passage should be checked for pestivirus contamination. Standard procedures may be used, with the expectation for harvesting noncytopathic virus on days 4–7 after inoculation of cultures. The optimal yield of infectious virus will depend on several factors, including the cell culture, isolate used and the initial seeding rate of virus. These factors should be taken into consideration and virus replication kinetics investigated to establish the optimal conditions for large-scale virus production. Whether a live or inactivated vaccine, the essential aim will be to produce a high-titred virus stock. This bulk antigen preparation can subsequently be prepared according to the type of vaccine being considered.

2.2.2. Requirements for ingredients

BDV vaccines have usually been grown in cell cultures of ovine origin that are frequently supplemented with medium components of animal origin. The material of greatest concern is bovine serum due to the potential for contamination with BVD viruses and antibodies to these viruses. These adventitious contaminants not only affect the efficiency of production but also may mask the presence of low levels of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all materials should be tested for sterility and freedom from contamination with other agents, especially viruses as described in chapters 1.1.8 and 1.1.9. Furthermore, materials of bovine or ovine origin should originate from a country with negligible risk for transmissible spongiform encephalopathies (see chapter 1.1.9).

2.2.3. In-process controls

In-process controls are part of the manufacturing process. Cultures should be inspected regularly to ensure that they remain free from gross bacterial contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate. While the basic requirement for efficacy is the capacity to induce an acceptable neutralising antibody response, during production, target concentrations of antigen required to achieve an acceptable response may be monitored indirectly by assessment of the quantity of infectious virus or antigen mass that is produced. Rapid diagnostic assays such as the ELISA are useful for monitoring BVDV antigen production. Alternatively, the quality of a batch of antigen may be determined by titration of the quantity of infectious virus present, although this may underestimate the quantity of antigen. For inactivated vaccines, infectivity is evaluated before inactivation. For inactivated vaccines the inactivation kinetics should be established so that a suitable safety margin can be determined and incorporated into the routine production processes. At the end of production, *in-vitro* cell culture assays should be undertaken to confirm that inactivation has been complete. These innocuity tests should include a sufficient number of passages and volume of inoculum to ensure that very low levels of infectious virus would be detected if present.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Identity tests should demonstrate that no other strain of BDV is present when several strains are propagated in a facility producing multivalent vaccines.

iii) Safety

Samples from inactivated vaccines should be tested rigorously for viable virus. Samples of the product should be passaged for a minimum of three passages in sensitive cell cultures to ensure absence of live BDV. This *in-vitro* monitoring can be augmented by injecting two BDV-seronegative sheep with 20 doses of unformulated antigen as part of a standard safety test. Presence of live virus will result in the development of a more convincing serological response than will occur with inactivated virus alone. The sheep sera can also be examined for antibody to other prescribed agents.

Safety tests shall also consist of detecting any abnormal local or systemic adverse reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is demonstrated and approved in the registration dossier and production is consistent with that described in chapter 1.1.8. Vaccines must either be demonstrated to be safe in pregnant sheep (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals.

iv) Batch potency

Vaccine potency is best tested in seronegative sheep in which the development and level of antibody is measured. BVD vaccines must be demonstrated to produce adequate immune responses when used in their final formulation according to the manufacturer's published instructions. The minimum quantity of infectious virus or antigen required to produce an acceptable immune response should be determined. An indirect measure of potency is given by the level of virus infectivity prior to inactivation. *In-vitro* assays should be used to monitor individual batches during production. The antigen content following inactivation can be assayed by MAb-capture ELISA and related to the results of established *in-vivo* potency results. It should be demonstrated that the lowest recommended dose of vaccine can prevent transplacental transmission of BDV in pregnant sheep.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the relevant authorities. Unless otherwise specified by the authorities, information should be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

There is no standard method for the manufacture of a BDV vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine, formalin or beta-propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

2.3.2. Safety requirements

In-vivo tests should be undertaken using repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) and contain the maximum permitted antigen load and, depending on the formulation of the vaccine, the maximum number of vaccine strains.

i) Target and non-target animal safety

The safety of the final product formulation of inactivated vaccines should be assessed in susceptible young sheep that are free of maternally derived antibodies and in pregnant

ewes. They should be checked for any local reactions following administration, and, in pregnant ewes, for any effects on the unborn lamb.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

In the event that a live virus vaccine was developed for BDV, virus seeds that have been passaged at least up to and preferably beyond the passage limit specified for the seed should be inoculated into young lambs to confirm that there is no evidence of disease. If a live attenuated vaccine has been registered for use in pregnant animals, reversion to virulence tests should also include pregnant animals. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact' animals.

iii) Precautions (hazards)

BDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory. While the inactivated virus in a vaccine should be identified as harmless for people administering the product, adjuvants included in the vaccine may cause injury to people. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

The potency of the vaccine should be determined by inoculation into seronegative and virus negative lambs, followed by monitoring of the antibody response. Antigen content can be assayed by infectivity titration prior to inactivation and subsequently by ELISA and adjusted as required to a standard level for the particular vaccine. Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration. Each production batch of vaccine should undergo potency and safety testing as batch release criteria. BVD vaccines must be demonstrated to produce adequate immune responses, as outlined above, when used in their final formulation according to the manufacturer's published instructions.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

To date, there are no commercially available vaccines for BDV that support use of a true DIVA strategy.

2.3.5. Duration of immunity

Inactivated vaccines are unlikely to provide sustained levels of immunity and it is likely that after an initial course of two or three injections annual booster doses may be required. Insufficient information is available to determine any correlation between vaccinal antibody titres in the dam and fetal protection. As there are likely to be different commercial formulations and these involve a range of adjuvants, there are likely to be different periods of efficacy. Consequently, duration of immunity data must be generated separately for each commercially available product by undertaking challenge tests at the end of the period for which immunity has been claimed.

2.3.6. Stability

There are no accepted guidelines for the stability of BDV vaccines, but it can be assumed that an inactivated virus vaccine should remain potent for at least 1 year if kept at 4°C and probably longer. Lower temperatures could prolong shelf life but adjuvants in a killed vaccine may preclude this. Bulk antigens that have not been formulated into finished vaccine can be reliably stored frozen at low temperatures, but the antigen quality should be monitored with *in-vitro* assays prior to incorporation into a batch of vaccine.

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NB: At the time of publication (2017) there were no WOAH Reference Laboratories for border disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1996. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 3.8.2.

CAPRINE ARTHRITIS/ENCEPHALITIS & MAEDI-VISNA

SUMMARY

Caprine arthritis/encephalitis (*CAE*) and *maedi-visna* (*MV*) *are persistent lentivirus infections of goats and sheep. They are often grouped together as the small ruminant lentiviruses* (*SRLVs*). *Maedi-visna is also known as ovine progressive pneumonia* (*OPP*). *Phylogenetic analyses comparing nucleotide sequences of MV virus* (*MVV*) *and CAE virus* (*CAEV*) *have demonstrated that these are closely related lentiviruses. One source of CAEV and MVV transmission is colostrum and milk. The source of horizontal transmission in the absence of lactation remains unknown; however, faeces and lung fluids are known to harbour infectious virus. Ovine lentiviruses have been identified in most of the sheep-rearing countries of the world, with the notable exceptions of Australia and New Zealand. The distribution of CAEV is highest in industrialised countries, and seems to have coincided with the international movement of European breeds of dairy goats. Clinical and subclinical MV and CAE are associated with progressive, mononuclear cell inflammatory lesions in the lungs, joints, udder and central nervous system. Indurative mastitis is common in both host species, and its economic significance may be underestimated. Laboured breathing associated with emaciation caused by progressive pneumonitis is the predominant feature in clinically affected sheep, whereas polyarthritis is the main clinical sign in goats. However, most lentivirus-infected sheep and goats are largely asymptomatic, but remain persistent carriers of virus and are capable of transmitting infection via colostrum or milk and respiratory secretions. The most practical and reliable approach to confirming a diagnosis of MV or CAE is a combination of serology and clinical evaluation. Although serology represents the most cost-effective method of diagnosing infection in persistently infected, clinically normal animals, it should be understood that testing errors can occur. The frequency of error depends on several factors including but not limited to: 1) the assay format, 2) the homology between the strain of virus used in the assay and the strains of virus present in the tested populations, and 3) the viral antigen used in the assay.*

Identification of the agent: *Virus isolation can be attempted from live clinical or subclinical cases by co-cultivating peripheral blood or milk leukocytes with appropriate ovine or caprine cell cultures, such as choroid plexus* (*MVV*) *or synovial membrane* (*CAEV*) *cells. Virus isolation is very specific but has variable sensitivities. Following necropsy, virus isolation is most readily accomplished by establishing explant cultures of affected tissues, e.g. lung, choroid plexus, synovial membrane or udder. Also, alveolar macrophages may be obtained from the lung at post-mortem and co-cultivated with susceptible cells. The cytopathic effects are characteristic, consisting of the appearance of refractile stellate cells and syncytia. The presence of MVV or CAEV can be confirmed by immunolabelling methods and electron microscopy.*

Nucleic acid recognition methods: *Many standard and a few quantitative polymerase chain reaction* (*PCR*) *assays for detecting MV and CAE provirus have been described and are used routinely in many laboratories for the rapid detection, quantitation, and identification of the small ruminant lentivirus strains. Cloning and/or sequencing of PCR products is the most direct method to confirm the specificity of PCR results.*

Serological tests: *Most infected sheep and goats possess detectable specific antibodies that can be assayed by a number of different serological tests. The two most commonly used are the agar gel immunodiffusion test and the enzyme-linked immunosorbent assay* (*ELISA*). *Western blot analysis and radio-immunoprecipitation are also performed, but only in specialised laboratories. A milk antibody assay may be appropriate in dairy goat herds. The time required for seroconversion*

following infection can be relatively prolonged and unpredictable, being measured in months rather than in weeks. However, after seroconversion, the antibody response usually persists and antibody-positive sheep and goats are regarded as virus carriers.

Requirements for vaccines: *There are no vaccines* available.

A. INTRODUCTION

Caprine arthritis/encephalitis (CAE) of goats and maedi-visna (MV) of sheep are persistent virus infections caused by closely related lentiviruses (Minguijón *et al.,* 2015; Peterhans *et al*., 2004). Maedi-visna is also known as ovine progressive pneumonia (OPP). Sheep can be experimentally infected with CAE and goats can be experimentally infected with MV. In addition, phylogenetic analyses comparing nucleotide sequences of MV virus (MVV) and CAE virus (CAEV) show clear indications of the existence and epidemiological importance of cross-species transmission between sheep and goats without demonstrating clearly that one virus has emerged from the other (Shah *et al*., 2004a; 2004b). MV and CAE are characterised by lifelong persistence of the causal agent in host monocytes and macrophages, and a variable length of time between infection and induction of a serologically detectable antiviral antibody response. Most infected sheep and goats do not exhibit clinical disease, but remain persistently infected and are capable of transmitting virus (Adams *et al*., 1983; Crawford *et al*., 1980).

Maedi-visna is an Icelandic name that describes two of the clinical syndromes recognised in MV virus (MVV)-infected sheep. 'Maedi' means 'laboured breathing' and describes the disease associated with a progressive interstitial pneumonitis, and 'visna' means 'shrinkage' or 'wasting', the signs associated with a paralysing meningoencephalitis. Whereas progressive lung disease is the primary finding with MVV infection, chronic polyarthritis, with synovitis and bursitis is the primary clinical outcome of CAEV infection. Encephalitis occurs primarily in kids aged between 2 and 6 months following CAEV infection, but careful differential diagnoses need to be conducted to rule out other syndromes or infections in kids. Indurative mastitis occurs in both syndromes. The lungs of sheep affected by MV do not collapse when removed from the thorax and often retain the impression of the ribs. The lungs and lymph nodes increase in weight (up to 2–3 times the normal weight). The lesions are uniformly distributed throughout the lungs, which are uniformly discoloured or mottled grey-brown in colour and of a firm texture. Diagnosis of CAEV and MVV induced respiratory disease was reviewed by Chakraborty *et al.* (2014). Udders affected by MV are diffusely indurated and associated lymph nodes may be enlarged.

When MV or CAE is the suspected cause of clinical disease, confirmation of the diagnosis can be achieved by a combination of clinical evaluation, detection and identification of the viruses, or by serology and, when necessary, histological examination of appropriate tissues collected at necropsy. Important tissues to examine include lung for progressive interstitial pneumonitis, brain and spinal cord for meningoencephalitis, udder for indurative mastitis, affected joints and synovium for arthritis, and kidney for vasculitis (Crawford & Adams, 1981). The nature of the inflammatory reaction in each site is similar, consisting of an interstitial, mononuclear cell reaction, sometimes with large aggregates of lymphoid cells and follicle formation.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of caprine arthritis/encephalitis and maedi-visna and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Virus isolation	–	–	–	+	–	–
Antigen detection	–	–	–	+	–	–
PCR	+	+	++	++	++	-

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
AGID	+	+++	++	+++	+++	+
CFT	–	–	–	–	–	–
ELISA	+++	+++	+++	+	+++	+
VN	–	–	–	–	–	+++
IFAT	–	+	–	–	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; CFT = complement fixation test;
ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation; IFAT = indirect fluorescent antibody test.

1. Identification of the agent

Isolation and characterisation of MVV or CAEV would not normally be attempted for routine diagnostic purposes. Due to the persistent nature of these infections, the establishment of a positive antibody status is sufficient for the identification of virus carriers. However, due to a late seroconversion after infection, negative serology may occur in recently infected animals.

There are two approaches to the isolation of MVV and CAEV: one for use with the live animal, and the second for use with necropsy tissues.

1.1. Isolation from the live animal

1.1.1. Maedi-visna virus

The MV provirus DNA is carried in circulating monocytes and tissue macrophages. Virus isolation from the live animal therefore requires the establishment of leukocyte preparations, with aseptic precautions, from peripheral blood or milk during lactation, culturing them together with indicator cells. Sheep choroid plexus (SCP) cells are commonly used for this purpose. These indicator cells can be prepared as primary explant cultures from fetal or new-born virus-free lambs, and their number can be multiplied over three to four passages for storage in liquid nitrogen. The recovered SCP cells are suitable for co-cultivation for up to 10 or 15 passages. Although the cells continue to grow well thereafter, their susceptibility to MVV may become reduced.

Leukocyte preparations can be made from peripheral blood as buffy coats by the centrifugation at 1000 ***g*** of heparinised, ethylenediamine tetra-acetic acid (EDTA) or citrated samples for 15 minutes. The cells are aspirated, suspended in Hanks' balanced salt solution (HBSS), and further purified by centrifugation at 400 ***g*** on to a suitable cushion of density medium for 40 minutes. The interface cells are spin-washed once or twice in HBSS at 100 ***g*** for 10 minutes, and the final cell pellet is resuspended in medium to a concentration of approximately 10^6 cells/ml; cells are generally cultured for 10–12 days in Teflon bags and are then added to a washed monolayer of slightly subconfluent SCP cells in a flask with an area of 25 cm^2.

Leukocytes can be similarly deposited from milk by centrifugation, when they are spin-washed, resuspended and finally added to SCP monolayer cultures.

These cultures are maintained at 37°C in a 5% CO_2 atmosphere, changing the medium and passaging as necessary. They are examined for evidence of a cytopathic effect (CPE), which is characterised by the appearance of refractile stellate cells with dendritic processes accompanied by the formation of syncytia. The cultures should be maintained for several weeks before being discarded as uninfected. Once a CPE is suspected, cover-slip cultures should be prepared. These are fixed, and evidence of viral antigen is sought by immunolabelling, usually by means of indirect fluorescent antibody or by the use of indirect immunoperoxidase methods. In addition, the cells of any suspect monolayers are deposited by centrifugation, and preparations are made for the identification of any characteristic lentivirus particles by transmission electron microscopy. Reverse transcriptase in the supernatant of the cell culture is indicative of the presence of retroviruses.

1.1.2. Caprine arthritis/encephalitis virus

The same principles that apply to the isolation of MVV also apply to the isolation of CAEV. CAEV was originally isolated by explantation of synovial membrane from an arthritic goat (Crawford & Adams, 1981). With live CAEV-infected goats, peripheral blood, milk, and possibly joint fluid aspirate represent the most suitable specimens from which leukocyte preparations can be established. Goat synovial membrane (GSM) cells are suitable indicator cells. If a CPE is suspected, tests for detection of viral antigen should be carried out, as described above.

1.2. Isolation from necropsy tissues

1.2.1. Caprine arthritis/encephalitis virus and maedi-visna virus

Samples of suspect tissues, collected as fresh as possible, such as lung, synovial membranes, udder, etc., are collected aseptically into sterile HBSS or cell culture medium and minced finely in a Petri dish using scalpel blades. Individual fragments are collected by Pasteur pipette and transferred to flasks of 25 cm^2, approximately 20–30 fragments per flask, and a drop of growth medium is placed carefully on each. The flasks are then incubated at 37°C in a humid 5% CO_2 atmosphere, and left undisturbed for a few days to allow the individual explants to adhere to the plastic. Fresh medium can be added with care, after which rafts of cells will gradually grow out from the fragments. When there is sufficient cell out-growth, the cultures are trypsin dispersed to allow the development of cell monolayers. These can be examined for CPE, and any suspected virus growth is confirmed in the same way as for the co-cultivations.

Adherent macrophage cultures are easy to establish from lung-rinse material (post-mortem broncho-alveolar lavage) and can be tested for virus production by serology, electron microscopy, or reverse transcriptase assay within 1–2 weeks. Virus isolations can be done by co-cultivation of macrophages and SCP or GSM cells as described for leukocytes above.

1.3. Nucleic acid recognition methods

Nucleic acid recognition methods may be used for the detection, quantitation, and identification of MV and CAE proviral DNA using the standard polymerase chain reaction (PCR) followed by Southern blotting, *in situ* hybridisation, or cloning and/or sequencing of the PCR product (Alvarez *et al*., 2006; Herrmann-Hoesing *et al*., 2007; Johnson *et al*., 1992). Standard PCR techniques for the detection of MV and CAE proviral DNA in cells and tissues are routinely used in many laboratories and are generally used as supplemental tests for determining infection status of those animals that cannot be definitively diagnosed by serology (Deandres *et al*., 2005). Real-time or quantitative PCR techniques are used in some laboratories and these tests, in addition to determining infection status, also quantify the amount of MV or CAE provirus in an animal (Alvarez *et al*., 2006; De Regge & Cay, 2013; Herrmann-Hoesing *et al*., 2007). Furthermore, molecular techniques such as PCR, cloning and sequencing also provide knowledge on a country's or region's specific MV and CAE strains, which may influence which serological assay and corresponding MV or CAE antigen to use. Phylogenetic analyses of MV and CAE proviral DNAs from SRLV strains throughout the world have suggested that in some areas, MV may have naturally infected goats, and CAE may have naturally infected sheep (Shah *et al*., 2004a; 2004b). Recently, loop-mediated isothermal amplification (LAMP) was applied to the detection of CAE provirus (Balbin *et al.,* 2014). LAMP uses 4–6 primers that amplify 6–8 regions of the target gene (Notomi *et al.,* 2000). In the future, molecular diagnostic tests along with phylogenetic analyses of MV and CAE provirus may be used to track transmission.

An important issue in the use of PCR is specificity. Because of the possibility of amplifying unrelated sequences from the host's genomic DNA (false positives), the amplified product should be checked by either hybridisation, restriction endonuclease digestion patterns, or sequencing. Sequencing provides the best proof of specificity in the validation of PCR-based tests and is recommended by the WOAH. Sensitivity of PCR-based tests can be improved by the use of nested PCR, but specificity of the nested PCR test should be checked using hybridisation, restriction endonuclease digestion patterns, or sequencing methods.

2. Serological tests

Ovine and caprine lentivirus infections are frequently persistent, so antibody detection is a valuable serological tool for identifying virus carriers. The close antigenic relationship between MVV and CAEV does not extend to detection of heterologous antibody in some serological assays (Knowles *et al*., 1994).

The assays most commonly used to serologically diagnose the presence of a small ruminant lentivirus infection are agar gel immunodiffusion (AGID) and the enzyme-linked immunosorbent assay (ELISA). AGID was first developed and reported in 1973 (Terpstra & De Boer, 1973), and the ELISA was first developed and reported in 1982 (Houwers *et al*., 1982). The AGID is specific, reproducible and simple to perform, but experience is required for reading the results. The ELISA is economical, quantitative and can be automated, thus making it useful for screening large numbers of sera. The sensitivity and specificity of both the AGID assay and ELISA depend upon the virus strain used in the assay, viral antigen preparation, and the standard of comparison assay. Western blot analysis and/or radio-immunoprecipitation are the standards of comparison used to access sensitivity and specificity of new AGID tests and ELISAs.

2.1. Agar gel immunodiffusion

There are two MV and CAE viral antigens of major importance in routine serology, a viral surface envelope glycoprotein commonly referred to as SU or gp135, and a nucleocapsid protein referred to as CA or p28. These are both conserved in an antigen preparation consisting of medium harvested from infected cell cultures and concentrated approximately 50-fold by dialysis against polyethylene glycol. As an example the WLC-1 strain of MV virus is commonly used in the AGID assay in the United States (Cutlip *et al*., 1977)[1] and a Canadian MV field strain is used for AGID tests in Canada (Simard & Briscoe, 1990b).

It is important to recognise that the sensitivity of the AGID test for detecting anti-CAEV antibody is dependent on both the virus strain and the viral antigen used (Adams & Gorham, 1986; Knowles *et al*., 1994). It was demonstrated that an AGID test with CAEV gp135 afforded greater sensitivity than an AGID test with CAEV p28 (Adams & Gorham, 1986). Also, it was shown that when compared with radio-immunoprecipitation, the sensitivity of the AGID test for anti-CAEV antibody was 35% greater using CAE virus antigen over using MV virus antigen (Knowles *et al*., 1994). The most likely explanation for this difference in sensitivity between the CAE and MV virus antigen for the detection of anti-CAEV antibody is that although the radio-immunoprecipitation assay requires only the binding of a single epitope by antibody to obtain a positive result, precipitation in an agar gel requires multiple epitope–antibody interactions. Although the MV and CAE viruses have 73–74.4% nucleotide sequence identity in the envelope gene, this amount of identity may not be sufficient to produce sufficient antibody to CAE and MV mutually common epitopes resulting in undetectable antibody/antigen precipitin lines using MV virus antigen. When the appropriate antigen is used, the AGID test performance is high. When compared with immunoprecipitation, the AGID for the detection of anti-CAEV antibody, if CAEV antigen was used, had 92% sensitivity and 100% specificity (Knowles *et al*., 1994). In addition, the AGID for detection of anti-MVV antibody, if MVV antigen was used, had 99.3 and 99.4% sensitivity and specificity, respectively.

In adult persistently MVV-infected sheep and CAEV-infected goats, the predominant immunoprecipitating antibody response is directed against gp135 antigen. An anti-p28 response is usually present at a lower titre than the anti-gp135 response in persistently infected adult small

1 This virus has been distributed by Dr Howard Lehmkuhl, National Animal Disease Center, United States Department of Agriculture, P.O. Box 70, Ames, Iowa, USA.

ruminants using immunoprecipitation. In some CAEV-infected goats there is evidence to suggest that an anti-gp135 antibody response is produced, in the absence of an anti-p28 response and vice versa, in a proportion of individuals (Rimstad *et al*., 1994). Hence, for validation of a test, standard sera producing both anti-gp135 and anti-p28 precipitin lines are required.

The gel medium is 0.7–1% agarose in 0.05 M Tris buffer, pH 7.2, with 8.0% NaCl. The test is conveniently performed in plastic Petri dishes, or in 10 cm^2 plastic trays. The pattern and size of the wells will determine the number of sera tested per plate. Various well patterns can be adopted, but a hexagonal arrangement with a central well is usual: for example, a pattern with alternating large (5 mm in diameter) and small (3 mm in diameter) peripheral wells, 2 mm apart and 2 mm from a central antigen well that is 3 mm in diameter. The large peripheral wells are used for test sera and the small ones for standard sera. A weak positive control must be included in each test. The plates are incubated overnight at 20–25°C in a humid chamber, and then examined for precipitin lines. Plates may be incubated at 2–8°C for another 24 hours to enhance the precipitin lines.

An important consideration is the need for experienced personnel to interpret the AGID. Interpretation of AGID results is dependent on the antigen used. Examples of AGIDs with different antigen preparations and a guide for interpretation of the results can be found in Adams *et al.,* 1983.

2.2. Enzyme-linked immunosorbent assay

Currently, there are over 30 different ELISAs reported for the detection of anti-MVV or anti-CAEV antibodies in the sera of sheep or goats, respectively (Deandres *et al*., 2005). Most of these ELISAs are indirect ELISAs (I-ELISA) although there are three reported competitive ELISAs (C-ELISA) using monoclonal antibodies (Herrmann *et al*., 2003; Houwers & Schaake, 1987). Half of I-ELISAs use purified whole virus preparations for antigen whereas the other half use recombinant protein and/or synthetic peptide antigens. A few of the I-ELISAs have shown high sensitivity and specificity against a standard of comparison, western blot analysis or radio-immunoprecipitation (Rosati *et al*., 1994; Saman *et al*., 1999). When compared with radio-immunoprecipitation, one C-ELISA has shown high sensitivity and specificity both in sheep and goats in the USA suggesting that this one test can be used for both MVV and CAEV US surveillance (Herrmann *et al*., 2003). ELISAs have been used for several years in some European countries in control and eradication schemes for MVV in sheep and CAEV in goats (Motha & Ralston, 1994; Pépin *et al*., 1998), AGID is useful to confirm positive ELISA results due to its high specificity.

Whole-virus antigen preparations are produced by differential centrifugation of supernatants from infected cell cultures and detergent treatment of purified virus, and are coated on microplates (Dawson *et al*., 1982; Simard & Briscoe, 1990a; Zanoni *et al*., 1994). Whole-virus preparations should contain both gp135 and p28. Recombinant antigens or synthetic peptides are usually produced from whole or partial segments of the gag or envelope genes and may be used in combination (Power *et al*., 1995; Rosati *et al*., 1994; Saman *et al*., 1999). Thus, recombinant gag or envelope gene products fused with glutathione S-transferase fusion protein antigen that have been produced in Escherichia coli provide a consistent source of antigen for international distribution and standardisation.

The ELISA technique is also applicable to colostrum or milk, and some studies have evaluated paired serum and milk samples. Because colostrum and milk are sources of CAEV transmission, the testing of milk samples for anti-CAEV or anti-MVV antibody would not provide timely information for the prevention of transmission, especially to offspring from the immediate gestation.

The ELISA is performed at room temperature (~25°C) and is easy to perform in laboratories that have the necessary equipment (microplate reader) and reagents. It is convenient for large-scale screening, as it is a reliable and quantitative technique for demonstrating small ruminant lentiviruses (SRLVs) antibodies in sheep and goats. It requires relatively pure antigen. One disadvantage of several ELISAs is that many have not been validated against a standard of comparison such as western blot analysis or radio-immunoprecipitation. The test method should be validated in accordance with Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* using a standard of comparison such as western blot analysis or radio-immunoprecipitation. To date, only one ELISA has met these testing standards (Zanoni *et al*., 1994).

For I-ELISA, wells of the microplate are coated with antigen. Diluted serum samples are added to the wells and react to antigens bound to the solid support. Unbound material is removed by washing after a suitable incubation period. Conjugate (e.g. horseradish-peroxidase-labelled anti-ruminant Ig) reacts with specific antibodies bound to the antigen. Unreacted conjugate is removed by washing after a suitable incubation period. Enzyme substrate is added. The rate of conversion of substrate is proportional to the amount of bound antibodies. The reaction is terminated after a suitable time and the amount of colour development is measured spectrophotometrically. A disadvantage of the I-ELISA is that test sera typically need to be diluted 1/50 or greater in order to lower the number of false positives.

Specific MAbs have been used in a C-ELISA for SRLVs to capture gp135 or p28 as antigen (Frevereiro *et al*., 1999; Herrmann *et al*., 2003; Houwers & Schaake, 1987; Ozyoruk *et al*., 2001). C-ELISA overcomes the problem of antigen purity, as the specificity of this test depends on the MAb epitope. For C-ELISA, sample sera containing anti-SRLV antibodies inhibit binding of enzyme–labelled MAb to SRLV antigen coated on the plastic wells. Binding of the enzyme-labelled MAb conjugate is detected by the addition of enzyme substrate and quantified by subsequent colour product development. Strong colour development indicates little or no blockage of enzyme-labelled MAb binding and therefore the absence of SRLV antibodies in sample sera. In contrast, weak colour development due to the inhibition of the enzyme-labelled MAb binding to the antigen on the solid phase indicates the presence of SRLV antibodies in sample sera. The format of the C-ELISA requires that serum antibodies must bind to or bind in close proximity to the specific MAb epitope.

2.2.1. Materials and reagents

Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with SRLV antigen; microplate reader (equipped with 405, 450, 490 and 620 nm filters); 37°C humidified incubator; 1-, 8- and 12-channel pipettes with disposable plastic tips; microplate shaker (optional); fridge; freezer.

Positive and negative control sera; conjugate (e.g. ruminant anti-immunoglobulin labelled with peroxidase); tenfold concentration of diluent (e.g. phosphate buffered saline/Tween); distilled water; 10× wash solution; substrate or chromogen (e.g. ABTS [2,2'-azino-bis-(3-ethylbenzo-thiazoline-6-sulphonic acid)] or TMB [3,3',5,5'-tetramethylbenzidine]); stop solution (e.g. detergent, sulfuric acid).

2.2.2. Indirect ELISA: test procedure

i) Dilute the serum samples, including control sera, to the appropriate dilution (e.g. 1/20) and distribute 0.1–0.2 ml per well (in duplicate if biphasic ELISA). Control sera are positive and negative sera provided by the manufacturer and an internal positive reference serum from the laboratory in order to compare the titres between different tests.

ii) Cover the plate with a lid and incubate at room temperature or 37°C for 30–90 minutes. Empty the contents and wash three times in washing solution at room temperature.

iii) Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well). Cover each plate and incubate as in step ii. Wash again three times.

iv) Add 0.1 ml of freshly prepared or ready-to-use chromogen substrate solution to each well (e.g. ABTS in citrate phosphate buffer, pH 5.0, and 30% H_2O_2 solution [0.1 µl/ml]).

v) Shake the plate; after incubation, stop the reaction by adding stopping solution to each well (e.g. 0.1 ml sulphuric acid).

vi) Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 450–620 nm (TMB). The absorbance values will be used to calculate the results.

vii) Interpretation of the results

For commercial kits, interpretations and validation criteria are provided with the kit.

Interpretation criteria should be developed and validated for the individual procedures and reagents used in the laboratory. The following is given as an example:

Calculate the mean absorbance (Ab) of the sample serum and of the positive (Ab_{pos}) and negative (Ab_{neg}) control sera, and for each serum, calculate the percentage:

$$\frac{Ab - Ab_{neg}}{Ab_{pos} - Ab_{neg}} \times 100$$

if a test sample has mean absorbance of <30% it is classed as negative, 30-40% is classed as doubtful, and >40% as positive.

2.2.3. Competitive ELISA: test procedure

i) Add 0.05 ml of undiluted serum and positive/negative controls to antigen-coated plate.

ii) Incubate for 1 hour at room temperature.

iii) Empty the plate and wash the plate three with diluted wash solution.

iv) Add 0.05 ml of diluted antibody-peroxidase conjugate to each well. Mix well and incubate for 30 minutes at room temperature.

v) After the 30-minute incubation, empty the plate and repeat the washing procedure described in step iii.

vi) Add 0.05 ml of substrate solution (ex: TMB) to each well. Mix and cover plate with aluminium foil. Incubate for 20 minutes at room temperature. Do not empty wells.

vii) Add 0.05 ml of stop solution to each well. Mix. Do not empty wells.

viii) Immediately after adding the stop solution, the plate should be read on a plate reader (620, 630 or 650 nm).

ix) Interpretation of results

Interpretation criteria should be developed and validated for the individual procedures and reagents used in the laboratory. The following is given as an example:

Calculation: 100 – [(Sample Ab × 100)/(Mean negative control Ab)] = % inhibition.

For goats, if a test sample causes >33.2% inhibition, it is positive; if a test sample causes <33.2% inhibition, it is negative. For sheep, if a test sample causes >20.9% inhibition, it is positive; if a test sample causes <20.9% inhibition, it is negative.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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NB: At the time of publication (2017) there were no WOAH Reference Laboratories for caprine arthritis/encephalitis & maedi-visna (please consult the WOAH Web site for the current list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: CAPRINE ARTHRITIS/ENCEPHALITIS FIRST ADOPTED IN 1990; MAEDI-VISNA FIRST ADOPTED 1989.
MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 3.8.3.

CONTAGIOUS AGALACTIA

SUMMARY

Description and importance of the disease: *Contagious agalactia is a serious disease syndrome of sheep and goats that is characterised by mastitis, arthritis, keratoconjunctivitis and, occasionally, abortion.* Mycoplasma agalactiae (Ma) *is the main cause of the disease in sheep and goats, but* M. capricolum *subsp.* capricolum (Mcc), M. mycoides *subsp.* capri (Mmc) *and* M. putrefaciens *produce a clinically similar disease, more often in goats, which may be accompanied by pneumonia.* Ma *and* Mcc *have been isolated from wild small ruminants such as ibex and mountain goats. Antibodies to* Mmc *and* Mcc *have been detected in South American camelids (alpacas, llamas and vicunas), but no mycoplasmas have yet been isolated.*

Identification of the agent: *Definitive diagnosis requires the isolation of the causative mycoplasmas from the affected animals, which are then identified by biochemical, serological and, increasingly, molecular tests such as the polymerase chain reaction. Samples of choice include milk, conjunctival and ear swabs, and joint fluid. The sampling of bulk milk tank provides a convenient way of monitoring whole herds/flocks for causative mycoplasmas. All four mycoplasmas grow relatively well in most mycoplasma media although* M. agalactiae *shows a preference for organic acids such as pyruvate as substrates.*

Serological tests: *Detection of antibodies in serum by enzyme-linked immunosorbent assay (ELISA) provides rapid diagnosis of disease, but may not be very sensitive in chronically affected herds and flocks. Indirect ELISAs have been used routinely in control programmes for screening herds for* Ma*. Confirmation of infection by isolation and identification or detection by polymerase chain reaction is usually necessary in areas believed to be free of contagious agalactia. Serological tests are not widely available for* M. putrefaciens*.*

Requirements for vaccines: *Commercial vaccines for* Ma*, inactivated with formalin, are widely used in southern Europe, but are not considered to be very efficacious. Under experimental conditions,* Ma *vaccines inactivated with saponin have been shown to be more protective than formalised preparations. Live vaccines for* Ma *are used in Turkey, where they are reported to be more protective than inactivated vaccines. A commercial vaccine containing* Ma, Mmc *and* Mcc *is available. Autogenous vaccines for* Mmc *and, occasionally, for* Mcc *are believed to be used in some countries. No vaccines exist* for M. putrefaciens*, as the disease it causes is not considered to be sufficiently serious or widespread.*

A. INTRODUCTION

Contagious agalactia is a disease of sheep and goats that is characterised by mastitis, arthritis and keratoconjunctivitis, and has been known for nearly 200 years. It occurs wherever pastoralism and small ruminant dairy production is located in Europe, particularly in the Mediterranean regions, Asia, North Africa, and sporadically in the United States of America (USA). It is mainly caused by *Mycoplasma agalactiae* (*Ma*) (reviewed by Bergonier *et al*., 1997). An upsurge of contagious agalactia, caused by *Ma*, has been seen in sheep in Spain and France recently with increased cases reported in and around the Pyrenees and new outbreaks being reported in Corsica (Chazel *et al*., 2010). Italy still reports more than 50 outbreaks every year, mainly notified on two of its Islands[1]. Frequent and numerous outbreaks occur in Iran and Mongolia (Nicholas *et al*., 2008). *Mycoplasma*

1 Data from WOAH World Animal Health Information System: https://wahis.woah.org/#/home

capricolum subsp. *capricolum* (*Mcc*) and *M. mycoides* subsp. *capri* (formerly *M. mycoides* subsp. *mycoides* LC [LC = large colonies]) have also been isolated from sheep and goats with mastitis and arthritis in many countries including Italy, where the disease is spreading (Marogna *et al*., 2015), South America (Nascimento *et al.,* 1986) and Australasia (Cottew, 1971).

The clinical signs of infections caused by *Mcc, Mmc* and *M. putrefaciens* (*Mp*) are sufficiently similar to be considered indistinguishable from contagious agalactia caused by *Ma*. In addition, *Mp* also causes mastitis and arthritis in goats, which is very similar to that caused by *Ma*, *Mmc* and *Mcc* (Rodriguez *et al*., 1994). Furthermore, the consensus of the working group on contagious agalactia of the EC COST[2] Action 826 on ruminant mycoplasmoses, which met in Toulouse, France, in 1999, was that all four mycoplasmas should be considered as causal agents of contagious agalactia. In France, *Mmc*, *Mcc* and *Mp* constitute over 80% of the mycoplasma isolations from goats with *Ma* accounting for less than 2%. *Ma* and *Mcc* have been isolated from wild small ruminants such as ibex and mountain goats in the Pyrenees and Alps (Chazel *et al*., 2010; Verbsick *et al*., 2008). There are occasional reports of the isolation of *Ma* (Chazel *et al.,* 2010) and *Mcc* from apparently healthy cattle (Pinho *et al.,* 2009), although Catania *et al.* (2016) isolated *Ma* from eyes, ears and brain spinal fluid of bulls.

Clinically, the disease caused by *Ma* is recognised by elevated temperature, inappetence and alteration in the consistency of the milk in lactating ewes with decline and subsequent failure of milk production, often within 2–3 days, as a result of interstitial mastitis (Bergonier *et al*., 1997); lameness and keratoconjunctivitis affects about 5–10% of infected animals. Fever is common in acute cases and may be accompanied by nervous signs, but these are rare in the more frequently observed subacute and chronic infections where the disease is endemic. Pregnant animals may abort. *Ma* may occasionally be found in lung lesions (Loria *et al*., 1999), but pneumonia is not a consistent finding. Bacteraemia is common, particularly for *Mmc* and *Mcc* and could account for the isolation of the organism from sites where it is only present transiently.

Arthritis, pleurisy, pneumonia, mastitis and keratoconjunctivitis may all result from infection with *Mmc,* which has one of the widest geographical distribution of ruminant mycoplasmas, being found on all continents where small ruminants are kept and wherever contagious agalactia and caprine pleuropneumonia are reported (DaMassa *et al*., 1983; Nicholas, 2002); however the lack of diagnostic facilities for mycoplasma diseases in many countries means that it is probably under reported. *Mmc* is mostly confined to goats but has occasionally been isolated from sheep with reproductive disease and cattle with arthritis or respiratory disease. Cases usually occur sporadically and the disease may persist and spread slowly within a herd; nevertheless, in free areas, the disease shows high morbidity and mortality of kids. In Sicily a reported outbreak showed more than 40% mortality (Agnello *et al*., 2012). After parturition, the opportunity for spread in milking animals increases, and kids ingesting infected colostrum and milk become infected. The resulting septicaemia, with arthritis and pneumonia, causes high mortality in kids (Bergonier *et al*., 1997; DaMassa *et al*., 1983).

Mcc is widely distributed and highly pathogenic, particularly in North Africa, but the frequency of occurrence is low (Bergonier *et al*., 1997). Goats are more commonly affected than sheep, and clinical signs of fever, septicaemia, mastitis, and severe arthritis may be followed rapidly by death (Bergonier *et al*., 1997; Bolske *et al*., 1988). Pneumonia may be seen at necropsy. The severe joint lesions seen in experimental infections with this organism are accompanied by intense periarticular subcutaneous oedema affecting tissues some distance from the joint (Bolske *et al*., 1988). In 2015 the first human isolate of *Mcc* was reported from a man with septicaemia (Seersholm *et al*., 2015).

Mp is common in milking goat herds in western France where it can be isolated from animals with and without clinical signs (Mercier *et al*., 2001). It has also been associated with a large outbreak of mastitis and agalactia leading to severe arthritis in goats accompanied by abortion and death without pyrexia in California, USA (Bergonier *et al*., 1997). *Mp* was the major finding in an outbreak of polyarthritis in kids in Spain (Rodriguez *et al*., 1994).

Antibodies to *Mmc* and *Mcc*, but not *Ma*, have been detected in South American camelids, including llamas, alpacas and vicunas, but as yet no mycoplasmas have been isolated (Nicholas, 1998). These camelids are affected by a range of mycoplasma-like diseases, including polyarthritis and pneumonia, so it is likely that mycoplasmas including *Mmc* and *Mcc* may be found in the future.

2 European Cooperation in the field of Scientific and Technical Research.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of contagious agalactia and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Culture and identification of the organism	++	+++	+++	+++	–	–
Conventional PCR	++	+++	–	+++	++	–
Real-time PCR	++	+++	–	+++	++	–
Detection of immune response						
CFT	+++*	+	+++	+++*	+++*	+++*
ELISA	+++**	+	+++	+++**	+++**	+++**
Immunoblotting	+	++	+	++	+	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
*for *Mcc* and *Mmc* only; **for *Ma* only.
PCR = polymerase chain reaction; CFT = complement fixation test; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agents

1.1. Selection of samples

Preferred samples from living animals include: milk from mastitic females or from apparently healthy females; nasal swabs and secretions; where there is a high rate of mortality/morbidity in kids; joint fluid from arthritic cases; conjunctival swabs from cases of ocular disease; and blood for antibody detection from affected and non-affected animals (Nicholas & Baker, 1998). The sampling of bulk milk tank provides a convenient way of monitoring flocks and herds for causative mycoplasmas. A distinctive smell of putrefaction in the milk is often the first sign of the presence of *Mp* infection in a herd. The ear canal has also been shown to be a source of pathogenic mycoplasmas, although in practice the presence of non-pathogenic mycoplasmas at this site may make confirmation difficult (Nicholas & Baker, 1998). Mycoplasmas may be isolated from the blood during the acute stage of the disease when there is mycoplasmaemia. From dead animals, samples should include: udder and associated lymph nodes, joint fluid, lung tissue (at the interface between diseased and healthy tissue) and pleural/pericardial fluid. Samples should be dispatched quickly to a diagnostic laboratory in a moist and cool condition. All four causative mycoplasmas are relatively easy to isolate from internal organs, joints and milk and grow well in most mycoplasma media, producing medium to large colonies in 3–4 days.

1.2. *Mycoplasma* isolation

The usual techniques used in the isolation of mycoplasmas apply to all four causative organisms (Nicholas & Baker, 1998). Many media have been reported to grow the causative mycoplasmas. Improved growth rates of *Ma* have been seen in media containing organic acids such as pyruvate and isopropanol (Khan *et al*., 2004). The formulation of PRM medium (Khan *et al*., 2004) is as follows:

Heat-inactivated porcine serum 100 ml/litre, special peptone 20 g/litre, yeast extract 5 g/litre, glycerol 5 g/litre, sodium chloride 5 g/litre, HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) 9 g/litre, fresh yeast extract 100 ml/litre, sodium pyruvate 5 g/litre, 12.5 ml of 0.2% phenol red and ampicillin (200,000 International Units/ml. Make up to 1 litre in distilled water and sterilise by filtration. Adjust the pH of the broth medium to 7.6. Prepare solid medium by adding 10 g of LabM agar No. 1 (Bury UK, or agar of equivalent quality) and dispense into sterile Petri dishes.

Thallium acetate (250 mg/litre), which is toxic and inhibitory to some mycoplasmas but not those causing contagious agalactia, may be a necessary component of the transport medium to reduce bacterial contamination from clinical samples, but should be omitted once the mycoplasmas begin to grow *in vitro*. A satisfactory alternative to thallium acetate may be colistine sulphate (37.5 mg/litre).

1.2.1. Test procedure

i) Make tenfold dilutions (10^1–10^6) of the liquid sample (milk, synovial fluid, conjunctival and ear swabs) or tissue homogenate in appropriate broth medium.

ii) Spread a few drops of each sample on the agar medium and dispense a 10% (v/v) inoculum into broth medium.

iii) Streak swabs directly on to agar medium.

iv) Incubate inoculated broths (optimally with gentle shaking) and agar media at 37°C in humidified atmosphere with 5% carbon dioxide.

v) Examine broths daily for signs of growth (indicated by a fine cloudiness or opalescence) or changes in pH indicated by a colour change and examine agar media under ×35 magnification for typical 'fried egg' colonies.

vi) If no mycoplasma growth is seen after 7 days, subculture a 10% (v/v) inoculum of broth into fresh broth and spread about 50 µl of this on to agar media.

vii) Repeat as for step v. If no mycoplasmas are seen after 21 days' incubation, consider the results to be negative.

viii) If bacterial contamination results (seen as excessive turbidity), filter sterilise by passing 1 ml of contaminated broth through a 0.45 µm filter into fresh broth medium.

Clinical samples frequently contain more than one mycoplasma species so clone purification of colonies is often considered necessary before performing biochemical and serological identification, in particular the growth and film inhibition tests (GIT and FIT, respectively). However, cloning is a lengthy procedure taking at least 2 weeks. The immunofluorescence test (Bradbury, 1998), dot immunobinding tests (Poumarat, 1998) and, more recently, polymerase chain reaction (PCR) tests (see Section B.1.5) do not require cloning as these tests can detect the pathogenic mycoplasmas in mixed cultures, saving a great deal of time.

1.3. Biochemical tests

The first test that should be performed on the cloned isolates is sensitivity to digitonin, which separates mycoplasmas from acholeplasmas; the latter are ubiquitous contaminants that can overgrow the mycoplasmas of interest. Growth in liquid medium containing glucose (1%), arginine (0.2%), and phenolphthalein diphosphate (0.01%), on solid medium containing horse serum or egg yolk for the demonstration of film and spots, and on casein agar or coagulated serum agar to test for proteolysis, are among the most useful tests for differentiating the four mycoplasmas (Poveda, 1998). These biochemical characteristics, however have been increasingly found to be variable for the individual mycoplasmas and have little diagnostic value. The most impressive biochemical characteristic that differentiates *Mp* from all other mycoplasmas is the odour of putrefaction it produces in broth culture. Other features that may be helpful include: film and spot production seen on the surface of the broth and solid media caused by *Ma* and to a lesser extent by *Mp*; and the proteolytic activity of *Mcc* and *Mmm*LC on casein and coagulated serum.

1.4. Serological identification

Identification of isolates using specific antisera is usually carried out with the GIT, FIT (Poveda & Nicholas, 1998) or the indirect fluorescent antibody (IFA) test (Bradbury, 1998). A dot immunobinding test that is carried out in microtitre plates offers many improvements over the other serological tests such as rapidity and higher throughputs (Poumarat, 1998) but requires subjective judgements of staining intensity. For *Ma*, film inhibition may often be more reliable as growth inhibition is not seen with all isolates; it can also be used for serodiagnosis. Film production by the mycoplasma may be enhanced by the incorporation of 10% egg yolk suspension into the solid medium.

1.4.1. Test procedure

i) Inoculate at least two dilutions of 48-hour cloned broth cultures (10^{-1} and 10^{-2}) on to predried agar media by allowing 50 µl of the cultures to run down the tilted plates using the 'running drop' technique (Poveda & Nicholas, 1998). Remove any excess liquid with a pipette.

ii) Allow the plates to dry. It is possible to apply two or three well separated running drops to each 90 mm diameter plate.

iii) Apply predried filter paper discs containing 30 µl of specific antiserum to the culture; ensure good separation of discs (at least 30 mm).

iv) Incubate the plates as for mycoplasma culture and examine daily by eye against a light background.

1.4.2. Interpretation of the results

A zone of inhibition over 2 mm, measured from the paper disc to the edge of mycoplasma growth is considered to be significant. Partial inhibition can occur with weak antiserum or where there are mixed cultures. Stronger reactions can be obtained if about 60 µl of antisera is added to 6 mm diameter wells made in the agar with a cork borer or similar device (Poveda & Nicholas, 1998).

In the IFA test, specific antisera are applied to colonies on solid medium. Homologous antiserum remains attached after washing and is demonstrated by adding fluorescein-conjugated antiglobulin, washing, and viewing the colonies with an epifluorescence microscope (Bradbury, 1998). Controls should include known positive and known negative control organisms, and a negative control serum. However like the immunobinding tests subjective judgements are required to assess staining intensity.

Antisera for these serological tests have traditionally been prepared against the type strains of the various *Mycoplasma* species, and most field isolates have been readily identified using these antisera. As more strains have been examined, however, some have been found to react poorly with these antisera, while reacting well with antisera to other representative strains of the species. Intraspecies variation in antigenic composition has not been reported for *Mp,* but occurs to some degree with *Ma* and with *Mcc* strains. Thus, diagnostic laboratories may need to have several antisera to enable all strains of the species to be identified.

1.5. Nucleic acid recognition methods

1.5.1. Polymerase chain reaction assays

PCR assays are routinely used in many laboratories and are extremely sensitive. They can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when results are positive. However negative results should not be considered definitive, as culture of bulk tank milk samples may be more sensitive (Tatay-Dualde *et al*., 2015). Several PCRs specific for *Ma* have been developed and show similar levels of sensitivity, although they are based on different gene sequences (Dedieu *et al*., 1995; Subrahamaniam *et al*., 1998; Tola *et al*., 1997a). They can be used directly on nasal, conjunctival, synovial and tissue samples; they have been used on milk samples where they have been reported to be more sensitive than culture (Tola *et al*., 1997a), although occasionally undefined inhibitors may interfere with the test. PCRs can also be used, more reliably, on mycoplasmas growing in culture; a 24 hour enrichment of the mycoplasma in the appropriate medium greatly

facilitates PCR detection even in the presence of bacterial contamination (Nicholas, 2002). The use of immunomagnetic capture-PCR methods may be more rapid than culture enrichment for detecting *Ma* from milk samples (Sanna *et al*., 2014). A PCR based method that uses denaturing gradient gel electrophoresis (DGGE) and mycoplasma-specific primers is capable of identifying the majority of small ruminant mycoplasmas including all the causative agents of contagious agalactia by their migration pattern (McAuliffe *et al*., 2005). Isothermal PCR methods may be applicable; and has been described for detecting *Ma* (Rekha *et al*., 2015).

A positive PCR result, particularly in an area previously free of contagious agalactia, should be confirmed by isolation and identification of the mycoplasma using standard procedures.

Individual PCRs have been reported for *Mmc* (Bashiruddin, 1998) and *Mcc* (Monnerat *et al.,* 1999) and *Mp* (Nicholas *et al.,* 2008; Peyraud *et al*., 2003) respectively. In addition a multiplex test has been described that can detect simultaneously *Ma*, *Mcc* and *Mmc* (Greco *et al*., 2001). Cillara *et al.* (2015) describe a PCR and restriction fragment length polymorphism PCR method based on the *lpd*A gene to discriminate between *Mmc* and *Mcc*.

1.5.2. Real-time PCRs

Several rapid real-time PCRs have been reported for *Ma* that provide advantages of speed, sensitivity and sample handling (Lorusso *et al*., 2007). More recently a multiplex real time test has been described which detects all four mycoplasmas simultaneously (Becker *et al*., 2012).

1.5.3. Micro-array analysis

Micro-array analysis has been applied to the detection of mycoplasmas. Using probes derived from the 23 rRNA genes and *tuf* gene target regions, Schnee *et al.* (2012) have described the identification of 37 mycoplasma species including all four contagious agalactia pathogens. At the time of publication some cross reaction was seen between *Ma* and the closely related bovine pathogen, *M. bovis*. The advantages of the test over PCR include ease of operation, high information content and cost-effectiveness.

1.5.4. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF)

Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF) has been used to identify bacteria (Randall *et al*., 2015), including *Mycoplasma* species and the causative organisms of contagious agalactia (Pèreyre *et al*., 2013). The organisms need to be cultured first, and sometimes only the predominant species is identified in mixed cultures.

1.5.5. Test procedure

The PCR detailed for *Mycoplasma agalactiae* has been evaluated across several laboratories (Bashiruddin *et al.,* 2005) whereas other molecular methods referred to in this chapter for *M. agalactiae* and other contagious agalactia-causing species have not been evaluated to the same extent and are therefore not detailed. The following primers based on the uvrC gene have been shown to be specific for *Ma* (Subrahamaniam *et al*., 1998). PCRs may need to be optimised in each laboratory. Positive and negative control DNA should be run in each assay.

MAGAUVRC1-L	CTC-AAA-AAT-ACA-TCA-ACA-AGC
MAGAUVRC1-R	CTT-CAA-CTG-ATG-CAT-CAT-AA

i) Extract DNA from *Mycoplasma* isolates or clinical material using the appropriate method (Bolske *et al*., 1988).

ii) Carry out PCR methods in 50 µl reaction mixtures containing: 1 µl of sample DNA, 20 pmol of each primer (see above), 1 mM each dNTP, 10 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 1.25 mM U *Taq* DNA polymerase.

iii) Subject the mixture to 35 amplification cycles in a thermal cycler with the following parameters: 30 seconds at 94°C, 30 seconds at 50°C annealing temperature and 1 minute at 72°C.

iv) Analyse the PCR products by electrophoresis on a 0.7% agarose at 110 V for 2 hours and visualise by staining with ethidium bromide. A 1.7 kb fragment is indicative of the presence of *Ma.*

2. Serological tests

2.1. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) using sonicated or Tween-20-treated antigens have been reported to be more sensitive than the CFT for the detection of antibody to *Ma* in serum (Bergonier *et al*., 1997). Problems of nonspecificity have been overcome by the use of monoclonal or protein G conjugates in the ELISA (Lambert *et al*., 1998). The use of these conjugates enables the testing of sera from a wide range of mammalian species, including camelids.

Two commercial ELISA kits for *Ma* are available, one using a fusion protein (sensitivity 54% and specificity 100%) and the other using whole cells as target antigens (sensitivity 84% and specificity 96% in sheep or 90% in goats) (Poumarat *et al.,* 2012). There also appeared to be differences in the ability of the tests to detect the responses to different strains equally. The choice of test depends on the objectives of the proposed study, i.e. a less sensitive test would be sufficient for prevalence study where the disease was endemic while a more sensitive test would be required for disease detection in a disease-free region.

ELISAs are not widely available for the other three causative mycoplasmas.

2.2. Complement fixation

A standard complement fixation test (CFT) for *Ma* has also been applied to other mycoplasmas involved in the contagious agalactia syndrome (Bergonier *et al*., 1997). Antigens are prepared from washed organisms, standardised by opacity, and lysed, either ultrasonically or by using sodium lauryl sulphate followed by dialysis. Sera are inactivated at 60°C for 1 hour, and the test is carried out in microtitre plates with overnight fixation in the cold or at 37°C for 3 hours. The haemolytic system is added, and the test is read after complete lysis is shown by the antigen control. A positive result is complete fixation at a serum dilution of 1/40 or greater for the following mycoplasmas: *Ma, Mcc*, and *Mmc.* The CFT is regarded as a herd test and at least ten sera are tested from each herd, preferably from acute and convalescent cases.

Some sera from healthy flocks react in the CFT using *Ma* up to a serum dilution of 1/20, but rarely react with the other two antigens. However, in flocks infected with *Ma*, sera giving a homologous reaction at 1/80 may cross-react at up to 1/40, the positive threshold, with the other two antigens. It is often difficult to perform the CFT if the quality of the test sera is poor; where possible, the ELISA is preferred.

2.3. Immunoblotting test

Immunoblotting tests have also been described for *Ma* and are considered as confirmatory tests for outbreaks in Italy (Nicholas, 1998; Tola *et al*., 1997b). Strong bands at approximately 80 and 55 kDa were seen with sera with antibodies to *Ma,* while sera from healthy flocks show no bands or very faint bands of different sizes. Diluting the sera to 1/50 improves the discrimination between positive and negative sera (Nicholas, 1998). On the other hand, Poumarat *et al.* (2012), using a 1/5 dilution of serum, considered a serum positive for *Ma* from a flock/herd in France if it contained 4 bands: 80, 48, 40 and 30 kDa suggesting there may be some geographic differences in humoral responses.

C. REQUIREMENTS FOR VACCINES

Vaccines for the prevention of contagious agalactia due to *Ma* are used widely in the Mediterranean countries of Europe and in western Asia. No single vaccine has been universally adopted, and no standard methods of preparation and evaluation have been applied.

1. Vaccines for *Mycoplasma agalactiae* infection

1.1. Inactivated vaccines for *Mycoplasma agalactiae* infection

In Europe where live vaccines for *Ma* are not acceptable, attention has focused on the use of killed organisms, mostly using formalin and an adjuvant such as aluminium hydroxide in an oil emulsion. The titres of the preparations, before inactivation, are very high (10^8–10^{10} colony-forming units per ml) and are derived from laboratory strains. Some products are available commercially including a trivalent preparation containing *Ma, Mcc* and *Mmc* but there are few data on their efficacy. A formalin-inactivated oil emulsion vaccine was shown to be immunogenic and protective in a small trial in lactating sheep and also prevented transmission of *Ma* (Greco *et al*., 2002). However, in a small trial, no potency was evident in a similar commercial product following contact challenge (Agnone *et al*., 2013).

It is possible that in some instances the apparent lack of protection given by vaccines could be the result of animals being infected with one of the other four mycoplasmas involved in the contagious agalactia syndrome (Gil *et al*., 1999). A multivalent formalin inactivated vaccine incorporating all four causative mycoplasmas and adjuvanted with saponin and aluminium hydroxide appears beneficial in preliminary trials (Ramirez *et al*., 2001).

More recently vaccines inactivated with phenol or with saponin have given superior protection against experimental infections compared with formalin, sodium hypochlorite or heat-inactivated vaccines (Tola *et al*., 1999).

1.2. Live attenuated vaccines for *Mycoplasma agalactiae* infection

Live attenuated vaccines against *Ma* have been used in Turkey for many years and have been reported to provide better protection in ewes and their lambs than inactivated vaccines (Nicholas, 2002). However they can produce a transient infection with shedding of mycoplasma. Live vaccines should not be used in lactating animals and should be part of a regional plan in which all flocks from which animals are likely to come into contact be vaccinated at the same time.

2. Vaccines for *Mycoplasma mycoides* subsp. *capri* infection

There is little recent published information on the availability of vaccines for *Mmc* (Marogna *et al*., 2015) although it is believed that inactivated vaccines are widely used in many Mediterranean countries and in Asia suggesting that their production and use is localised (Bergonier *et al*., 1997). Saponised vaccines have been reported in India which provoke a strong antibody response and show some protection (Sunder *et al*., 2002).

3. *Mycoplasma capricolum* subsp. *capricolum* and *M. putrefaciens*

Although infections with *Mcc* and *Mp* can be severe, their prevalence is relatively low and, as might be expected, little or no work appears to have been carried out on preventive vaccination for these infections.

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NB: There are WOAH Reference Laboratories for contagious agalactia (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for contagious agalactia

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.8.4.

CONTAGIOUS CAPRINE PLEUROPNEUMONIA

SUMMARY

Description of the disease: *Contagious caprine pleuropneumonia (CCPP) is a disease affecting goats and some wild ruminant species, caused by* Mycoplasma capricolum *subsp.* capripneumoniae (Mccp). *In goats it is manifested by anorexia, fever and respiratory signs such as dyspnoea, polypnea, cough and nasal discharges. The acute and subacute disease is characterised by unilateral sero-fibrinous pleuropneumonia with severe pleural effusion. Diagnosis is carried out by clinical and necropsy observations that should be confirmed by laboratory tests. As the isolation of* Mccp *is difficult, molecular techniques should be the methods of choice for laboratory confirmations.*

Detection of the agent: *Samples to be taken from live animals are broncho-alveolar washings or pleural fluid obtained by puncture. Samples to be taken at necropsy are lung lesions, lymph nodes, and pleural fluid. For cultivation of the pathogen, the tissues are ground in buffered solution and inoculated into selective broth and solid media with antibiotics or other inhibitors to prevent the growth of other bacteria. Growth of* Mccp *requires very rich media containing high percentages of serum. Isolation is hampered by the very slow growth of* Mccp*, up to 15 days, and the presence of other mycoplasma species such as* M. ovipneumoniae*.*

In broth, growth is visible within 4–15 days but turbidity is always very faint. Mccp *sometimes produces 'comets' in unshaken liquid cultures. On agar media, typical 'fried egg' colonies are always very small, 0.1–0.5 mm and differ from* M. ovipneumoniae *colonies that are centerless. Molecular techniques can be used for the rapid and specific identification of* Mccp*.*

Serological tests: *Goats are often infected by other* Mycoplasma *species that are closely related to Mccp and that will induce cross-reactions in nonspecific tests. Polysaccharide-latex agglutination can be used to confirm outbreaks in the field and specific competition enzyme-linked immunosorbent assay can be used for sero-prevalence studies or monitoring vaccination campaigns.*

Requirements for vaccines: *CCPP vaccines are inactivated and adjuvanted. The antigen is composed of whole* Mccp *cells that are concentrated and semi-purified, the minimum content is 0.15 mg of* Mccp *protein per dose. The adjuvant of choice is saponin with an indicative content of 3 mg per dose. The adjuvant quantity may vary according to batches of saponin.*

A. INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats occurring in many countries in Africa and Asia caused by *Mycoplasma capricolum* subsp. *capripneumonlae* (*Mccp*). The acute form of the disease is characterised by unilateral sero-fibrinous pleuropneumonia with severe pleural effusion (Thiaucourt, 2018; Thiaucourt & Bolske, 1996). Synonyms include: pleuropneumonie contagieuse caprine (France), bou-frida (Algeria), and abu-nini (Sudan).

From a taxonomic point of view, *Mccp* belongs to the so-called "mycoides cluster" (Manso-Silvan *et al.*, 2007) and was acknowledged as a subspecies in 1993 (Leach *et al.*, 1993). Its closest relatives are *Mycoplasma capricolum* subsp. *capricolum* and *M. leachii,* which may cross-react with *Mccp,* but the other members of the mycoides cluster, such as *M. mycoides* subsp. *capri* or *M. mycoides* subsp. *mycoides,* may also share similarities. *Mccp* is highly fastidious and faint turbidity in liquid medium or colonies on solid medium may appear only after 5–15 days. Isolation is often unsuccessful and detection may be easier with specific molecular methods such as the PCR (Woubit *et al.,* 2004).

The disease was first described in 1873 in Algeria. Shortly after, in 1881, the disease was introduced to South Africa by a shipment of Angora goats. The disease was eradicated using a policy of slaughter of the infected goats coupled with a traditional vaccination procedure for the in-contact goats (Hutcheon, 1889). This organism was first isolated and shown to cause CCPP in Kenya (MacMartin *et al*., 1980; MacOwan & Minette, 1976); it has subsequently been isolated in the Chad, Eritrea, Ethiopia, Niger, Oman, Sudan, Tanzania, Tunisia, Turkey, Uganda, the United Arab Emirates, and more recently in Mauritius (Srivastava *et al.,* 2010), China (People's Rep. of) (Chu *et al.,* 2011) Tajikistan (Amirbekov *et al*., 2010) and Saudi Arabia (El Deeb *et al.,* 2017). CCPP was first reported in mainland Europe in 2004, when outbreaks were confirmed in the Thrace region of Turkey, with losses of up to 25% of kids and adults in some herds (Ozdemir *et al.,* 2005). However, the exact distribution of the disease is not known and it may be much more widespread than the zone represented by the countries where *Mccp* has been isolated, as CCPP is often confused with other respiratory infections and also because the isolation of the causative organism is difficult.

In CCPP outbreaks in mixed goat and sheep herds, sheep may also be infected, as verified by isolation of *Mccp* (Bolske *et al.,* 1995) or detection of antibodies from clinically affected sheep. *Mccp* has also been isolated from healthy sheep (Litamoi *et al*., 1990) and the role of sheep as a reservoir for the disease has to be considered.

CCPP was first confirmed in wild ruminants kept in a wildlife preservation reserve in Qatar. The disease affected wild goats (*Capra aegagrus*), Nubian Ibex (*Capra ibex nubiana*), Laristan mouflon (*Ovis orientalis laristanica*) and Gerenuk (*Litocranius walleri*) with significant morbidity and mortality in these species (Arif *et al.,* 2005). It has also been reported in gazelles in the United Arab Emirates. There is growing evidence that a number of wild ruminant species are susceptible, such as the Tibetan antelope (*Pantholops hodgsonii*) (Yu *et al.,* 2013) and Arabian oryx (*Oryx leucoryx*) (Chaber *et al.,* 2014). In sand gazelles (*Gazella Marica*), the mortality rate reached up to 70% and the basic reproductive number was evaluated at 2.3 (Lignereux *et al.,* 2018).

CCPP is not a zoonotic infection. There is no known risk of human infection with *Mccp*. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

Differential diagnosis may be difficult in the field as goats may be infected with a number of mycoplasma species that may induce similar signs. However CCPP may be suspected when lesions are restricted to the respiratory tract, affect only one lung and when animals present a conspicuous pleurisy with profuse effusion of pleural fluid. CCPP could also be confused with peste des petits ruminants or pasteurellosis.

B. DIAGNOSTIC TECHNIQUES

The diagnosis of outbreaks of respiratory disease in goats, and of CCPP in particular, is complicated, especially where it is enzootic. It must be differentiated from similar clinico-pathological syndromes caused by other mycoplasma species belonging to the Mycoides cluster but also peste des petits ruminants, to which sheep are also susceptible; pasteurellosis, which can be differentiated on the basis of distribution of gross lung lesions; and contagious agalactia syndrome (Nicholas & Churchward, 2011; Thiaucourt & Bolske, 1996). The disease caused by *Mccp* is readily contagious and fatal to susceptible goats of all ages and both sexes, rarely affects sheep and does not affect cattle.

Other mycoplasmas such as *M. mycoides* subsp. *capri* (Mmc) may induce lung lesions that could be mistaken for CCPP lesions upon post-mortem examination. However Mmc usually induces other lesions such as arthritis mastitis or keratitis. Mmc is one of the causative agent of contagious agalactia (see chapter 3.8.3) and its rapid growth in vitro ensures that it cannot be confused with Mccp.

Table 1. Laboratory methods currently used for diagnosis of CCPP and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
In-vitro culture[b]	–	–	–	++	–	–
Molecular tests (PCR)	–	–	–	+++	–	–
Detection of immune response						
CFT	–	–	+	++	+	–
Latex agglutination	+	+	–	+++	+	–
C-ELISA	+++	++	–	++	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; CFT = complement fixation test; C-ELISA = competitive enzyme-linked immunosorbent assay.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]Organisms isolated should be subjected to confirmatory molecular,
biochemical or immunological methods as described below.

1. Detection of the agent

1.1. Microscopy of lung exudates, impression smears or sections

Mccp is characterised histopathologically by an interstitial pneumonia with interstitial, intralobular oedema of the lung (Kaliner & MacOwan, 1976). It shows a branching filamentous morphology *in vivo* that can be observed by dark-field microscopy in exudates or tissue suspensions from lesions or pleural fluid. Alternatively, smears made from cut lung lesions can be stained by the May–Grünwald–Giemsa method and examined by light microscopy. The other caprine mycoplasmas show a short filamentous or coccobacillary morphology. None of these techniques provides a definitive diagnosis.

1.2. Molecular identification and typing: polymerase chain reaction

Two polymerase chain reaction (PCR) assays for the specific identification of *Mccp* have been published. The first one (Bascunana *et al*., 1994) is based on the amplification of the 16S rRNA gene of the mycoides cluster. The PCR product is then analysed by restriction enzyme cleavage for the identification of the *Mccp* amplicon. The second one (Woubit *et al*., 2004) is based on a specific amplification; the primer sequences (Mccp-spe-F/R) are shown hereafter.

Mccp-spe-Forward: 5'-ATC-ATT-TTT-AAT-CCC-TTC-AAG-3'

Mccp-spe-Reverse: 5'-TAC-TAT-GAG-TAA-TTA-TAA-TAT-ATG-CAA-3'

PCR conditions consist of an initial denaturation step of 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 15 seconds at 47°C and 15 seconds at 72°C and a final extension step of 5 minutes at 72°C. The expected amplified product is 316 bp long.

The same primers can be used in a quantitative PCR method (Lorenzon *et al.,* 2008).

A field-applicable recombinase PCR (Liljander *et al.,* 2015) and a one-step multiplex real-time reverse transcription PCR assay that includes the detection of Mccp (Settypalli *et al.,* 2016) can also be used.

These PCR techniques can be used directly on clinical materials such as lung tissue and pleural fluid (Bölske *et al*., 1996), including pleural fluid dried on filter papers. Due to the difficulty in isolating *Mccp*, PCR is the technique of choice for the diagnosis of CCPP. However, isolation of *Mccp* remains the confirmatory test. All mycoplasmas of the mycoides cluster can be assigned a precise phylogenetic position by using a multilocus sequence typing approach (Manso-Silván *et al*., 2007).

1.3. Gel precipitin tests to detect antigen in tissue specimens

Mccp releases an antigenic polysaccharide to which a specific monoclonal antibody (MAb) (WM-25) has been produced (Rurangirwa *et al*., 1987c). This MAb immunoprecipitates in agar gel with the polysaccharide produced by *Mccp*, and is used to identify the causative agent in cases of CCPP, particularly when specimens are no longer suitable for culture because of deterioration during transit. The MAb may be replaced by goat CCPP convalescent sera provided they are precipitating. This precipitation test may not be completely specific and could give some cross-reactions, notably with *M. leachii.* Extracted exopolysaccharide was characterised by nuclear magnetic resonance, resulting in the identification of a homopolymer of β(1→2)-glucopyranose (glucan) in *Mccp* and *M. leachii* (Bertin *et al.,* 2015).

1.4. Isolation of mycoplasmas

1.4.1. Selection of samples

The necropsy samples of choice are lung lesions, particularly from the interface between consolidated and unconsolidated areas, pleural fluid, and mediastinal lymph nodes. If microbiological examination cannot be performed immediately, samples or whole lungs can be stored at –20°C for considerable periods (months) with little apparent loss of mycoplasma viability. During transport, samples should always be kept as cool as possible, as mycoplasma viability diminishes rapidly with increasing temperature. Lung samples can be dispatched to other laboratories in frozen form.

1.4.2. Treatment of samples

Swabs are suspended in 2–3 ml of culture medium. Tissue samples are best minced using scissors, and then shaken vigorously, or pulverised in medium using 1 g of tissue to 9 ml of medium. Tissues should not be ground. The suspension is usually prepared with a mycoplasma medium, but if parallel bacteriological examination is to be carried out, a high quality bacteriological medium, such as nutrient broth, may be used to provide a suspension suitable for both forms of examination. Pleural fluid, or a tissue suspension or swab, is serially diluted through at least three tenfold steps (to a nominal 10^{-4}) in the selected mycoplasma medium. Dilutions should also be plated on to solid medium.

1.4.3. Mycoplasma media

The medium used by MacOwan & Minette to culture *Mccp* organisms (MacOwan & Minette, 1976), is termed 'viande foie goat' (VFG), and includes goat-meat liver broth and goat serum. Alternative suitable media are WJ (Jones & Wood, 1988), modified Hayflick's, and modified Newing's tryptose broth (Kibor & Waiyaki, 1986) Media enriched with 0.2% (or up to 0.8%) sodium pyruvate perform considerably better, both for primary isolation and antigen production of *Mccp* (Mohan *et al*., 1990; Thiaucourt *et al*., 1992).

i) CCPP medium

a) Autoclaved portion (121°C for 15 minutes)

PPLO (pleuropneumonia-like organisms) broth without crystal violet (21 g); deionised water (700 ml).

b) Membrane-filtered portion

Horse (alternatively pig or donkey) serum inactivated at 56°C for 30 minutes (200 ml); fresh yeast extract (100 ml); glucose (sterile solution 0.5 g/ml) (2 ml); and sodium pyruvate (sterile solution 0.5 g/ml) (8 ml).

Part B is added to A aseptically. Ampicillin (0.1 g/litre) and thallium acetate (250 mg/litre) can be added to prevent contamination in primary isolations. The final pH of the medium should be 7.4–7.6.

ii) Modified CCPP medium

a) Autoclaved portion (121°C for 15 minutes)

PPLO broth without crystal violet (17.5 g); glass distilled water (650 ml).

b) Membrane-filtered portion

Horse (alternatively pig or donkey) serum inactivated at 56°C for 30 minutes (250 ml); fresh yeast extract (100 ml); 50% glucose (4 ml); 25% sodium pyruvate (8 ml); 5% thallium acetate (4 ml); ampicillin (250 mg); and 0.5% phenol red (4 ml). The pH is adjusted to 7.8 with sodium hydroxide or hydrochloric acid. Part B is added to A aseptically.

1.4.4. Medium production, storage and quality control

Certain medium components, particularly serum, yeast extract and deionised water, should be regularly monitored for growth-promoting capacity before incorporation into mycoplasma media. Low-passage field isolates should be used for this screening purpose.

Broth media may be stored for at least 6 months at –25°C before use, but penicillin or its analogues should not be added until final dispensing. Broth media are dispensed into bijoux (1.8 ml or 2.7 ml) or screw-capped tubes (4.5 ml), and stored for up to 3 weeks at 4°C. Solid media are best made with agarose (0.9% [w/v]), Noble agar (1.5% [w/v]), or purified agar (0.6% [w/v]). Plates, which are poured to a depth of 6–8 mm, should be as fresh as possible when used, and should be stored for no more than 2 weeks at 4°C before use. All culture media should be subjected to quality control and must support the growth of *Mycoplasma* spp. from small inocula. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

1.4.5. Cultivation

Cultures are incubated at 37°C. Plates are best incubated in a humid atmosphere of 5% CO_2, 95% air or N_2, or in a candle jar with a moisture source. Cultures can also be incubated anaerobically.

Broth cultures are examined daily for evidence of growth – colour change and the appearance of floccular material. Gross turbidity indicates bacterial contamination; cultures showing this should be passed through a 0.45-μm membrane filter before subculture. Broth cultures are subcultured by inoculation of fresh broth medium with one-tenth of their volume, or by streaking agar medium with a loop.

Plate cultures are examined every 1–3 days using a stereo microscope (×5–50 magnification) and transmitted and incident light sources. If negative, the plates are discarded after 15 days. Subculture is carried out by the transfer of excised agar blocks bearing isolated colonies to either agar (on which the blocks are pushed, face down) or broth media. Alternatively, an agar plug bearing one colony is drawn into a Pasteur pipette and discharged into fresh broth medium.

Cloning and purification of isolates is performed by repeated transfer of single colonies representing each morphological type seen. Colony morphology varies with the medium used, the mycoplasma species, its passage level and the age of the culture.

In early passage, many mycoplasma species produce colonies of bizarre morphology, often small, centreless, and of irregular shape. This effect is often associated with the use of marginally suitable medium. With passage, such isolates demonstrate conventional ‘fried egg’ colony

morphology, except *M. ovipneumoniae*, which retains centreless colonies. Colonies of *M. mycoides* subsp. *capri* may be up to 3 mm in diameter.

Filtration of broth cultures through 0.45 μm filters before subculture aids purification by excluding cell aggregates.

Cultures suspected of being L-forms of bacteria should be examined for reversion to bacterial form by three to five passages on solid mycoplasma medium from which antibiotics and thallium acetate have been omitted.

Broth media used for primary isolation and which have shown no indication of growth by 7 days, should be subcultured blind.

Broth cultures of each sample, including one blind subculture, should be examined for a minimum of 3 weeks before being discarded. Titrations in broths, if performed in full (to 10^{-10}), are also read at 3–4 weeks and are expressed as colour-changing units per transfer volume. Growth on plates is expressed as colony-forming units (CFU) per ml.

1.5. Identification of mycoplasmas

Wild strains should be passaged, and preferably cloned, three times before identification is attempted.

1.5.1. Polymerase chain reaction

Once the organism has been cultured, verification of *Mccp* can be achieved in 1 day by PCR. See B.1.2 of this chapter.

PCR and sequencing has been used to establish the molecular epidemiology of CCPP. A multilocus sequence analysis (MLSA) method is also available (Manso-Silvan *et al.,* 2011) that revealed two main lineages comprising five groups from a representative set of *Mccp* strains.

Identification of *Mccp* strains by PCR (and sequencing) has now superseded all other techniques because of its rapidity and reliability. However PCR reactions must be performed with great care to prevent contamination (refer to Chapter 2.1.2. *Biotechnology advances in the diagnosis of infectious diseases*). Sequencing whole *Mccp* genomes is the ultimate typing tool. Some *Mccp* genomes are already publicly available (Chen *et al.,* 2017; Dupuy & Thiaucourt 2014; Falquet *et al.,* 2014) and more should be available in the coming years (https://www.ncbi.nlm.nih.gov/genome/neighbors/521?&genome_assembly_id=300226).

1.5.2. Biochemical tests

Biochemical tests cannot identify an isolate unequivocally, which at present can only be done by genetic means.

The tests most commonly used are glucose breakdown (*Mccp:* positive), arginine hydrolysis (*Mccp:* negative), 'film and spots' formation (*Mccp:* negative), reduction of tetrazolium chloride aerobically and anaerobically (*Mccp:* +/++), phosphatase activity (*Mccp:* negative), serum digestion (*Mccp:* negative) and digitonin sensitivity (*Mccp:* positive).

1.5.3. Serological identification

Mycoplasmal antigens used in hyperimmune serum production are often contaminated with medium constituents. The antibodies stimulated by these contaminants can cause false-positive reactions in serological identification tests. This problem is avoided by absorption of the antiserum with the medium used to produce the antigen (10 mg lyophilised medium per ml of antiserum), or by growing the mycoplasmas to be used as antigens in medium containing homologous animal components, e.g. growth in VFG medium to immunise goats.

1.5.3.1. Growth inhibition test

The growth inhibition test (GIT) is the simplest and most specific of the tests available. It depends on the direct inhibition of growth on solid medium by specific hyperimmune serum, and detects primarily surface antigens.

Mccp appears to be highly homogeneous serologically and wide zones of inhibition free of 'breakthrough' colonies are observed with antiserum to the type strain, regardless of the source of the test strain (Jones & Wood, 1988). *Mccp* cross-reacts with *M. leachii* (PG50), *M. equigenitalium* and *M. primatum* in the GIT when polyclonal antisera are used, but an MAb specific for *Mccp* in the GIT has been produced (Rurangirwa *et al.,* 1987c). The MAb reagent, WM25, has been reported to be specific for *Mccp* isolates by the disc growth inhibition method, which will exclude *M. agalactiae*, *Mcc* and the other members of the 'mycoides cluster' associated with goats, but not bovine group 7 (not usually found in goats): the latter can be excluded, however, by colony indirect fluorescence tests. A small proportion of *Mccp* isolates also cross-react in the GIT with antiserum to *Mcc*. *Mycoplasma leachii* strains can sometimes be found in goats although it is rare. Results should be interpreted carefully as some bovine strains have been misidentified by the GIT using the 'specific' antiserum.

- **Test procedure**

i) Broth culture in mid-to-late logarithmic phase is used at three tenfold dilutions, the selection of which is related to the vigour of growth of the isolate on agar.

ii) Agar plates are dried for 30 minutes at 37°C.

iii) Sterile paper disks of 6–7 mm in diameter are impregnated with a drop (10–20 µl) of undiluted antiserum. Disks may be used wet, in which form they can be stored at –20°C, or they can be lyophilised (Dighero *et al.,* 1970), which allows storage at 4°C.

iv) Using a separate plate for each dilution of culture, 1 ml or 2.5 ml volumes are pipetted on to 5 cm or 10 cm diameter plates, respectively. The inoculum is dispersed evenly over the plate, then the excess is removed.

v) The plates are dried at 20–30°C for 15–20 minutes, preferably under a protective hood, until no visible liquid is present on the surface. Sufficient residual moisture should remain to enable freeze-dried disks to adhere to the agar surface.

vi) Several disks, each impregnated with a different antiserum (selected on the basis of sample source and the biochemical reactions and colony morphology of the isolate), are carefully placed on the agar plates; isolates from CCPP cases should be screened with antisera against Mccp, *M. mycoides* subsp. *capri* and *M. ovi-pneumoniae*. A disk containing 1.5% digitonin should also be included on the plates.

vii) The plates are incubated at 37°C for 2–6 days. Initial overnight incubation at 27°C can increase the sensitivity of the test. Inhibition by digitonin is generally readily apparent; however, inhibition by antiserum may be more difficult to interpret, with suppression rather than total inhibition of growth, depending on the species of mycoplasma, colony density and potency of the antiserum. 'Breakthrough' colonies are commonly observed within zones of inhibition. Circular precipitin bands are occasionally seen around disks. Positive inhibition is regarded as a zone of 2 mm or more.

2. Serological tests

Serology has not been widely applied to identifying the cause of outbreaks of pleuropneumonia in goats and sheep. Three methods are currently available: CFT, the latex agglutination test and the competitive enzyme-linked immunosorbent assay (ELISA) with a specific MAb ("4.52" C-ELISA). Goats are frequently infected by mycoplasmas of the mycoides cluster, which may induce cross-reactions with tests such as the CFT that use crude antigenic preparations. In addition, the CFT detects mostly IgMs, which are short-lived. For this reason, the CFT is not recommended.

Seroconversion to *Mccp* in experimentally infected animals is observed by the CFT to start 7–9 days after the appearance of clinical signs, to peak between days 22 and 30, and to decline rapidly thereafter. These various

observations indicate that serology should be applied on a herd, not an individual basis, and that whenever possible, paired serum samples collected 3–8 weeks apart, should be examined.

2.1. Complement fixation test (MacOwan & Minette, 1976)

To prepare the antigen, 2 litres of culture of titre higher than 10^9 CFU/ml is centrifuged at 12,000 ***g*** for 1 hour at 5°C. The deposit is resuspended and washed three times in physiological saline prior to storage in 0.5–1.0 ml volumes at –20°C.

Sterile broth treated as above constitutes a control antigen, and a freeze-dried broth reconstituted at 200 mg/ml constitutes a second control antigen. Prior to testing, the antigen is diluted 1/60 and ultrasonicated for 3 minutes at low power in a container of iced water. The sonicate is centrifuged at 1250 ***g*** for 30 minutes to remove any debris, and stored at –20°C. If stored for more than 2–3 weeks the antigen should be recentrifuged.

2.1.1. Test procedure

Microtitre plate tests are performed using 0.025 ml volumes, two volumes containing three mean haemolytic doses of complement, and a 1.5% (v/v) final concentration of sheep red blood cells (SRBCs) in U-bottomed microtitre plates as follows:

i) The following are mixed and incubated at 37°C for 45 minutes:

   a) 25 µl of doubling dilutions of test serum (heat inactivated at 56°C for 30 minutes) starting with 1/2 dilution;

   b) 25 µl of antigen containing two units of antigen (the dilution of the antigen must be determined in a chequer-board titration using a known positive serum. One unit of antigen corresponds to the highest antigen dilution yielding the highest titre with the positive reference serum;

   c) 25 µl of complement (3 haemolytic units).

ii) 25 µl of sensitised SRBCs, at a final concentration of 1.5% (v/v), is mixed and the plates are incubated at 37°C for 45 minutes.

iii) The plates are incubated at 4°C for 1 hour to allow the intact SRBCs to sediment.

iv) Reading the results: The titre will be the highest serum dilution that will fix 50% of the complement, i.e. 50% haemolysis.

2.1.2. Controls

In all CF tests a number of controls are required:

i) Indicator systems (RBCs + haemolysin) alone to ensure that RBCs do not lyse spontaneously.

ii) Indicator system with complement only to show that enough complement is present to lyse the cells.

iii) Indicator system with antigen only and no complement to show that antigen alone does not lyse the cells.

iv) Indicator system with serum alone and no complement to show that the serum alone does not lyse the cells.

v) Indicator system with complement and antigen to detect any anticomplementary activity of the antigen.

vi) Indicator system with the complement and serum to detect any anticomplementary activity of the serum.

NB: as many mycoplasmas, notably those belonging to the mycoides cluster, are expected to induce cross-reactions in the CFT, additional tests should be performed when finding CF positive titres in a CCPP-free country or zone. Suspicious sera should be tested in parallel with antigens prepared with various mycoplasma species and notably *M. capricolum*, *M. mycoides* subsp. *mycoides, M. leachii* and

M. mycoides subsp. *capri*. The antigen yielding the highest titre should indicate which species was infecting the animal/herd.

2.1. Latex agglutination test

Latex beads sensitised with the polysaccharide produced by *Mccp* and present in culture supernatant have been used in a slide agglutination test (Rurangirwa *et al.,* 1987a). It is a very useful test in an outbreak because it can be performed at the penside using a drop of whole blood.

This test is sensitive at an early stage of the disease as long as IgM persists in the serum. Its specificity is not well characterised. Cross-reactions may occur as *Mccp* polysaccharides are similar to those produced by *M. leachii* and *M. capricolum* subsp. *capricolum* (Bertin *et al.,* 2015) and may be found in other bacteria.

2.2. Competitive enzyme-linked immunosorbent assay

A C-ELISA was developed (Thiaucourt *et al*., 1994) and proved both specific and sensitive. This test has recently been reformatted as a kit containing pre-coated plates and ready-made reagents, including MAb 4/52. It is now a strict competition assay instead of a semi-blocking test as in the original publication. The new kit has been re-validated to establish its cut-off value, 55% inhibition (PI), to obtain a strict specificity of 99.9%. It allows the detection of positive sera in CCPP-infected herds, but its true sensitivity at the individual level has not yet been fully evaluated. As it is highly specific, it can be used to evaluate herd status using targeted sampling of recovered animals in the tested herds should greatly enhance the sensitivity without any specificity problem. In the WOAH Reference Laboratory in France, the uncertainty of measurement for this C-ELISA has been evaluated at ±8 PI.

This test can be used to evaluate the CCPP vaccine quality as the seroconversion measured 1 and 2 months post-vaccination is proportional to the *Mccp* antigen or saponin content. However, the correlation between C-ELISA titre and protection has not yet been established (Peyraud *et al.,* 2014).

C. REQUIREMENTS FOR VACCINES

1. Background

Successful eradication of CCPP has already been achieved in Southern Africa in 1889 by applying a strategy based on the slaughter of affected animals and the inoculation of all in-contact goats. At that time, inoculation consisted in the subcutaneous injection of preparations containing pleural fluid or affected lungs homogenates. Contrary to what is observed in contagious bovine pleuropneumonia, the subcutaneous injection of live *Mccp* into naïve goats is not followed by any untoward inflammatory reaction. This may have contributed to the success of the eradication programme in spite of the crude nature of the preparations. However the respective contributions of slaughter and inoculations to the eradication process have not been evaluated.

Few experiments have been performed to develop live vaccines and evaluate their potency. Studies have focused on inactivated preparations containing saponin as an inactivating agent and adjuvant (Rurangirwa *et al*., 1987b). The optimum dose of antigen was established at 0.15 mg of *Mccp* protein and 3 mg of saponin. In the original publication, the concentrated antigen was freeze dried and reconstituted extemporaneously with a diluent containing 3 mg of saponin per ml. Such a procedure ensured a very long shelf life for the concentrated antigen (>14 months) and the duration of protection exceeded 12 months.

Due to the fastidious nature of *Mccp*, the production of CCPP vaccines is costly. *Mccp* requires very rich media, the yield is limited, the procedure involves a purification process, and inactivated vaccines also require larger amounts of antigen compared with live vaccines.

CCPP vaccines should be safe. The fact that live *Mccp* strains do not induce post-vaccine reactions could be an advantage for this kind of vaccine. For inactivated vaccines containing saponin, the pro-inflammatory effect of the saponin has to be verified as it may vary according to the producers or batches. It is not recommended to vaccinate pregnant animals because of a possible reaction to saponin. Because of the immune response triggered by the saponin adjuvant, serology, and notably the specific C-ELISA, can be used either for batch quality control or for sero-

monitoring of vaccination campaigns (Peyraud *at al.,* 2014). Few vaccine producers undertake these controls. In 2018, a new mass spectrometry technique was designed to analyse the vaccine composition. It allows *Mccp*-specific peptides to be quantified by comparison with those of other origins, including those coming from medium components (Thiaucourt *et al.,* 2018).

CCPP vaccine efficacy should last at least 1 year and protect vaccinated animals from clinical disease.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production.* The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Any local isolate of *Mycoplasma capricolum* subsp. *capripneumoniae* can be used because of the homogeneity of this subspecies. The choice of strain will mostly depend on growth characteristics: rapid growth, ease of concentration and purification, etc.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Seed lots must be shown to be:

i) Pure

Purity can be tested by randomly selecting 10 *Mccp* clones originating from the seed and verifying the identity of these 10 clones, for example using a specific PCR technique (other tests may not be specific enough). Due to the fastidious nature of *Mccp* strains, any contaminant is likely to overgrow *Mccp* and be detectable by this procedure.

ii) Safe

No inflammatory reaction is normally observed when injecting *Mccp* strains subcutaneously into susceptible goats. It is therefore difficult (if necessary at all) to establish the seed's safety.

iii) Efficacious

Vaccines produced with the selected strain must induce protection when produced according to standard procedures and injected into naïve susceptible goats.

2.1.3. Validation as a vaccine strain

As there is no laboratory animal model for CCPP, seed validation as a suitable vaccine strain will have to be performed on susceptible goats at least once. The vaccine has to be prepared according to standard procedures and injected into susceptible goats. Vaccinated and naïve goats should be put in contact with CCPP-infected animals at least 3 months after vaccination. The expected level of protection should be at least 90% with a number of animals per group yielding statistically significant results.

If the inactivation process or the presentation of the final vaccine differs from the original presentation where protection has been demonstrated, additional tests must be performed to show the immunogenicity of the new final product.

2.2. Method of manufacture

2.2.1. Procedure

For vaccine production, a working seed is first established by amplifying an aliquot of the freeze dried master seed bank that has been shown to induce protection. There is no specific requirement for the type of medium used provided it ensures satisfactory growth of the *Mccp*

strain. The number of passages from master seed bank to batch production should be less than five (here, a passage corresponds to a 1/200 dilution of the inoculum).

Mccp cells have to be concentrated and purified. Again there is no specific requirement for this step and producers may choose any method deemed necessary, provided the end product is pure and devoid of extraneous products originating from the culture medium. For example, cultures can be centrifuged at high speed (> 12,000 ***g***) for 20 minutes, the pellet resuspended in an adequate volume of sterile PBS for washing, and *Mccp* pelleted again.

Washed concentrated *Mccp* antigen can be diluted to adjust the protein content, and freeze dried. The content of each vial is adjusted so that the final quantity of protein should be 0.15 mg per dose once the vaccine is reconstituted. A primary inactivation of the concentrated antigen by saponin can take place at this stage.

The final vaccine is obtained by reconstituting the freeze dried product with the necessary volume of diluents containing 3 mg of saponin per dose. Saponin acts as an inactivating agent for *Mccp* and as an adjuvant.

NB: Any production procedure that modifies the antigen content and characteristics or the adjuvant type warrants a new validation/registration verification.

2.2.2. Requirements for ingredients

No specific requirements. For general requirements, please refer to chapter 1.1.8, with special focus on products of biological origin originating from a country with negligible risk for transmissible spongiform encephalopathies.

2.2.3. In-process controls

The purity of the *Mccp* growth can be assessed regularly by rapid methods such as phase-contrast observation of cultures. It will ensure that there is no cell-walled bacterial contamination (mycoplasmas appearing as tiny grey spots barely visible while cell-walled bacteria appear bigger and brighter).

The absence of medium contaminants in the final concentrated *Mccp* product may be assessed by techniques such as SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) with controls including *Mccp* antigen and diluted production medium. The absence of medium contaminant ensures that the protein dosage refers to mycoplasma antigen and not to contaminants.

The amount of mycoplasma antigen in the concentrated product may be evaluated by its protein content provided that the percentage of extraneous proteins is limited. Any suitable technique is acceptable, e.g. the bicinchoninic acid technique, provided the proper controls are included in the testing, for example, bovine albumin standards or reference *Mccp* antigen.

Mass spectrometry may be used to evaluate the final composition of the concentrated *Mccp* antigen (Thiaucourt *et al.,* 2018). This technique allows the detection and quantification of medium contaminants still present in the concentrated antigen. It is the responsibility of the vaccine producer to define, in the registration/validation dossier, which level of extraneous protein content is acceptable without jeopardising the immunogenicity of the vaccine.

Once the antigen is inactivated by saponin, sterility can be assessed by seeding a sample on suitable media allowing *Mccp* growth.

2.2.4. Final product batch tests

i) Sterility

Standard procedures have to be used for testing sterility on a representative number of vials (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

ii) Safety

Target animal batch safety tests (TABST) may be waived if safety of the product has been demonstrated in pre-licensing testing and approved in the registration dossier, and consistency with the production process is approved in accordance with chapter 1.1.8.

Where necessary, a TABST is conducted to detect any abnormal local or systemic adverse reactions. For the purposes of batch release, each of at least three healthy seronegative goats is inoculated by the recommended route of administration with at least twice the recommended dose of vaccine. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days. Any adverse reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. If the potency test is performed in the target species, observation of the safety during this test can also be considered as an alternative to conducting the batch safety test described here.

iii) Batch potency

It is impractical to test the potency of the batches in susceptible hosts because of the difficulties encountered in reproducing CCPP. For batch release, indirect tests can be used for practicability and animal welfare considerations.

The three naïve goats that are vaccinated for the safety test must demonstrate a persistent specific and high titre seroconversion to *Mccp* antigen for at least 2 months post-vaccination. Unfortunately the serological tests that are used for CCPP have not been evaluated for a correlation between post-vaccination seroconversion and protection. However, the demonstration of a suitable specific seroconversion ensures that the tested product contains the correct antigen and that it can induce an immune response in vaccinated animals. For the time being, a western blot analysis (Abdo el *et al.,* 1998) may be used to evaluate this seroconversion. Alternative tests could be CFT or the specific C-ELISA that detects antibodies directed towards a single epitope. The latex agglutination test may not be used as it detects antibodies to a polysaccharide. This polysaccharide can be found in other mycoplasmas of the mycoides cluster and there is also no vaccine requirement for the polysaccharidic content of the vaccine (the possible protective role of the immune response towards polysaccharides has not yet been established).

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

2.3.2. Safety requirements

i) Target and non-target animal safety

For the purpose of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by the recommended route of administration in an *in-vivo* test in eight susceptible goats. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days after injection. Any adverse reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine. Due to the presence of saponin in the vaccine, a transient feverish reaction may be observed as well as a localised swelling.

ii) Precautions (hazards)

Mccp is not pathogenic for humans. Accidental self-injection may induce a local irritation because of the presence of saponin in the vaccine.

2.3.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

Efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to live pathogen challenge and in comparison with nonvaccinated control animals.

Efficacy will be evaluated 3 months post-vaccination by an in-contact method whereby artificially infected goats are put in contact with vaccinated and naïve animals. Protection rate will be based on the observation of clinical signs (days with fever) and of lesions when animals are disposed of, 1 to 2 months after the initial onset of disease in the control group. The protection rate should reach at least 90% (± 10).

Due to the conserved nature of *Mccp* strains, any field pathogenic strain can be used to assess vaccine protection, provided it proves pathogenic in the control group.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable to current CCPP vaccines.

2.3.5. Duration of immunity

As part of the regulatory approval procedure, the manufacturer should be required to demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test at the end of the claimed period of protection. In the case of saponin inactivated CCPP vaccine, this period is estimated at 1 year.

2.3.6. Stability

As part of registration/licensing procedure, the manufacturer should be required to demonstrate the stability of all the vaccine's properties at the end of the claimed shelf-life period. Storage temperature shall be indicated and warnings should be given if product is damaged by freezing or ambient temperature.

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NB: There is a WOAH Reference Laboratory for contagious caprine pleuropneumonia (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratory for any further information on diagnostic tests, reagents and vaccines for contagious caprine pleuropneumonia

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.8.5.

ENZOOTIC ABORTION OF EWES (OVINE CHLAMYDIOSIS) (INFECTION WITH *CHLAMYDIA ABORTUS*)

SUMMARY

***Description and importance of the disease**: Ovine chlamydiosis, also known as enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA), is caused by the bacterium* Chlamydia abortus. *Chlamydial abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and inflamed placentas. However, infection can also result in the delivery of full-term stillborn lambs or weak lambs that do not survive longer than 48 hours. Infected ewes can also give birth to healthy lambs. There are rarely any predictive signs that abortion is going to occur, although behavioural changes and a vulval discharge can be observed in the last 48 hours of pregnancy.*

Diagnosis of enzootic abortion depends on the detection of antigen or nucleic acid of the causative agent in the products of abortion or vaginal excretions of freshly aborted females. A humoral antibody response may be detected following abortion. Goats as well as sheep and, less commonly, cattle, pigs, horses and wild ruminants, can be affected. Chlamydiosis of small ruminants caused by C. abortus *is zoonotic and the organism must be handled with appropriate biosafety precautions. Pregnant women are particularly at risk.*

***Identification of the agent:** The basis for a positive diagnosis of infection with* C. abortus *depends on a history of abortion in sheep or goats (often in late pregnancy), evidence of purulent to necrotising placentitis with vasculitis, and the demonstration of large numbers of the organism in affected placentae by quantitative polymerase chain reaction (PCR) or antigen tests or in stained smears. The still moist fleece of fetuses or their abomasal content or vaginal swabs of females that have freshly aborted are also useful. It is important to distinguish cotyledonary damage caused by* Toxoplasma gondii *and, in stained smears, to be aware of the morphological similarities between* C. abortus *and* Coxiella burnetii*, the agent of Q fever.*

Chlamydial organisms in tissues and smears can be detected by staining, or antigen-detection methods (immunohistochemistry or immunofluorescence), whereas chlamydial DNA can be detected by PCR-based methods including real-time PCR and DNA microarray. Some of these methods are available in commercial kit form.

Chlamydia abortus *can be isolated only in living cells; thus facilities for growth in cell cultures or chicken embryos, with appropriate biohazard containment, are required.*

***Serological tests:** A rise in antibody titre to* C. abortus*, which can be detected by enzyme-linked immunosorbent assay (ELISA), is common after abortion or stillbirth, but this does not occur in every case.* Chlamydia abortus *shares common antigens with other* Chlamydia *species and some Gram-negative bacteria, so that the complement fixation (CF) test or crude ELISAs are not specific and no longer recommended. Serological screening during the period after parturition helps to identify infected flocks, to which control measures can then be applied. Serological tests to differentiate between vaccinated and naturally infected sheep or goats (DIVA tests) are not currently available.*

***Requirements for vaccines:** Inactivated and live vaccines are available that have been reported to prevent abortion and to reduce excretion. They assist in control of the disease but will not eradicate it.*

A. INTRODUCTION

1. Description and impact of the disease

Ovine chlamydiosis (enzootic abortion of ewes [EAE] or ovine enzootic abortion [OEA]) is caused by the bacterium *Chlamydia abortus.* Chlamydial abortion in late pregnancy causes serious economic loss in many sheep-rearing areas of the world, particularly where flocks are closely congregated during the parturient period (Aitken & Longbottom, 2007; Longbottom & Coulter, 2003). Abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and grossly inflamed placentas. Infection can also result in the delivery of full-term stillborn lambs and weak lambs that generally fail to survive beyond 48 hours. It is also not uncommon in multiple births for an infected ewe to produce one dead lamb and one or more weak or healthy lambs. Infection is generally established in a 'clean' (immunologically naïve) flock through the introduction of infected replacements and results in a small number of abortions in the first year, followed by an 'abortion storm' in the second year that can affect around 30% of ewes.

Infected animals show no clinical illness prior to abortion, although behavioural changes and a vulval discharge may be observed in ewes within the last 48 hours of pregnancy. Pathogenesis commences around day 90 of gestation coincident with a phase of rapid fetal growth when chlamydial invasion of placentomes produces a progressively diffuse inflammatory response, thrombotic vasculitis and tissue necrosis. Milder changes occur in the fetal liver and lung and, in cases with severe placental damage, there may be evidence of hypoxic brain damage (Buxton *et al*., 2002; Longbottom *et al.*, 2013). Abortion probably results from a combination of impairment of materno-fetal nutrient and gaseous exchange, disruption of hormonal regulation of pregnancy and induced cytokine aggression (Entrican, 2002).

Chlamydial abortion also occurs to a similar extent in goats and, less frequently, cattle, pigs, horses and wild ruminants may be affected. In sheep, abortion in late pregnancy with expulsion of necrotic fetal membranes are diagnostic indicators.

2. Nature and classification of the pathogen

Taxonomically, the family *Chlamydiaceae* comprises a group of Gram-negative, obligate intracellular bacteria within the single genus *Chlamydia,* which includes eleven species: *C. trachomatis* (humans), *C. suis* (swine), *C. muridarum* (mouse and hamster), *C. psittaci* (avian), *C. felis* (cat), *C. abortus* (sheep, goat and cattle), *C. caviae* (guinea-pig), *C. pecorum* (sheep, cattle and, koala), *C. pneumoniae* (humans), *C. avium* and *C. gallinaceae* (both in birds) (Sachse *et al*., 2015) as well as two candidate species named *Candidatus* Chlamydia ibidis and *Candidatus* Chlamydia sanzinia (Taylor-Brown *et al*., 2016; Vorimore *et al*., 2013).

Infected ewes shed vast numbers of infective *C. abortus* at the time of abortion or parturition, particularly in the placenta and uterine discharges, thus providing an infection source. Ewes having aborted do not usually abort again from *C. abortus* infection. Recent evidence suggests that the proportion of infected ewes is reduced at the subsequent breeding season and only low levels of chlamydial DNA are detected during the periovulation period and at lambing, so that this would not have significant impact on the epidemiology (Gutierrez *et al.,* 2011; Livingstone *et al.,* 2009).

3. Zoonotic risk and biosafety requirements

Human infection may be acquired from infected products of abortion or parturition or from carelessly handled laboratory cultures of the organism, with manifestations ranging from subclinical infection to acute influenza-like illness. Cultures and potentially infected tissues should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Authenticated cases of human placentitis and abortion caused by *C. abortus* of ovine/caprine origin indicate that pregnant women are at special risk and should not be exposed to sources of infection (Longbottom & Coulter, 2003; Sillis & Longbottom, 2011).

4. Differential diagnosis

Specific experience is needed to distinguish the diffuse pattern of necrosis and inflammation caused by *C. abortus* infection from necrosis caused by *Toxoplasma gondii,* which is limited to the cotyledons. Differentiation from other infectious causes of abortion, such as brucellosis (see Chapter 3.1.4), coxiellosis (see Chapter 3.1.16) or other

bacterial pathogens (*Campylobacter* [see Chapter 3.10.4], *Listeria* [see Chapter 3.10.5], *Salmonella* [see Chapter 3.10.7]), can be achieved by conducting further agent-specific diagnostic tests. Recently, other chlamydial species, such as *C. pecorum* and *C. psittaci,* have been implicated as abortigenic agents in ruminants (Berri *et al*., 2009; Lenzko *et al*., 2011).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of enzootic abortion of ewes and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[(a)]						
Stained smears	–	–	–	+	–	–
Bacterial isolation	–	–	–	++	–	–
Antigen detection by IHC	–	–	–	++	+	–
ConventionalPCR	–	–	–	+++	++	–
Real-time PCR	–	–	-	+++	++	–
Detection of immune response						
CFT	+	+	+	+	+	+
ELISA	+++	++	+++	++	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
IHC= immunohistochemisty; PCR=polymerase chain reaction; CFT=complement fixation test;
ELISA=enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Smears

Where the clinical history of the flock and the character of lesions in aborted placentae suggest enzootic abortion, a diagnosis can be attempted by microscopic examination of smears made from affected chorionic villi or adjacent chorion. Smears are stained according to modified Machiavello, Giemsa, *Brucella* differential, or modified Ziehl–Neelsen (Stamp *et al*., 1950). In positive cases stained by the latter method and examined under a high-power microscope, large numbers of small (300 nm) coccoid elementary bodies are seen individually or in clumps stained red against the blue background of cellular debris. Under dark-ground illumination, the elementary bodies appear pale green. Fluorescent antibody tests (FATs) using a specific antiserum or monoclonal antibody may be used for identification of *C. abortus* in smears. However, polymerase chain reaction (PCR)-based tests are superior to stained or FAT smears regarding sensitivity and specificity and should therefore be applied if available. Stained smears might be useful as an initial screening test, but confirmation by molecular methods is highly recommended due to inferior sensitivity of staining and lack of species specificity.

If placental material is not available, smears may be prepared from vaginal swabs of ewes that have aborted within the previous 24 hours, or from the moist fleece of a freshly aborted or stillborn lamb that has not been cleaned by its mother, or from the abomasal content of the aborted or stillborn lamb. In general, such preparations contain fewer organisms than placental smears.

In terms of morphology and staining characteristics, *C. abortus* resembles *Coxiella burnetii* (see chapter 3.1.16 *Q fever*), which, in some circumstances, may provoke abortion and which causes Q fever in humans. Care must be taken to differentiate between these two organisms in cases lacking a good history or evidence of chlamydia-induced placental pathology.

1.2. Isolation of the agent – cell culture

Cell culture is the method of choice for isolation of the organism. The causative agent of ovine chlamydiosis is zoonotic and thus isolation and identification procedures must be carried out with appropriate biosafety and containment procedures as determined by biorisk analysis (see chapter 1.1.4).

Tissue samples, such as cotyledons, placental membranes, fetal lung or liver, or vaginal swabs, that may be subject to delay before laboratory isolation, should be maintained in a suitable transport medium in the interim period. For optimal recovery, such samples should be stored frozen, preferably at –80°C. The most satisfactory medium is sucrose/phosphate/glutamate or SPG medium (sucrose [74.6 g/litre], KH_2PO_4 [0.52 g/litre], K_2HPO_4 [1.25 g/litre], L-glutamic acid [0.92 g/litre]) supplemented with bovine serum albumin – fraction V (1 g/litre), antibiotics (streptomycin and gentamycin are suitable, but not penicillin), and a fungal inhibitor. A tissue-to-medium ratio of 1:10 is commonly employed. Alternatively, approximately 1 g of tissue can be ground with sterile sand in 8 ml of transport medium.

Chlamydia abortus of ovine origin can be isolated in a variety of cell types. McCoy, Buffalo Green Monkey (BGM) or baby hamster kidney (BHK) cells are most commonly used. For confirmatory diagnosis, cultured cell monolayers are suspended in growth medium at a concentration of 2×10^5 cells/ml. Aliquots of 2 ml of the suspension are dispensed into flat-bottomed vials, each containing a single 12 mm coverslip. Confluent coverslip monolayers are achieved after incubation at 37°C for 24 hours. The growth medium is removed and replaced with 2 ml of test inoculum, which is then centrifuged at 2500–3500 ***g*** for 30–60 minutes onto the coverslip monolayer and incubated at 37°C and 5% CO_2 for 2 hours. The inoculum is removed and replaced with serum-free or cycloheximide (0.5 μg/ml) containing tissue culture medium, and then incubated at 37°C for 2–3 days. The coverslip monolayers are fixed in methanol and stained using Giemsa or Gimenez procedures (Arens & Weingarten, 1981; Gimenez, 1964), or are detected by immunofluorescence using species- or genus-specific antibodies (Sachse *et al.,* 2009). After methanol fixation, infected cultures contain basophilic (Giemsa) or eosinophilic (Gimenez) fluorescent intracytoplasmic inclusions. Similar procedures are used in culturing *C. abortus* for antigen preparation.

1.3. Isolation of the agent – chicken embryos

Test samples are prepared as 10% suspensions in nutrient broth containing streptomycin (not penicillin) (200 μg/ml); 0.2 ml of suspension is inoculated into the yolk sac of 6- to 8-day old embryos, which are then further incubated at 37°C. Infected embryos die between 4 and 13 days after inoculation. Smears prepared from their vascularised yolk sac membranes reveal large numbers of elementary bodies.

1.4. Antigen detection in tissue sections

In histopathological sections, antigen detection can be performed using commercially available anti-*Chlamydiaceae* antibodies directed against lipopolysaccharide (LPS) or MOMP (major outer membrane protein) (Borel *et al*., 2006). Immunohistochemistry is an indispensable tool to show the association of chlamydial agent and pathological lesions in tissues. Genus- or species-specific antibodies in combination with streptavidin–biotin are used to detect the chlamydial antigen within histological lesions of the placenta or inner organs (mostly lung and liver) of aborted fetuses (Sachse *et al*., 2009).

Intracellular chlamydial inclusions can be demonstrated by Giemsa staining of thin (≤4 μm) sections taken from target tissues that have been suitably fixed in fluids such as Bouin or Carnoy. However, unambiguous immunological staining procedures as described above are more suitable.

1.5. Detection of DNA by conventional PCR, real-time PCR and DNA microarray

Amplification of chlamydial DNA by PCR for verifying the presence of chlamydiae in biological samples is the method of choice because of high sensitivity and specificity of PCR. Conventional PCR protocols for *C. abortus* DNA detection target the 16S–23S rRNA region (Everett & Andersen, 1999) or *pmp* genes (Laroucau *et al*., 2001) and can be combined with restriction fragment length polymorphism (RFLP) analysis for discriminating between amplified DNA sequences originating from *C. abortus, C. psittaci* and *C. pecorum*.

Real-time PCR has become the preferred method in diagnostic laboratories due to its high specificity, rapidity, high throughput and ease of standardisation (Sachse *et al.,* 2009). A hierarchical approach is recommended including a *Chlamydiaceae*-specific screening PCR based on the sequences of 23S rRNA (Ehricht *et al*., 2006), and, in positive cases, followed by a *C. abortus*-specific PCR assay based on sequences of the outer membrane protein (*omp*A) (Livingstone *et al.*, 2009; Pantchev *et al.*, 2009) or DNA microarray hybridisation assays (Sachse *et al*., 2005). Both real-time PCR and DNA microarray have been validated for the direct detection and identification of organisms from clinical samples (Borel *et al.,* 2008; Pantchev *et. al*., 2010).

PCR assays in combination with RFLP analysis or HRM (high resolution melting) analysis have been developed with the aim of differentiating naturally infected from vaccinated animals (DIVA) (Laroucau *et al*., 2010; Vorimore *et al*., 2012; Wheelhouse *et al.,* 2010).

***Table 1. Real-time PCR assays for screening and specification of* C. abortus**

Reference	Ehricht *et al.,* 2006	Livingstone *et al.,* 2009	Pantchev *et al.,* 2009
Specificity	*Chlamydiaceae*	*C. abortus*	*C. abortus*
Target	23S rRNA	*omp*A	*omp*A
Amplicon size	111 bp	86 bp	82 bp
Primer forward 5'–3'	CTG-AAA-CCA-GTA-GCT-TAT-AAG-CGG-T	GCG-GCA-TTC-AAC-CTC-GTT	GCA-ACT-GAC-ACT-AAG-TCG-GCT-ACA
Primer reverse 5'–3'	ACC-TCG-CCG-TTT-AAC-TTA-ACT-CC	CCT-TGA-GTG-ATG-CCT-ACA-TTG-G	ACA-AGC-ATG-TTC-AAT-CGA-TAA-GAG-A
Probe 5'–3'	FAM-CTC-ATC-ATG-CAA-AAG-GCA-CGC-CG-TAMRA	FAM-TGT-TAA-AGG-ATC-CTC-CAT-AGC-AGC-TGA-TCA-G-TAMRA	FAM-TAA-ATA-CCA-CGA-ATG-GCA-AGT-TGG-TTT-AGC-G-TAMRA
Cycling conditions	95°C/10 minutes 45 × (95°C/15 seconds, 60°C/60 seconds)	95°C/10 minutes 45 × (95°C/15 seconds, 60°C/60 seconds)	95°C/10 minutes 45 × (95°C/15 seconds, 60°C/60 seconds)

2. Serological tests

Sheep and goats are generally tested serologically within 3 months of abortion or parturition. Infection is evident through *C. abortus*-specific antibody response principally during active placental invasion by the pathogen in the last month of gestation and following the bacteraemia that often accompanies abortion. Consequently, serum collected after abortion will reveal an elevated antibody titre resulting from current or previous infection.

2.1. ELISA

Several ELISAs are commercially available for *Chlamydia* diagnosis in ewes (overview in Sachse *et al.,* 2009). Care must be taken to select an appropriate ELISA for each diagnostic problem considering different specificities and sensitivities. LPS or EB (elementary body) antigen-based ELISAs cannot differentiate between animals infected with *C. pecorum* and *C. abortus*, but were proven to be more

sensitive primary screening tools for EAE compared with the CF test. Specific detection of anti-*C. abortus* antibodies can be accomplished by the use of ELISAs based on synthetic peptides of MOMP, recombinant MOMP (Salti-Montesanto *et al*., 1997), or POMP90 (polymorphic outer membrane protein) (Longbottom *et al*., 2002; Wilson *et al*., 2009). Most recently, a new indirect ELISA based on POMP90 has been commercialised and shown to be both sensitive and specific for *C. abortus,* in particular in differentiating animals infected with *C. pecorum* (Anon, 2015; Essig & Longbottom, 2015).

2.2. Complement fixation test

Complement fixation (CF) has traditionally been the most widely used procedure for detecting EAE. However, antigenic cross-reactivity between *C. abortus* and *C. pecorum*, which is endemic in small ruminants, as well as with some Gram-negative bacteria (e.g. *Acinetobacter*), can give rise to false-positive CF test results. This is because chlamydial antigen contains LPS as an immunodominant component, which is common to all *Chlamydiaceae* species. Furthermore, CF has been shown to be less sensitive than alternative tests. Therefore, CF is no longer recommended as the method of choice for serological diagnosis of EAE, but might be used for herd diagnosis when no alternative tools are available and the limitations mentioned above are taken into consideration.

Antigen is prepared from heavily infected yolk sac membranes obtained from chicken embryos that have been inoculated in the same manner as for isolation of the organism from field material. The preparation of the antigen should be carried out in a biosafety cabinet with the appropriate biosecurity precautions to prevent human infection (see chapter 1.1.4). Chopped and ground membranes are suspended in phosphate buffer, pH 7.6, at the rate of 2 ml per g membrane. After removal of crude debris, the supernatant fluid is centrifuged at 10,000 ***g*** for 1 hour at 4°C, the deposit is resuspended in a small volume of saline, and a smear of this is examined to ensure a high yield of chlamydiae. The suspension is held in a boiling water bath for 20 minutes, or is autoclaved, and sodium azide (0.3%) is added as a preservative. Antigen may also be prepared from cell cultures infected with *C. abortus*. Infected monolayers are suspended in phosphate buffer, pH 7.6, and the cells are disrupted by homogenisation or ultrasonication. Gross debris is removed and subsequent procedures are as for the preparation of antigen from infected yolk sacs. In either case, CF tests with standardised complement and antisera will establish the optimal working dilution for each batch of antigen. Antigen for CF testing of ruminant sera is commercially available.

Samples are tested at twofold dilutions from 1/32 to 1/512. CF titres are expressed as the highest serum dilution giving 50% or less haemolysis: 50% haemolysis is graded 2+, and 0% haemolysis is graded 4+. A titre of 4+ at a dilution of 1/32 or greater is assumed to be positive, whereas a titre of 2+ at a dilution of 1/32 is assumed to be equivocal (Stamp *et al*., 1950).

None of the serological tests available to date can differentiate vaccination titres from those acquired as a result of natural infection (DIVA tests).

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Currently, two types of vaccine (inactivated and attenuated live vaccines) are available commercially, to be administered intramuscularly or subcutaneously at least 4 weeks before breeding to aid in the prevention of abortion. A multi-component recombinant vaccine against *C. abortus* remains a future goal of chlamydial vaccine research (Longbottom & Livingstone, 2006).

Inactivated vaccines can be prepared from infected yolk sacs or cell cultures (Jones *et al*., 1995) and incorporate whole organisms or fractions of them (Tan *et al*., 1990) using the appropriate biosecurity precautions to prevent human infection (see chapter 1.1.4). Operator care should be observed in handling commercial inactivated vaccines that incorporate mineral oil-based adjuvants, as self-injection can result in severe local inflammation and tissue necrosis. The commercial live attenuated vaccine is based on a chemically induced temperature-sensitive mutant strain of the organism that grows at 35°C but not at 39.5°C, the body temperature of sheep (Rodolakis, 1986). This vaccine is

supplied lyophilised and must be reconstituted in diluent immediately before administration. Operator care should be observed in handling and administering this live vaccine, particularly by immunocompromised individuals and pregnant women. Importantly, the live vaccine must not be given to animals being treated with antibiotics, particularly tetracyclines. Inactivated vaccines are safe for administration during pregnancy, whereas live vaccines cannot be used in pregnant animals.

Both types of vaccine have a role to play in controlling disease, but neither confers absolute protection against challenge or completely reduces the shedding of infective organisms. However, vaccinates exposed to infection do experience significantly lower abortion rates and reduced excretion of chlamydiae for at least two to three lambings after vaccination. It has been claimed that the live vaccine could be an aid to eradication of disease (Nietfeld, 2001). In addition, the live vaccine strain 1B has been detected in the placentas of vaccinated animals that have aborted as a result of OEA, suggesting a possible role for the vaccine in causing disease (Wheelhouse *et al.,* 2010), but despite this the use of live vaccine remains the most effective method of protecting from the disease (Essig & Longbottom, 2015; Stuen & Longbottom, 2011).

Vaccine stored under refrigeration (5±3°C) should remain stable for at least 1 year. No firm data are available, but revaccination is recommended every 1–3 years, according to the exposure risk.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

One or more ovine abortion isolates that consistently grow productively in the chosen substrate are suitable, and an early passage of the seed stock can be established. Alternatively, an isolate that has been adapted to the chicken embryo by multiple passage (>100) can be used. Although adaptation to the embryo may diminish the isolate's virulence for sheep, there is no evidence that such change reduces its protective efficacy as an inactivated vaccine.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Before inoculation of large numbers of embryos or cell cultures, the viability and freedom from contamination (e.g. other pathogens, fungi, mycoplasma, toxins, etc.) of seed stock should be verified. It may be convenient to collect the total harvest in separate manageable lots. In this case, the infectivity of an aliquot of each lot should be separately titrated to ensure that each matches the requirements (see below). Store under refrigeration.

2.2. Method of manufacture

2.2.1. Procedure

For production, cell monolayers or chicken embryos are infected with *C. abortus*. Once the final harvest suspension is obtained, an aliquot is removed for titration of its infectivity. The bulk is treated with formalin to a final concentration of 4%, and stored until sterility tests confirm complete inactivation.

2.2.2. Requirements for substrates and media

The inactivated harvest is centrifuged and resuspended in phosphate buffered saline containing 0.2% formalin to a volume representing a preinactivation infectivity titre of approximately 10^8 infectious units/ml. Usually, the aqueous suspension is blended with an oil adjuvant, either directly or after precipitation by potassium alum ($AlK[SO_4]_2.12\ H_2O$). A preservative, such as 0.01% thiomersal, may also be added.

2.2.3. In-process controls

The main requirements are to ensure adequate growth of *C. abortus*, avoidance of extraneous infection of the culture substrate, completeness of inactivation and biohazard awareness by process workers.

2.2.4. Final product batch tests

Each separate batch of manufactured vaccine should be tested for sterility, safety and potency.

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

Subcutaneous inoculation into two or more seronegative sheep of twice the standard dose of manufactured vaccine should elicit no systemic reaction, but oil-adjuvant vaccines can cause a nonharmful swelling at the inoculation site.

iii) Batch potency

At present, potency is judged by the occurrence of a serological response in previously unvaccinated sheep given 1 ml of vaccine subcutaneously. Blood samples taken before and 28 days after vaccination are compared. Ultimately, potency has to be determined by a controlled vaccination-challenge study or field performance. No *in-vitro* correlation of protective efficacy has yet been established.

2.3. Requirements for authorisation

2.3.1. Safety requirements

See Chapter 1.1.8 *Principles of veterinary vaccine production*.

2.3.2. Efficacy requirements

See chapter 1.1.8.

2.3.3. Stability

See chapter 1.1.8.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

No biotechnology-based vaccines are currently in use for this disease.

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* *

NB: There are WOAH Reference Laboratories for enzootic abortion of ewes
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for enzootic abortion of ewes

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.8.6.

NAIROBI SHEEP DISEASE

See Chapter 3.10.1. Bunyaviral diseases of animals (*excluding Rift Valley fever and Crimean–Congo haemorrhagic fever*)

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CHAPTER 3.8.7.

OVINE EPIDIDYMITIS (*BRUCELLA OVIS*)

SUMMARY

Brucella ovis *infects sheep causing a clinical or subclinical disease that is characterised by genital lesions and reduced fertility in rams, placentitis and abortions in ewes, and increased perinatal mortality in lambs. The disease has been reported in American and European countries as well as Australia, New Zealand and South Africa, but probably occurs in most sheep-raising countries.*

Identification of the agent: *Clinical lesions (epididymitis and orchi-epididymitis) in rams may be indicative of the existence of infection, but laboratory examinations are required to confirm the disease. Laboratory confirmation may be based on direct or indirect methods. Direct diagnosis is made by means of bacteriological isolation of* B. ovis *from semen samples or tissues of rams, or vaginal discharges, milk and tissues of ewes, on adequate selective media. Molecular methods have been developed for complementary identification based on specific genomic sequences. Polymerase chain reaction (PCR) based methods can provide additional means of detection. However, indirect diagnosis based on serological tests is preferred for routine diagnosis.*

Serological tests: *The complement fixation test (CFT), agar gel immunodiffusion (AGID) test and indirect enzyme-linked immunosorbent assays (I-ELISA) using soluble surface antigens obtained from the* B. ovis *REO 198 strain, should be used for diagnosis. The sensitivities of the AGID test and I-ELISA are similar and may be higher than that the CFT. A combination in parallel of the AGID test and I-ELISA seems to give the best results in terms of sensitivity, but with regard to simplicity and cost, the AGID test is the most practicable test for diagnosing* B. ovis *in non-specialised laboratories. However, because of the lack of standardised methods recognised at the international level for AGID and I-ELISA, the test that is most suitable for certifying individual animals prior to movement, including for international trade, remains the CFT.*

Requirements for vaccines: *Seed cultures for vaccine production should be obtained from internationally recognised laboratories. A single standard dose (10^9 colony-forming units) of the live* B. melitensis *Rev.1 vaccine, administered subcutaneously or, better, conjunctivally, can be used safely and effectively in rams, for the prevention of* B. ovis *infection. This vaccine strain should meet minimal quality standards: safe in the host animal, of adequate concentration, with absence of dissociation, adequate residual virulence and possess immunogenicity (efficacy) and be free of extraneous agents (see Chapter 3.1.4 Brucellosis* [Brucella abortus, B. melitensis *and* B. suis]).

A. INTRODUCTION

1. Definition of the disease

Brucella ovis produces a disease unique to sheep and is one of the most common causes of epididymitis in rams and a rare cause of infertility and abortion in ewes and neonatal mortality in lambs.

2. Causal pathogen

Brucella ovis and *B. canis* are the two presently known *Brucella* species naturally in the rough phase. *Brucella ovis* is similar to the other *Brucella* spp. in its morphology, staining properties and cultural characteristics, except that it gives negative reactions to the oxidase and urease tests. The microbiological and serological properties of all

Brucella species and biovars are given in detail in the Chapter 3.1.4 *Brucellosis* (Brucella abortus, B. melitensis *and* B. suis).

3. Description of the disease

Brucella ovis infects sheep causing genital lesions (epididymitis and orchi-epididymitis) and infertility in rams, placentitis, abortions and infertility in ewes, and increased perinatal mortality in lambs. *Brucella ovis* is usually excreted in semen in infected rams. Passive venereal transmission via the ewe appears to be the most frequent route of infection, but ram-to-ram transmission is also very common (Blasco, 1990; 2010). Under the semi-extensive production systems (most common in European Mediterranean countries), rams are usually housed together. Direct ram-to-ram transmission during non-breeding periods is thus quite frequent and has been suggested to take place by several routes, including anal intercourse and, more frequently, through oral-genital contact (preputial licking).

Moreover, infected ewes may excrete *B. ovis* in vaginal discharges and milk and, accordingly, ewe-to-ram and lactating ewe-to-lamb transmission could also be determinant mechanisms of infection. Accordingly, ewes should be considered relevant in the epidemiology of infection, and this should be taken into account for the effective eradication of *B. ovis* in infected flocks (Blasco, 2010; Grilló *et al.,* 1999).

The disease has been reported in American and European countries as well as Australia, New Zealand and South Africa, but probably occurs in most sheep-raising countries.

The demonstration of genital lesions (unilateral or bilateral epididymitis and orchi-epididymitis) by palpating the testicles of rams may suggest the presence of this infection in a given flock. However, clinical diagnosis lacks sensitivity because not all rams infected with *B. ovis* present palpable genital lesions (Blasco, 1990). Moreover, clinical diagnosis lacks specificity since many other bacteria may cause genital lesions in rams. The most frequently reported pathogens such lesions in rams include *Actinobacillus seminis*, *A. actinomycetemcomitans*, *Histophilus ovis*, *Haemophilus* spp., *Corynebacterium pseudotuberculosis ovis*, *B. melitensis*, *Chlamydia abortus* and *Pasteurella* spp. (Bulgin & Anderson, 1983; Garcia-Pastor *et al.,* 2009; Livingstone & Hardy, 1964). Moreover, it must be emphasised that many palpable testicular lesions in rams are sterile, trauma-induced spermatic granulomas.

Although cattle, goats and deer have been proved susceptible to *B. ovis* in artificial transmission experiments, natural cases have been reported only in red deer reared in close contact with infected rams (Ridler *et al.,* 2012).

4. Zoonotic risk and biosafety requirements

To date, no human cases have been reported, and *B. ovis* is considered to be non-zoonotic. However, in areas where *B. melitensis* infection co-exists with *B. ovis*, special care is required when handling samples, which should be transported to the laboratory in leak-proof containers (for further details see Chapter 3.1.4 Brucellosis (*Brucella abortus, B. melitensis* and *B. suis*) and Chapter 1.1.3 *Transport of biological materials*). All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of infection with **Brucella ovis**

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement[a]	Contribute to eradication policies[b]	Confirmation of clinical cases[c]	Confirmation of suspect cases[d]	Herd/flock prevalence of infection – Surveillance
Identification of the agent[e]						
Staining methods	–	–	–	+	–	–
Culture	–	–	–	+++	+/++[d]	–
PCR[f]	–	–	–	+/++	+/++	–
Detection of immune response						
CFT	+++	+++	+++	++	++	++
I-ELISA	+++	+++	+++	++	++	+++
AGID	++	++	+++	++	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; CFT = complement fixation test; I-ELISA = indirect enzyme-linked immunosorbent assay; AGID = agar gel immunodiffusion test.
[a]This applies only to herds/flocks, countries or zones free from infection with *Brucella ovis*.
[b]To improve the efficiency of eradication policies in infected herds/flocks it is recommended to associate tests in parallel to increase the sensitivity of the diagnosis, i.e. two serological tests at least, e.g. CFT (or AGID) and I-ELISA;
[c]In low-prevalence or almost-free zones, the predictive value of positive results to serological tests may be very low. In such situation, the agent identification is usually needed for confirming clinical cases. In infected herds/flocks, a positive result to any serological test may be considered as a confirmation of a clinical case.
[d]In infected herds/flocks, any reactor in any serological test should be considered as infected. In low-prevalence or almost-free zones, singleton serological reactors may be confirmed by culture (and/or PCR). In free countries or zones, suspect animals are those positive to both a screening and a confirmatory serological test (tests in series, e.g. I-ELISA and CFT respectively) and should be confirmed by culture (and/or PCR).
[e]A combination of agent identification methods applied on the same clinical sample is recommended.
[f]False-positive results may occur.

1. Identification of the agent

1.1. Collection of samples

The most valuable samples for the isolation of *B. ovis* from live animals are semen, vaginal swabs and milk. For the collection of vaginal swabs and milk, see the instructions given in Chapter 3.1.4. Semen (genital fluids) can be collected in swabs taken from the preputial cavity of rams after electro-ejaculation. Alternatively, swabs can be taken directly from the vagina of brucellosis-free ewes immediately after being mated by the suspect ram. Clinically or sub-clinically infected rams may excrete *B. ovis* intermittently in their semen for years (Blasco, 2010). Vaginal swabs taken after abortion or premature lambing and milk samples are highly recommended samples to isolate *B. ovis* from infected ewes (Grilló *et al.,* 1999).

For the isolation of *B. ovis* after necropsy, the preferred organs in terms of probability of isolation are the epididymides, seminal vesicles, ampullae, and inguinal lymph nodes in rams, and the uterus, iliac and supra-mammary lymph nodes in ewes. However, to obtain maximum sensitivity, a complete search that

includes other organs and lymph nodes (spleen, cranial, scapular, pre-femoral and testicular lymph nodes) should be performed (Blasco, 2010). Dead lambs and placentas can also be examined. The preferred culture sites in aborted or stillborn lambs are abomasal content and lung.

Samples for culture should be refrigerated and transported to the laboratory to be cultured as soon as possible after collection. The organism remains viable for 48–72 hours at room temperature but if culture has to be delayed survival is enhanced by refrigerating or, preferably, freezing the tissue samples.

1.2. Staining methods

Semen or vaginal smears from clinically affected animals can be examined following staining by Stamp's method (Alton *et al.,* 1988) (see Chapter 3.1.4), and characteristic coccobacilli can be demonstrated in many infected animals. Examination of Stamp-stained smears of suspect tissues (ram genital tract, inguinal lymph nodes, placentas, and abomasal content and lung of fetuses) may also allow a rapid presumptive diagnosis. However, other bacteria with similar morphology or staining characteristics (*B. melitensis*, *Coxiella burnetii*, and *Chlamydia abortus*) can also be present in such samples, making the diagnosis difficult for inexperienced personnel. For such reason, microscopy results should always be confirmed by culture of the microorganism.

1.3. Culture

Due to its specificity, the isolation and identification of *B. ovis* in sheep fluids and tissues is the best direct method of diagnosis and, if positive, the only incontestable demonstration of *B. ovis* infection in a given animal or flock. Semen, vaginal swabs, or milk samples can be smeared directly onto plates containing adequate culture media and incubated at 37°C ± 2°C in an atmosphere of 5–10% CO_2. Tissues should be macerated and ground in a small amount of sterile saline or phosphate buffered saline (PBS) with a stomacher or blender, before plating. It is important to take into account that the larger the amount of tissue homogenates and the higher number of culture plates inoculated per diagnostic sample, the higher will be the final diagnostic sensitivity obtained.

Growth normally appears after 3–4 days of incubation, but cultures should not be discarded as negative until 7 days have elapsed. Colonies of *B. ovis* become visible (0.5–2.5 mm) after 3–4 days of incubation, and are in rough phase, round, shiny and convex.

Brucella ovis can be isolated in non-selective media, such as blood agar base enriched with 10% sterile ovine or bovine serum, or in blood agar medium with added 5–10% sterile ovine blood. However, since primary isolation requires 4–7 days of incubation, overgrowing fungi and commensal and environmental bacteria frequently contaminate the non-selective culture plates, and result in a reduced diagnostic sensitivity. Thus, the use of selective culture media is of paramount importance for a proper bacteriological diagnosis of *B. ovis* infection. The modified Farrell's selective medium used widely for the isolation of the smooth *Brucella* (see Chapter 3.1.4), inhibits the growth of *B. ovis* and should not be used (Marin *et al.,* 1996). Various selective media have been described, but modified Thayer–Martin's (mTM) medium (Marin *et al.,* 1996) has been used classically for isolating *B. ovis*. Briefly, this medium can be prepared with GC medium base (38 g/litre Difco, USA) supplemented with haemoglobin (10 g/litre) and colistin methane-sulphonate (7.5 mg/litre), vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre = 17.7 mg) and amphotericin B (2.5 mg/litre). Working solutions are prepared as follows:

Solution A: Add 500 ml of distilled water to the GC medium base, heat carefully to avoid burning the medium while stirring continuously and autoclave at 120°C for 20 minutes.

Solution B: Suspend the haemoglobin in 500 ml of purified water, adding the water slowly to avoid lumps. Once dissolved, add a magnetic stirrer and autoclave at 120°C for 20 minutes.

Antibiotic solutions (*prepared freshly*): colistin, nystatin and vancomycin are suspended in a mixture of methanol/water (1/1); nitrofurantoin is suspended in 1 ml of a 0.1 M NaOH sterile solution. For amphotericin B, it is recommended to prepare a stock solution of 10 mg/ml amphotericin B with 10 mg dissolved first in 1 ml sterile dimethyl sulphoxide (C_2H_6OS, analytical grade) and then added to 9 ml of sterile PBS (10 mM, pH 7.2 ± 0.1). Any stock solution remaining can be stored 5 days at 5°C ± 3°C. All

antibiotic solutions must be filtered through 0.22 μm filters before addition to the culture medium. Another suitable, but less effective, antibiotic combination can be: vancomycin (3 mg/litre); colistin (7.5 mg/litre); nystatin (12,500 IU/litre); and nitrofurantoin (10 mg/litre).

Once autoclaved, stabilise the temperature (45–50°C) of both solutions A and B with continuous stirring. Mix both solutions (adding A to B), avoiding bubble formation. Add the antibiotic solutions while stirring continuously and carefully, then distribute into sterile plates. Once prepared, the plates should not be stored for long periods, and freshly prepared medium is always recommended.

However, the mTM is not translucent due to the haemoglobin incorporated as a basal component, being thus unsuitable for the direct observation of colonial morphology. This has important practical consequences since this is probably the most widely used procedure for the presumptive identification of *Brucella* (Alton *et al.,* 1988). Having this in consideration, a new culture medium (named CITA) has been recently formulated using blood agar base as a basal component, and supplemented with 5% of sterile calf serum and the following antibiotics: vancomycin (20 mg/litre), colistin methanesulfonate (7.5 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 IU/litre), and amphotericin B (4 mg/litre). This antibiotic mixture can be prepared as indicated above for the preparation of the mTM medium. This new CITA medium inhibits most contaminant microorganisms but allows the growth of all *Brucella* species. Moreover, CITA medium outperforms mTM for isolation of *B. ovis*, and is more sensitive than both mTM and Farrell's media for isolating all smooth *Brucella* species from field samples, and is therefore the selective medium of choice for general *Brucella* isolation (De Miguel *et al.,* 2011).

All culture media used should be subjected to quality control with reference strains, to demonstrate that it performs properly.

1.4. Identification and typing

Brucella ovis colonies are not haemolytic. They are circular, convex, have unbroken edges, are always of the rough type when examined by oblique illumination, and test positive in the acriflavine test (Alton *et al.,* 1988). For growth, *B. ovis* needs an incubating atmosphere containing 5–10% CO_2. It lacks urease activity, fails to reduce nitrate to nitrite, and is oxidase negative. It does not produce H_2S and, although it does not grow in the presence of methyl violet, it usually grows in the presence of standard concentrations of thionin. The cultures are not lysed by *Brucella*-phages of the Tbilisi (Tb), Weybridge (Wb) and Izatnagar (Iz_1) groups at the routine test dilution (RTD) or 10^4 RTD, while they are lysed by phage R/C (Alton *et al.,* 1988). Most laboratories are not equipped enough for a complete identification of *Brucella* at species and biovar levels, and a practical schedule for presumptive identification is needed. Most *B. ovis* isolates can be correctly identified on the basis of growth characteristics, direct observation using obliquely reflected light, Gram or Stamp's staining, catalase, oxidase, urease and acriflavine tests. However, it is recommended that the definitive identification be carried out by reference laboratories with experience in identification and typing of *Brucella.*

The polymerase chain reaction (PCR) and other recently developed molecular methods provide additional means of detection and identification of *Brucella* sp. (see Chapter 3.1.4), and are becoming routine in many diagnostic laboratories. The existence of semen samples heavily contaminated with overgrowing organisms or containing dead *B. ovis*, could also justify the use of PCR as a supplementary direct diagnostic test. In fact, several PCR procedures have been reported to have similar sensitivity to standard bacteriological culture when applied to semen samples from *B. ovis* infected rams (Xavier *et al.,* 2010). However, the sensitivity and specificity of these PCR-based direct diagnostic procedures remain to be properly determined on other clinical samples and, for the moment, classical bacteriology should be considered the method of choice for the bacteriological diagnosis of *B. ovis*. By contrast, the use of the Bruce-ladder multiplex PCR (see Chapter 3.1.4) on DNA samples extracted from culture plate colonies is a rapid and highly specific procedure for the proper identification of most *Brucella* species including *B. ovis*.

2. Serological tests

The most efficient and widely used tests are the complement fixation test (CFT), the double agar gel immunodiffusion (AGID) test and the indirect enzyme-linked immunosorbent assay (I-ELISA). Several countries have adopted various standard diagnostic techniques for *B. ovis*, but the only test that is recognised by WOAH and the European Union (EU) as the test that is most suitable for certifying individual animals prior to movement,

including for international trade is the CFT. However, it has been demonstrated that the AGID test shows similar sensitivity to the CFT, and it is a simpler test to perform. Moreover, although international standardisation is lacking, numerous independent validation studies have shown that the I-ELISA is more sensitive than either the CFT or AGID test. AGID test and I-ELISA have been reported as more sensitive than the CFT. Conversely I-ELISA was sometimes reported as a less specific method, but this greatly depends on the protocol used (Estein *et al.,* 2002; Nielsen *et al.,* 2004; Praud *et al.,* 2012).

The International Standard anti-*Brucella ovis* Serum (ISaBoS, International Standard 1985[1]) is the one against which all other standards are compared and calibrated. This reference standard is available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.

HS antigens for use in serological tests should be prepared from. *Brucella ovis* strain REO 198[2] is CO_2- and serum-independent.

2.1. Antigens

When rough *Brucella* cells are heat-extracted with saline (hot-saline method, HS), they yield water-soluble antigenic extracts, the major component of which precipitates with sera to rough *Brucella* (Diaz & Bosseray, 1973; Myers *et al.,* 1972). For this reason, the HS extract has been referred to as the 'rough-specific antigen' or, when obtained from *B. ovis*, as the *'B. ovis*-specific antigen'. However, chemical characterisation of the HS extract from *B. ovis* has shown that it is enriched in rough lipopolysaccharide (R-LPS), group 3 outer membrane proteins and other outer membrane components (Riezu-Boj *et al.,* 1986). Thus, the HS extract contains LPS determinants specific for *B. ovis*, but also additional antigenic epitopes, some of them being shared by rough and smooth *Brucella* (Santos *et al.,* 1984). Such epitopes account for the cross-reactivity that is sometimes observed with the HS method and sera of sheep infected with *B. melitensis* or vaccinated with *B. melitensis* Rev.1 (Riezu-Boj *et al.,* 1986). The HS extract is the most widely and currently used for the serological diagnosis of *B. ovis* infection. Its water solubility and high content in relevant cell surface epitopes explain its good performance in *B. ovis* serological tests. However, in areas where *B. melitensis* infection also exists or vaccination with *B. melitensis* Rev.1 is applied in sheep, the specificity of the diagnosis with regard to *B. ovis* has to be carefully interpreted taking into account the results of serological tests for smooth *Brucella* (Blasco, 2010).

Solid basal non-selective media described in Section B.1.3 are satisfactory for the growth of *B. ovis* REO 198.

2.1.1. Preparation of HS antigen

i) Exponentially grow the REO 198 *B. ovis* strain in one of the following ways: for 48 hours in trypticase–soy broth flasks in an orbital incubator at 37 C ± 2 C and 150 rpm; or in Roux bottles of trypticase–soy agar, or other suitable medium; or in a batch-type fermenter as described for *B. abortus.* Addition of 5% serum to the medium is optional as the REO 198 *B. ovis* strain is serum-independent.

ii) Cells are resuspended in 0.85% sterile saline or PBS, and then washed twice in 0.85% sterile saline (12 g of dried cells or 30 g of wet packed cells in 150 ml).

iii) The cell suspension is then autoclaved at 120°C for 15–30 minutes.

iv) After cooling, the suspension is centrifuged (15,000 ***g***, 5°C ± 3°C, 15 minutes) and the supernatant fluid is filtered and dialysed against purified water using 100 times the volume of the suspension, at 4°C; the water should be changed three times over a minimum of 2 days.

v) The dialysed fluid can be ultracentrifuged (100,000 ***g***, 4°C, 6–8 hours), and the sediment is resuspended in a small amount of purified water and freeze-dried. When produced to be

1 Obtainable from the WOAH Reference Laboratory for brucellosis in the United Kingdom: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3.

2 Obtainable from the WOAH Reference Laboratory for brucellosis in France.

used in the CFT, the addition of control process serum replacement II (CPSRII) prior to freeze-drying may assist in stability and anti-complementary activity.

HS is then resuspended either in purified water (for use in the AGID test), veronal buffered saline (for use in the CFT), or carbonate/bicarbonate buffer (for use in the I-ELISA) and titrated accordingly.

If it is to be used in the AGID test, the resuspended HS may be kept at 5°C ± 3°C adding optionally 0.5% phenol as preservative. Freezing and thawing of antigen suspensions should be avoided (Diaz & Bosseray, 1973).

The CFT antigen should be standardised against the ISaBoS to give 50% fixation at a 1/100 serum dilution. It must be emphasised that each CFT antigen batch must be titrated with the CFT procedure that is to be followed for the routine test. Therefore before using a CFT antigen (commercial or in-house) in a particular CFT procedure, the laboratory should ensure that the antigen titre has been established with the same CFT procedure.

In the absence of well-established standardisation rules, the I-ELISA and AGID antigens should be titrated against a set of appropriate positive and negative sera.

2.1.2. Standardisation of the I-ELISA

The following criteria for standardisation of the I-ELISA have been used in a recent work in which the I-ELISA has been validated in comparison with the CFT (Praud *et al.,* 2012):

i) A 1/64 pre-dilution of the ISaBoS made up in a negative serum (or in a negative pool of sera) must give a positive reaction;

ii) A 1/256 pre-dilution of the ISaBoS made up in a negative serum (or in a negative pool of sera) must give a negative reaction.

These criteria still need to be validated through an international ring-trial.

In any case, I-ELISA commercial or in-house kits must have been validated according to Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*.

2.2. Complement fixation test

There is no standardised method for the CFT and the use of an International Standard serum is therefore recommended (see Section B.2.2.2). The test is most conveniently carried out using the microtitration method. Some evidence shows that cold fixation is more sensitive than warm fixation (Ris *et al*., 1984), but that it is less specific. Anticomplementary reactions, common with sheep serum, are, however, more frequent with cold fixation.

Several methods have been proposed for the CFT using different concentrations of fresh sheep red blood cells (SRBCs) (a 2–3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-SRBC serum diluted to contain several times (usually from two to five times) the minimum concentration required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea-pig complement. The latter is independently titrated (in the presence or absence of antigen according to the method) to determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in a unit volume of a standardised suspension; these are defined as the 50% or 100% haemolytic unit of complement ($C'H_{50}$ or $C'H_{100}$), respectively. It is generally recommended to titrate the complement before each set of tests, a macromethod being preferred for an optimal determination of $C'H_{50}$. Usually, 1.25–2 $C'H_{100}$ or 5–6 $C'H_{50}$ are used in the test.

Barbital (veronal) buffered saline (VBS) is the standard diluent for the CFT. This is prepared from tablets available commercially, otherwise it may be prepared according to the formula described elsewhere (see Chapter 3.1.4). The test sera should be inactivated for 30 minutes in a water bath at 60–63°C, and then diluted (doubling dilutions) in VBS. The stock solution of HS antigen (2.5–20 mg/ml in VBS) is diluted in VBS as previously determined by titration (checkerboard titration). Usually, only one serum dilution is tested (generally 1/10).

2.2.1. Test procedure

Using standard 96-well microtitre plates with round (U) bottom, the technique is usually performed as follows:

i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first and second rows. The first row is an anti–complementary control for each serum. Volumes of 25 µl of VBS are added to the wells of the first row (anti–complementary controls) to compensate for lack of antigen. Volumes of 25 µl of VBS are added to all other wells except those of the second row. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the third row onwards; 25 µl of the resulting mixture in the last row are discarded

ii) Volumes of 25 µl of antigen, diluted to working strength, are added to each well except wells in the first row.

iii) Volumes of 25 µl of complement, diluted to the number of units required, are added to each well.

iv) Control wells are set up to contain 75 µl total volume in each case; the wells contain

a) diluent only,

b) complement + diluent,

c) antigen + complement + diluent.

A control serum that gives a minimum positive reaction should be tested in each set of tests to verify the sensitivity of test conditions.

v) The plates are incubated at 37°C ± 2°C for 30 minutes or at 5°C ± 3°C overnight, and a volume (25 or 50 µl according to the techniques) of sensitised SRBCs is added to each well. The plates are reincubated at 37°C ± 2°C for 30 minutes.

vi) The results are read after the plates have been centrifuged at 1000 ***g*** for 10 minutes at 5°C ± 3°C or left to stand at 5°C ± 3°C for 2–3 hours at least to allow unlysed cells to settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The titre of the serum under test is the highest dilution in which there is 50% or less haemolysis.

2.2.2. Standardisation of the results of the complement fixation test

There is a unit system that is based on the International Standard for anti-*Brucella* ovis Serum (ISaBoS or International Standard 1985 [see footnote 3]). This serum contains 1000 ICFTU per ml. If this serum is tested in a given method and gives a titre of, for example 200 (50% haemolysis), then the factor for an unknown serum tested by that method can be found from the formula: 1000/200 × titre of test serum = number of ICFTU (International CFT units) of antibody in the test serum per ml. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test by the same method, to allow the same level of sensitivity and specificity to be obtained against an adequate panel of sera from *B. ovis* culture positive and *Brucella*-free sheep. Results should always be expressed in ICFTU, calculated in relation to those obtained in a parallel titration with a standard serum, which itself may be calibrated against the International Standard.

2.2.3. Interpretation of the results

Sera giving a titre equivalent to 50 ICFTU/ml or more are considered to be positive.

2.3. Enzyme-linked immunosorbent assay

Several variations of this assay have been proposed. The assay described here is an indirect I-ELISA using ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) as chromogen, but other procedures are also suitable, and several commercial kits are now available.

Tests are performed on 96-well flat-bottomed ELISA plates.

Reagent and serum dilutions are made in PBS, pH 7.2 (± 0.2), with the addition of 0.05% Tween 20 (PBST).

Antigen dilutions are made for adsorption in a carbonate/bicarbonate buffer (pH 9.6 ± 0.2). Plates are washed after antigen coating and between incubations, where appropriate, usually with PBST (see below). The antigen (HS) and conjugate are checkerboard titrated, and dilutions are selected to give the best discriminating ratio between negative and positive standard sera. Secondary antibodies (e.g. anti-ovine IgG [H+L chains]) are usually conjugated to horseradish peroxidase (HRPO), although other enzymes or conjugates (such as recombinant Protein G/HRPO) can be used. A monoclonal antibody to bovine IgG_1–HRPO conjugate has also been found to be suitable for use in the I-ELISA (Vigliocco *et al.,* 1997). If a peroxidase conjugate is used, the chromogen, usually ABTS, is diluted in a substrate buffer (composed of sodium citrate and citric acid, see below)[3]. The substrate, hydrogen peroxide (H_2O_2), is added to this, and the plates are incubated for 15–30 min at room temperature (22°C ± 4°C). The reaction may be stopped with 1 mM sodium azide or other reagents, and the colour change is read at 405–414 nm (for further details see Chapter 3.1.4).

The antigen used in the I-ELISA is the HS in stock solution at 1 mg/ml in coating buffer, titrated in a checkerboard titration manner, with different dilutions of antigen, conjugate and substrate, against a standard serum or against serial dilutions of a panel of sera from *B. ovis* culture positive and *Brucella*-free sheep to determine the most sensitive and specific working concentration. Other antigens have been reported in the literature, in particular R-LPS (Nielsen *et al.,* 2004), but its extraction is cumbersome and dangerous, and it has no particular advantage compared with the HS that is also used in CFT and AGID.

A positive and a negative control are included in each plate. OD ranges to be obtained with these two controls must be established to define the criteria for validating each plate results. The positive control OD is the one to which each test serum OD is compared to establish the final result (negative or positive).

An additional positive serum (internal control) must be included in each plate to validate the repeatability of the test from plate to plate and from day to day.

2.3.1. Test procedure (example)

i) Microtitre plates of good quality polystyrene (this is important to obtain consistent results since there are differences in adsorption among different brands) are coated by the addition of 100 µl to each well of a predetermined antigen dilution in the adsorption buffer:

Adsorption buffer (0.06 M carbonate–bicarbonate buffer, pH 9.6 ± 0.2):

a) Solution A: 0.84 g $NaHCO_3$ in 10 ml purified water.

b) Solution B: 1.06 g Na_2CO_3 in 10 ml purified water.

Mix 4.53 ml of A with 1.82 ml of B and complete with purified water to 100 ml.

Sealed plates are incubated at 37°C ± 2°C overnight, preferably. Plates are then washed four times with the washing buffer to remove unbound antigen and dried by tapping firmly upside down on an absorbent paper.

Washing buffer (0.01 M PBS, pH 7.2 ± 0.2, and containing 0.05% Tween 20):

a) Stock solution:

Solution A: Na_2HPO_4: 10.96 g in 150 ml purified water

Solution B: NaH_2PO_4 (H_2O): 3.15 g in 150 ml purified water (3.5 g in 150 ml purified water if using NaH_2PO_4 2(H_2O)

3 TMB (3,3',5,5'-tetramethylbenzidine) is also a popular chromogenic substrate for HRP detection in ELISA and is available in several formats. It is not carcinogenic.

b) Mix A and B then complete to 400 ml with purified water.

c) Washing Buffer (PBST): 40 ml of Stock solution + 8.5 g NaCl and complete to 1000 ml with purified water, adding 0.05% Tween 20.

The coated and washed plates can be used immediately or dried and stored at 5°C ± 3°C (the stability in these conditions is usually adequate for at least 1 month). Most of HS batches perform properly when used at working concentrations of 2.5–15 µg/ml in adsorption buffer.

ii) *Sera*: Dilute test and positive and negative control serum samples (1/100 -1/200 are usually the optimal working dilutions, prepared by the addition of 10 µl of serum to 1–2 ml PBST, respectively). These working serum dilutions are usually the optimal when using either polyclonal or monoclonal anti-IgG conjugates. However, lower working dilutions (usually 1/50) are the optimal when using the protein G-HRPO conjugates (Marin *et al.,* 1998). Add 100 µl/well volumes of samples in duplicate to the microtitre plates. The plates are covered or sealed, incubated at 37°C ± 2°C for 40–60 minutes, and washed three times with the PBST washing buffer.

iii) *Conjugate*: The optimal working dilution of titrated conjugate (the most widely used are the protein G or polyclonal rabbit anti-sheep IgG (H+L), both coupled to HRPO) in PBST is added (100 µl) to the wells, and the plates covered and then incubated for 40–60 minutes at 37°C ± 2°C After incubation, the plates are washed again three times with PBST.

iv) *Substrate*: There are several possibilities but the substrate most widely used[4] is usually composed by a 0.1% solution (w/v) of ABTS (2-2'-azinobis 3- ethylbenzthiazoline sulfonic acid, diammonium salt) in citrate buffer containing 0.004% H_2O_2):

Citrate buffer (0.05 M, pH 4 ± 0.2):

a) Solution A: 22.97 g citric acid ($C_6H_8O_7.H_2O$) in 1000 ml purified water.

b) Solution B: 29.41 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$) in 1000 ml purified water.

Mix 660 ml of A with 470 ml of B and complete to 2000 ml with purified water. Add then a 0.004% of good grade and fresh H_2O_2.

The substrate solution is added (100 µl/well) and the plates incubated for 15–30 minutes at room temperature with continuous shaking)

v) *Reading and interpreting the results:* Absorbance is read automatically in a spectrophotometer at 405–414 nm. Mean absorbance values may be expressed as percentages of the mean absorbance values of the positive control or, preferably, transformed into I-ELISA units calculated either manually or by using a computer and a curve-fitting program from a standard curve constructed with the series of positive control dilution results. Duplicate readings of each serum should be similar. In case of significant discrepancies, the particular serum should be retested. Before calculating the final results, each plate must be validated taking into account the OD values obtained for the positive and negative controls as well as the transformed OD of the internal control according to pre-established expected ranges of values.

The cut-off threshold to differentiate the positive and negative results should be properly established using the appropriate validation techniques (see Chapter 1.1.6) and avoiding, if possible, cut-off thresholds resulting in inconclusive results. The ISaBoS or the corresponding secondary or national standards should be used to verify or calibrate the particular test method in question as mentioned above

4 TMB (3,3',5,5'-tetramethylbenzidine) is also a popular chromogenic substrate for HRP detection in ELISA and is available in several formats. It is not carcinogenic.

2.4. Agar gel immunodiffusion test

The AGID test (Blasco, 1990) uses the following reagents: Good grade Noble agar or agarose, sodium chloride (NaCl), and borate buffer (prepared with boric acid [12.4 g]; potassium chloride [14.5 g]; purified water [1600 ml]; adjusted to pH 8.3 ± 0,02 with 0.2 M NaOH solution and made up to 2000 ml with purified water).

2.4.1. Agar gel preparation

Dissolve 1 g of agarose (or Noble agar) and 10 g of NaCl in 100 ml of borate buffer (by boiling while stirring continuously).

On a flat surface, cover clean glass slides with the necessary amount of molten gel to form a bed of 2.5 mm depth (3.5 ml approximately for standard microslides).

After the gel has solidified (15–20 minutes), wells are cut in it using a gel puncher.

The wells should be 3 mm in diameter and 3 mm apart, and should be arranged in a hexagonal pattern around a central well that is also 3 mm in diameter.

The test can be adapted to Petri dishes and other patterns.

2.4.2. Test procedure

Sera to be examined are placed in alternate wells separated by a control positive serum (infection proved by bacteriology), with the antigen at its optimum concentration in the central well.

The results are read after incubation for 24 and 48 hours at room temperature in a humid chamber.

A positive reaction is evidenced by a clearly defined precipitin line between the central well and the wells of the test sera that gives total or partial identity with that of the positive controls.

Precipitin lines not giving total identity may also appear and correspond usually to minor antigenic components of HS extracts (antibodies to these components can also be common in infections due to *B. melitensis* or in case of vaccination with Rev.1). These reactions should also be considered as positive. Before a definitive reading, it is important to wash the slides for 1 h in a 5% sodium citrate solution in purified water to clean unspecific precipitin lines.

The HS (2.5–20 mg/ml) diluted in purified water (optionally containing 0.5% phenol as a preservative) is the most widely used antigen in the AGID test (the preserved antigen can be stored refrigerated for at least 1 month). Dilutions of antigen are tested with a panel of 20–30 sera from rams naturally infected with *B. ovis* and with a panel of *Brucella*-free sheep. The optimum working concentration of antigen is the one giving the clearest precipitation lines with all control sera from *B. ovis*-infected rams, resulting simultaneously negative with the sera from *Brucella*-free animals.

Comparative studies have shown that the I-ELISA has a better sensitivity than either the AGID test or the CFT (Blasco, 2010; Gall *et al.,* 2003; Praud *et al.,* 2012; Ris *et al.,* 1984). However, due to the existence of some I-ELISA-negative but AGID (or CFT) positive sera and vice versa, the parallel combination of the AGID (or CFT) and I-ELISA results usually in optimal sensitivity and may be helpful in eradication programmes in infected zones or flocks (Blasco, 2010; Praud *et al.,* 2012).

Moreover, the CFT has other important disadvantages such as complexity, obligatory serum inactivation, anti-complementary activity of some sera, the difficulty of performing it with haemolysed sera, and prozone phenomena. Because of their sensitivity, simplicity and easy interpretation, both the I-ELISA and AGID test are therefore preferred for surveillance in free or almost-free zones.

Little is known about the existence of false positive results in *B. ovis* serological tests as a consequence of infections due to bacteria showing cross-reacting epitopes with *B. ovis*. The foot rot agent (*Dichelobacter nodosus*) has been described as responsible for serological cross-reactions with *B. ovis*, but the extent and practical consequences of this cross-reactivity in *B. ovis* diagnostic tests is not well understood. In addition, *Arcanobacterium pyogenes* and *Corynebacterium ovis*, whose soluble extracts cross-react with sera from *B. ovis* infected rams, have been isolated from several lymph nodes of rams giving strong positive responses in both *B. ovis* AGID and I-ELISA tests (Blasco, 2010; Blasco & Moriyon, unpublished results).

C. REQUIREMENTS FOR VACCINES

As both rams and ewes can play a role in the transmission of infection (Blasco, 2010; Grilló *et al.,* 1999), vaccination of both rams and ewes is probably the most economical and practical means for medium-term control of *B. ovis* in areas with a high prevalence of infection. For long-term control, consideration should be given to the effect of vaccination on serological testing, and the potential complication of the implementation of *B. ovis*-free accreditation programmes.

There is no specific vaccine for *B. ovis*, however live *B. melitensis* strain Rev.1 (described in Chapter 3.1.4 including the quality requirements) is also suitable to stimulate immunity against *B. ovis* infection (Blasco, 1990). A single standard dose (10^9 colony-forming units) of Rev.1 administered subcutaneously (in a 1 ml volume) or, better, conjunctivally (in a 25–30 µl volume), to 3–5 month-old animals confer adequate immunity against *B. ovis*. Conjunctival vaccination has the advantage of minimising the intense and long-lasting serological response evoked by subcutaneous vaccination, thereby improving the specificity of serological tests (Blasco, 1990), and facilitating the interpretation of serological results after vaccination. When used in both young and adult males, the safety of the Rev.1 vaccine has been shown to be adequate and side-effects appear to be very rare (Marin *et al.,* 1990; Muñoz *et al.,* 2008). Therefore, in countries with extensive management and high levels of prevalence, it would be advisable to vaccinate both young and healthy adult animals (see Chapter 3.1.4). In countries affected by *B. ovis* but free of *B. melitensis,* before using the Rev.1 vaccine account should be taken of possible serological interferences and the conjunctival route should be preferred to minimise this problem.

The *B. abortus* RB51 vaccine has not been proven successful against *B. ovis* in sheep (Jiménez De Bagües *et al.,* 1995), and despite the promising results obtained with new generation subcellular vaccines (Cassataro *et al.,* 2007; Da Costa Martins *et al.,* 2010; Muñoz *et al.,* 2006), none has yet been licensed for field use.

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NB: There are WOAH Reference Laboratories for ovine epididymitis (*Brucella ovis*)
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for ovine epididymitis (*Brucella ovis*)

NB: FIRST ADOPTED IN 1991 AS BRUCELLOSIS IN SHEEP, GOATS AND SWINE; CHAPTER FIRST ADOPTED WITH CURRENT TITLE IN 1996.
MOST RECENT UPDATES ADOPTED IN 2015.

CHAPTER 3.8.8.

OVINE PULMONARY ADENOCARCINOMA (ADENOMATOSIS)

SUMMARY

Ovine pulmonary adenocarcinoma (OPA), also known as ovine pulmonary adenomatosis and jaagsiekte, is a contagious tumour of sheep and, rarely, of goats. It is a progressive respiratory disease, principally affecting adult animals. The disease occurs in many regions of the world. A betaretrovirus (jaagsiekte sheep retrovirus: JSRV), distinct from the non-oncogenic ovine lentiviruses, has been shown to cause the disease.

Detection of the agent: *JSRV cannot yet be propagated* in vitro*, therefore routine diagnostic methods, such as virus isolation, are not available for diagnosis. Diagnosis relies, at present, on clinical history and examination, as well as on the findings at necropsy and by histopathology and immunohistochemistry. Viral DNA or RNA can be detected in tumour, draining lymph nodes, and peripheral blood mononuclear cells by polymerase chain reaction. Lambs become persistently infected by JSRV at an early age, and, in an OPA-affected flock, most sheep are infected.*

Serological tests: *Antibodies to the retrovirus have not been detected in infected sheep; therefore, serological tests are not available for diagnosis.*

Requirements for vaccines: *There are no vaccines available.*

A. INTRODUCTION

Ovine pulmonary adenocarcinoma (OPA), also known as ovine pulmonary adenomatosis, jaagsiekte (Afrikaans = driving sickness) and ovine pulmonary carcinoma (OPC), is a contagious lung tumour of sheep and, more rarely, of goats. It is the most common pulmonary tumour of sheep and occurs in many countries around the world. It is absent from Australia and New Zealand and has been eradicated from Iceland.

Historically, a number of different viruses have been linked to OPA, including a herpesvirus and lentiviruses, which have been propagated from tumour tissue. However, the former does not have an aetiological role in OPA and the latter exhibit characteristics of non-oncogenic lentiviruses. It has been demonstrated clearly that OPA is caused by a betaretrovirus that cannot yet be cultured *in vitro,* but the virus has been cloned and sequenced. The term jaagsiekte sheep retrovirus (JSRV) is used in referring to this virus.

B. DIAGNOSTIC TECHNIQUES

At present, diagnosis of OPA usually relies on clinical and pathological investigations but, more recently, specific techniques to identify JSRV proteins or nucleic acid can be employed. In particular, polymerase chain reaction (PCR) offers hope for ante-mortem diagnosis of OPA as a flock test (Lewis *et al.,* 2011). In flocks in which the disease is suspected, its presence must be confirmed, at least once, by histopathological examination of affected lung tissue to identify the characteristic lesions and, preferably by immunohistochemistry or PCR, to confirm the presence of JSRV. For such an examination, it is imperative to take specimens from several affected sites and, if possible, from more than one animal. This is because secondary bacterial pneumonia, which might be the immediate cause of death, often masks the lesions (both macroscopic and microscopic) of the primary disease. In the absence of specific serological tests that can be used for the diagnosis of OPA in live animals, disease control relies on strict biosecurity to prevent the introduction of the disease to OPA-free countries and flocks; where the disease already exists, control relies on regular flock inspections and prompt culling of suspected cases and, in the case of ewes,

their offspring. However, clinical examination has been shown to be unreliable for detection of early OPA cases (Cousens *et al.,* 2008), although transthoracic ultrasound examination can detect a proportion of subclinical cases (Cousens & Scott, 2015). There is no known risk of human infection with JSRV. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

Table 1. Test methods available for the diagnosis of ovine pulmonary adenocarcinoma and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
PCR	++	+	+	+++	++	–
Histopathology	–	–	+	+++	+	–
Immuno-histochemistry	+	–	+	+++	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR: polymerase chain reaction in either blood or tumour samples.

1. Detection of the agent

JSRV has been designated as a betaretrovirus because of its genetic organisation and its structural proteins. Although the ovine genome contains approximately 20 copies of endogenous betaretroviruses that are highly related to JSRV (Spencer & Palmarini, 2012), JSRV is clearly exogenous and associated exclusively with OPA (Palmarini *et al*., 1996). JSRV is detected consistently in the lung fluid, tumour, peripheral blood mononuclear cells, somatic cells in colostrum and milk, and lymphoid tissues of sheep affected by OPA or unaffected in-contact flockmates, and never in sheep from unaffected flocks with no history of the tumour. Full-length proviral clones of JSRV have been obtained from OPA tumour DNA and cells. JSRV virus particles, prepared from these clones by transient transfection of a cell line, were used for intratracheal inoculation of neonatal lambs. OPA tumour was induced in the lambs, thus demonstrating that JSRV is the causal agent of OPA (DeMartini *et al*., 2001; Palmarini *et al*., 1999).

Although the endogenous betaretroviruses that are highly related to JSRV are not involved in the aetiology of OPA, their expression in the uteroplacental tissues appears to be beneficial (Dunlap *et al.,* 2006) and expression in the fetus may, by induction of tolerance, account for the apparent lack of immune response of mature animals to exogenous JSRV (Palmarini *et al*., 2004).

There are no permissive cell culture systems for propagation of JSRV. Some cell cultures prepared from the tumours occurring in young lambs can support virus replication for a short period (Jassim, 1988; Sharp *et al*., 1985).

1.1. Nucleic acid recognition methods

Single step and hemi-nested JSRV-specific PCRs have been developed, based on primers derived from the U3 region of the JSRV LTR (Table 2) (Palmarini *et al.,* 1997). These can detect JSRV in several tissues, including peripheral blood mononuclear cells and lung fluid, from OPA-affected sheep, as well as experimentally infected lambs (De las Heras *et al*., 2005; Holland *et al*., 1999; Salvatori *et al.,* 2004). Importantly, JSRV can be detected by PCR in blood (Gonzalez *et al*., 2001), colostrum and milk (Borobia *et al.,* 2016), and in bronchoalveolar lavage samples (Voigt *et al*., 2007) from unaffected in-contact sheep from flocks with OPA.

These PCRs have a high diagnostic specificity but a low diagnostic sensitivity when applied to individual animals, due to low concentrations of target DNA in the blood of clinically healthy animals (De las Heras *et al.,* 2005; Holland *et al*., 1999; Lewis *et al.,* 2011). Longitudinal studies in OPA-affected flocks, supported by studies with lambs fed artificially with colostrum and milk (Grego *et al.,* 2008), have shown that most lambs become infected at a very early age. A high proportion of animals in these flocks are infected, yet only a minority develops OPA (Borobia *et al.,* 2016; Caporale *et al*., 2005; Salvatori, 2005).

Table 2. Primers used in JSRV-specific PCRs

	Primer	**Sequence (5'→3')**
Single step PCR	P1	TGG-GAG-CTC-TTT-GGC-AAA-AGC-C
	PIII	CAC-CGG-ATT-TTT-ACA-CAA-TCA-CCG-G
Hemi-nested PCR (uses product from single-step PCR)	P1	TGG-GAG-CTC-TTT-GGC-AAA-AGC-C
	PVI	TGA-TAT-TTC-TGT-GAA-GCA-GTG-CC

A few reports have described techniques, such as reverse-transcription PCR (RT-PCR) and real-time RT-PCR, to detect JSRV RNA in lung fluid and tumour tissues (Cousens *et al.,* 2009; Lee *et al.,* 2017; Zhang *et al.,* 2014). Although these techniques have not been investigated further, they have the potential to increase the sensitivity of detection and future studies, including comparison with the established PCR protocols, are merited.

2. Clinical signs and pathology

2.1. Clinical signs

There is no reliable laboratory method for the ante-mortem diagnosis of OPA in individual animals, therefore flock history, clinical signs and post-mortem lesions are the primary method for the diagnosis of the disease. OPA has a long incubation period, clinical disease is encountered most commonly in sheep over 2 years of age, with a peak occurrence at the age of 3–4 years. In exceptional cases, the disease occurs in animals as young as 2–3 months of age. The cardinal signs are those of a progressive respiratory embarrassment, particularly after exercise; the severity of the signs reflects the extent of tumour development in the lungs. Accumulation of fluid within the respiratory tract is a prominent feature of OPA, giving rise to moist rales that are readily detected by auscultation. Raising the hindquarters and lowering the head of affected sheep may cause frothy mucoid fluid to run from the nostrils. Coughing and inappetance are not common but, once clinical signs are evident, weight loss is progressive and the disease is terminal within weeks or months. Death is often precipitated by a superimposed bacterial pneumonia, particularly that due to *Mannheimia* (formerly *Pasteurella*) *haemolytica*. In clinically affected animals, a peripheral lymphopenia characterised by a reduction in CD4+ T lymphocytes and a corresponding neutrophilia may assist clinical diagnosis, but the changes are not pathognomonic and are not detected during early experimental infection (Summers *et al*., 2002).

In some countries, another form of OPA (atypical OPA) occurs, which generally presents as an incidental finding at necropsy or the abattoir (De las Heras *et al*., 2003).

2.2. Necropsy

OPA lesions are in most cases confined to the lungs, although intra- and extrathoracic metastasis to lymph nodes and other tissues can occur. In typical cases, affected lungs are considerably enlarged and heavier than normal due to extensive nodular and coalescing firm grey lesions affecting much of the pulmonary tissue. Usually lesions are present in both lungs, although the extent on either side does vary. Tumours are solid, grey or light purple with a shiny translucent sheen and often separated from the adjacent normal lung by a narrow emphysematous zone. The presence of frothy white fluid in the respiratory passages is a prominent feature and is obvious even in lesions as small as a few millimetres. In advanced cases, this fluid flows out of the trachea when it is cut or pendant. Samples should be taken at necropsy for histopathology, immunohistochemistry or PCR for JSRV.

Pleurisy may be evident over the surface of the tumour and often abscesses are present in the adenomatous tissue.

In atypical OPA, tumours comprise solitary or aggregated hard white nodules that have a dry cut surface and show clear demarcation from surrounding tissues. The presence of excess fluid is not a prominent feature. Classical and atypical lesions may be found in individual sheep from the same flock and, on occasions, in the same lung.

Adult sheep, which on post-mortem examination appear to have died from acute pasteurellosis, should have their lungs examined carefully, as lesions of OPA may be masked by coexisting bronchopneumonia, verminous pneumonia, chronic progressive pneumonia (maedi-visna) or combinations of these. Samples should be taken at necropsy for histopathology.

2.3. Histopathology and immunohistochemistry

Although JSRV can infect several pulmonary cell types (Martineau *et al.,* 2011) and tumours are heterogeneous (De las Heras *et al.,* 2014), OPA originates from JSRV infection and transformation of type II pneumocytes (Murgia *et al.,* 2011). Histologically, the lesions are characterised by proliferation of mainly type II pneumocytes, a secretory epithelial cell in the pulmonary alveoli. Nonciliated club cells (formerly known as Clara cells) and epithelial cells of the terminal bronchioles may be involved. The cuboidal or columnar tumour cells replace the normal thin alveolar cells and sometimes form papilliform growths that project into the alveoli. Intrabronchiolar proliferation may be present. In advanced cases, extensive fibrosis may develop and, occasionally, nodules of loose connective tissue in a mucopolysaccharide substance may be present.

Several studies have employed rabbit polyclonal antibodies to JSRV CA (capsid protein) or mouse monoclonal antibodies to JSRV SU (surface protein) to demonstrate these proteins in the cytoplasm of the transformed epithelial cells by immunohistochemistry, using standard protocols, thus providing a definitive diagnosis, in addition to PCR, e.g. Palmarini *et al.* 1995 and Wootton *et al.* 2006. Antisera are not available commercially but may be made available through personal collaborations.

A prominent feature is the accumulation of large numbers of alveolar macrophages in the alveoli adjacent to the neoplastic lesions (Summers *et al*., 2005).

Where maedi-visna is concurrent, perivascular, peribronchiolar and interstitial lymphoid infiltrates may be prominent.

The histological appearance of atypical OPA is essentially the same as classical OPA, but with an exaggerated inflammatory response (mostly lymphocytes and plasma cells) and fibrosis (De las Heras *et al*., 2003).

For more detailed accounts of the clinical, post-mortem and histopathological aspects of OPA, the reader is referred elsewhere (De las Heras *et al*., 2003; Sharp & DeMartini, 2003; Summers *et al.,* 2012).

There appears to be a synergistic interaction between OPA and maedi-visna. Lateral transmission of maedi-visna virus appears to be enhanced in sheep affected by OPA (Dawson *et al*., 1985; Gonzalez *et al*., 1993).

3. Serological tests

At present, there are no laboratory tests to support a clinical diagnosis of OPA in the live animal. JSRV has been associated exclusively with both typical and atypical forms of OPA, but antibodies to this virus have not been detected in the sera of affected sheep, even with highly sensitive assays such as immunoblotting or enzyme-linked immunosorbent assay (Ortin *et al*., 1997; Summers *et al*., 2002).

C. REQUIREMENTS FOR VACCINES

There are no vaccines available at the present time.

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NB: At the time of publication (2021) there were no WOAH Reference Laboratories for ovine pulmonary adenocarcinoma (adenomatosis) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1990 AS SHEEP PULMONARY ADENOMATOSIS. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.8.9.

PESTE DES PETITS RUMINANTS (INFECTION WITH SMALL RUMINANT MORBILLIVIRUS)

SUMMARY

Description of the disease: *Peste des petits ruminants (PPR), is an acute contagious disease caused by the small ruminant* Morbillivirus *in the family* Paramyxoviridae*. It affects mainly sheep and goats and occasionally wild small ruminants. Based on the fact that PPR has been reported on a few occasions in camels, cattle and buffaloes, those animal species are considered to be susceptible although their potential role in the circulation of PPR virus has not been formally established. PPR occurs in Africa except Southern Africa, in the Arabian Peninsula, throughout most of the Near East and Middle East, and in Central and South-East Asia.*

The clinical disease resembles rinderpest in cattle. It is usually acute and characterised by pyrexia, serous ocular and nasal discharges, diarrhoea and pneumonia, and erosive lesions on different mucous membranes particularly in the mouth. At necropsy, erosions may be noted in the gastrointestinal and urogenital tracts. The lungs may show interstitial bronchopneumonia and often secondary bacterial pneumonia. PPR can also occur in subclinical form.

PPR must be confirmed by laboratory methods, as bluetongue, foot and mouth disease and other erosive or vesicular conditions, as well as contagious caprine pleuropneumonia, can cause clinically similar disease.

Detection of the agent: *The collection of specimens at the correct time is important to achieve diagnosis by virus isolation and they should be obtained in the acute phase of the disease when clinical signs are apparent. The recommended specimens from live animals are swabs of conjunctival discharges, nasal secretions, buccal and rectal mucosae, and anticoagulant-treated blood.*

Laboratory diagnosis is done by immunocapture enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction. Counter immunoelectrophoresis and agar gel immunodiffusion may also be used. A penside test is available for field use.

Serological tests: *The serological tests that are routinely used are virus neutralisation and competitive ELISA.*

Requirements for vaccines: *Effective live attenuated PPR virus vaccines are widely available. Since the global eradication of rinderpest, the use of rinderpest vaccines to protect against PPR is forbidden.*

A. INTRODUCTION

Peste des petits ruminants (PPR) is an acute viral disease of small ruminants characterised by fever, oculo-nasal discharges, stomatitis, diarrhoea and pneumonia with foul offensive breath. Because of the respiratory signs, PPR can be confused with contagious caprine pleuropneumonia (CCPP) or pasteurellosis. In many cases, pasteurellosis is a secondary infection of PPR, a consequence of the immunosuppression that is induced by the virus. The virus is transmitted mainly by aerosols between animals living in close contact (Lefevre & Diallo, 1990). Infected animals present clinical signs similar to those historically seen with rinderpest in cattle, although the two diseases are caused by distinct virus species.

On the basis of its similarities to rinderpest, canine distemper and measles viruses, the virus causing PPR has been classified within the genus *Morbillivirus* in the family *Paramyxoviridae* (Gibbs *et al.*, 1979). It is now officially named *Small Ruminant Morbillivirus* (SRMV) (ICTV, 2019). Pending further revision of this chapter, it is also referred to below as PPR virus (PPRV). Virus members of this group have six structural proteins: the nucleocapsid protein (N), which encapsidates the viral genomic RNA, the phosphoprotein (P), which associates with the polymerase (L for large protein) protein, the matrix (M) protein, the fusion (F) protein and the haemagglutinin (H) protein. The matrix protein, intimately associated with the internal face of the viral envelope, makes a link between the nucleocapsid and the virus external glycoproteins: H and F, which are responsible, respectively, for the attachment and the penetration of the virus into the cell to be infected. The PPRV genome also encodes two nonstructural proteins, C and V.

PPR was first described in Côte d'Ivoire (Gargadennec & Lalanne, 1942). Since the late 1990s, it has expanded its range to cover large regions of Africa, from North Africa to Tanzania, and the Middle East, and is also widespread in countries from central Asia to South and East Asia (reviewed in Banyard et *al.,* 2010).

The natural disease affects mainly goats and sheep. It is generally considered that cattle are only naturally infected subclinically, although in the 1950s, disease and death were recorded in calves experimentally infected with PPRV-infected tissue and PPRV was isolated from an outbreak of rinderpest-like disease in buffaloes in India in 1995. Antibodies to PPRV as well as PPRV antigen and nucleic acid were detected in some samples from an epizootic disease that affected dromedaries in Ethiopia and Sudan. PPR affects a number of wild species within the order *Artiodactyla* some of which species are highly endangered, e.g. the Mongolian saiga antelope. The American white-tailed deer (*Odocoileus virginianus*) can be infected experimentally with PPRV. Dual infections can occur with other viruses such as pestivirus or goatpox virus.

The incubation period is typically 4–6 days, but may range between 3 and 10 days. The clinical disease is acute, with a pyrexia up to 41°C that can last for 3–5 days; the animals become depressed, anorexic and develop a dry muzzle. Serous oculonasal discharges become progressively mucopurulent and, if death does not ensue, persist for around 14 days. Within 4 days of the onset of fever, the gums become hyperaemic, and erosive lesions develop in the oral cavity with excessive salivation. These lesions may become necrotic. A watery blood-stained diarrhoea is common in the later stage. Pneumonia, coughing, pleural rales and abdominal breathing also occur. The morbidity rate can be up to 100% with very high case fatality in severe cases. However, morbidity and mortality may be much lower in milder outbreaks, and the disease may be overlooked. A tentative diagnosis of PPR can be made on clinical signs, but this diagnosis is considered provisional until laboratory confirmation is made for differential diagnosis with other diseases with similar signs.

At necropsy, the lesions are very similar to those observed in cattle affected with rinderpest, except that prominent crusty scabs along the outer lips and severe interstitial pneumonia frequently occur with PPR. Erosive lesions may extend from the mouth to the reticulo–rumen junction. Characteristic linear red areas of congestion or haemorrhage may occur along the longitudinal mucosal folds of the large intestine and rectum (zebra stripes), but they are not a consistent finding. Erosive or haemorrhagic enteritis is usually present and the ileo-caecal junction is commonly involved. Peyer's patches may be necrotic. Lymph nodes are enlarged, and the spleen and liver may show necrotic lesions.

There are no known health risks to humans working with PPRV as no report of human infection with the virus exists. Laboratory manipulations should be carried out at an appropriate containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

A list including all the types of tests available for PPR is given in Table 1. Assays applied to individuals or populations may have different purposes, such as confirming diagnosis of clinical cases, determining infection status for trade and/or movement, estimates of infection or exposure prevalence (surveillance) or checking post-vaccination immune status (monitoring).

Table 1. Test methods available for the diagnosis of peste des petits ruminants and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
RT-PCR	–	++	++	+++	+	–
Real-time RT-PCR	–	++	+++	+++	+	–
Virus isolation in cell culture	–	–	–	++	–	–
Immunocapture ELISA		+	++	+++	+	–
Penside test (LFD)	–	–	++	++	–	–
AGID	–	–	+	+	–	–
Counter immune-electrophoresis	–	–	–	+	–	–
Detection of immune response						
Virus neutralisation	+++	–	–	++	++	++
Competitive ELISA	+++	–	+++	+	+++	+++
AGID	–	–	+	+	–	+
Counter immune-electrophoresis	–	–	–	+	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction;
ELISA = enzyme-linked immunosorbent assay; LFD = lateral flow device; AGID = agar gel immunodiffusion.
[(a)]A combination of agent identification methods applied to the same clinical sample is recommended.

1. Collection of samples

Samples for virus isolation must be kept chilled in transit to the laboratory. In live animals, swabs are made of the conjunctival discharges or from the nasal, buccal or rectal mucosae. During the very early stage of the disease, whole blood is also collected in anticoagulant for virus isolation, polymerase chain reaction (PCR) and haematology (either ethylene diamine tetra-acetic acid or heparin can be used as anticoagulant, though the former is preferred for samples that will be tested using PCR). At necropsy, samples from two to three animals should be collected aseptically from lymph nodes, especially the mesenteric and bronchial nodes, lungs, spleen and intestinal mucosae, chilled on ice and transported under refrigeration. Samples of organs collected for histopathology are placed in 10% neutral buffered formalin. It is good practice to collect blood for serological diagnosis at all stages, but particularly later in the outbreak.

2. Detection of the agent

2.1. Immunocapture enzyme-linked immunosorbent assay

The immunocapture enzyme-linked immunosorbent assay (IC-ELISA) (Libeau *et al.,* 1994) using two monoclonal antibodies (MAb) raised to the N protein allows rapid identification of PPRV.

Advice on the use and applicability of the IC-ELISA is available from the WOAH Reference Laboratories for PPR[1]. The assay is available as a commercial kit. The instructions provided by the kit supplier should be followed. The general principle of the kit is:

i) Wells are provided pre-coated with an anti-PPRV-N antibody.

ii) Samples to be tested, and included controls, are added to the wells. PPRV, if present, forms an antibody–antigen complex.

iii) After washing, an anti-PPRV-N MAb–horse radish peroxidase (HRP) conjugate is added, forming an antibody–antigen–MAb–HRP complex.

iv) After further washing to eliminate the excess conjugate, the substrate solution (TMB: tetramethyl benzidine) is added. The resulting coloration depends on the quantity of PPRV present in the sample tested. In the presence of PPRV, a blue coloration appears that becomes yellow after addition of the stop solution. In the absence of PPRV, no coloration appears. The microplate is read at 450 nm.

The test is very specific and sensitive (it can detect $10^{0.6}$ $TCID_{50}$/well of PPRV). The results are obtained in less than 2 hours.

A sandwich form of the IC-ELISA is widely used in India (Singh *et al.*, 2004): the sample is first allowed to react with the detection MAb and the immunocomplex is then captured by an MAb or polyclonal antibody adsorbed onto the ELISA plate. The assay shows high correlation to the cell infectivity assay ($TCID_{50}$) with a minimum detection limit of 10^3 $TCID_{50}$/ml.

2.2. Nucleic acid recognition methods

Reverse transcription PCR (RT-PCR) techniques based on the amplification of parts of the N and F protein genes have been developed for the specific diagnosis of PPR (Couacy-Hymann *et al.*, 2002). This technique is 1000 times more sensitive than classical virus titration on Vero cells (Couacy-Hymann *et al.,* 2002) with the advantage that results are obtained in 5 hours, including the RNA extraction, instead of 10–12 days for virus isolation. The two most commonly used protocols are given in some detail below. A multiplex RT-PCR, based on the amplification of fragments of N and M protein genes, has been reported (George *et al.*, 2006). Another format of the N gene-based RT-PCR has also been described (Saravanan *et al.,* 2004). Instead of analysing the amplified product – the amplicon – by agarose gel electrophoresis, it is detected on a plate by ELISA through the use of a labelled probe. This RT-PCR-ELISA is ten times more sensitive than the classical RT-PCR.

In recent years, nucleic acid amplification methods for PPR diagnosis have been significantly improved with quantitative real-time RT-PCR (e.g. Bao *et al.,* 2008; Batten *et al.,* 2011; Kwiatek *et al.,* 2010). This method is also ten times more sensitive than conventional RT-PCR, as well as minimising the risk of contamination; a real-time RT-PCR assay specific for lineage IV strains has also been described (Li *et al.*, 2016). An alternative to RT-PCR is loop-mediated isothermal amplification (Li *et al.*, 2010). The sensitivity of this assay seems to be similar to that of the real-time RT-PCR, it is simple to implement, rapid and the result can be read by the naked eye.

In all cases RNA must first be purified from blood or tissue samples. Viral RNA can be purified from spleen (not ideal because of its high blood content), lymph node or tonsil, lung tissue, whole blood, buffy coat or purified peripheral blood lymphocytes (PBLs), or swabs from eyes or nose. Tissue samples should be extracted with acidified guanidinum thiocyante phenol using one of the commercial preparations available. Solid tissues (0.5–1.0 g) are minced and homogenised with 10 ml reagent. Conjunctival, oral or nasal swabs can be extracted, and whole blood, buffy coat or purified PBLs homogenised with, the same reagent; RNA is then purified according to the manufacturer's instructions. For tissues, blood, white cells or swabs, RNA extraction based on magnetic beads or spin columns are also suitable. The resulting RNA is stored at –70°C (or –20°C if –70°C not available) until required.

Although a real-time RT-PCR assay is the method of choice for laboratories that have the necessary equipment, none of the existing assays has proven satisfactory against all PPRV isolates tested. Because

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

this is a rapidly developing field, users are advised to contact WOAH (see footnote 1) and FAO[2] Reference Laboratories for PPR for advice on the most appropriate techniques.

2.2.1. RT-PCR for the diagnosis of PPRV based on the amplification of part of the N gene

The tests described in this section and the next require: a one-step RT-PCR kit, distilled water, the relevant primers, a thermal cycler, plus reagents and equipment for agarose gel electrophoresis. In each case, the master mix recipe and thermal cycling conditions given are for a specific one-step RT-PCR kit, other reagents could also be used, but these should be optimised and validated for use in the relevant assay.

N gene amplification is based on the initial protocol described by Couacy-Hymann *et al.* (2002) in a one-step RT-PCR method. The conventional RT-PCR based on the primers described here is capable of detecting all four viral lineages. Other primers targeting the N gene could be used, but may not suitable for this aim.

i) Primer sequences used:

Primer	Sequence
NP3:	5'-GTC-TCG-GAA-ATC-GCC-TCA-CAG-ACT-3';
NP4:	5'-CCT-CCT-CCT-GGT-CCT-CCA-GAA-TCT-3'.

ii) Prepare each primer dilution by adding 5 µl of the primer stock solution (100 µM) to 45 µl of distilled water. A primer concentration of 10µM is obtained with a final volume of 50 µl.

iii) Add 5 µl of RNA template to 45 µl of PCR master mix containing:

Reagent	Mix (1 reaction)	Final concentration
Distilled water	15 µl	
5× RT-PCR Buffer	10 µl	1×
dNTP Mix	2 µl	
Q solution	10 µl	
Primer NP3 (10 µM)	3 µl	0.6 µM
Primer NP4 (10 µM)	3 µl	0.6 µM
Enzyme mix	2 µl	
Final volume	50 µl	

iv) Distilled water (5 µl) is used in place of RNA to provide a negative control which has to be included into each set of PCR tests.

v) Thermal cycler conditions are set as follows:

50°C for 30 minutes	1 cycle	reverse transcription step
95°C for 15 minutes	1 cycle	Inactivates RT and activates polymerase
94°C for 30 seconds 60°C for 30 seconds 72°C for 1 minute	40 cycles	PCR amplification of the cDNA
72°C for 5 minutes	1 cycle	Final extension
4°C (indefinite)	–	–

2 Food and Agriculture Organization of the United Nations.

vi) The RT-PCR gives an amplification product of 351 bp. 10 µl of these products are analysed by electrophoresis on a 1.5 % agarose gel. For all positive results, 40 µl of the final product may be directly used for sequencing.

2.2.2. RT-PCR for the diagnosis of PPRV based on the amplification of part of the F gene

This assay is based on that originally published in Forsyth *et al.* (2003). Like the N gene PCR, it detects virus from all of the four lineages.

i) Sequences of primers used in this protocol:

Primer	Sequence
F1b	5'-AGT-ACA-AAA-GAT-TGC-TGA-TCA-CAG-T-3'
F2d	5'-GGG-TCT-CGA-AGG-CTA-GGC-CCG-AAT-A-3'
F1	5'-ATC-ACA-GTG-TTA-AAG-CCT-GTA-GAG-G-3'
F2	5'-GAG-ACT-GAG-TTT-GTG-ACC-TAC-AAG-C-3'

ii) The first stage of this assay uses the consists of a one-tube RT-PCR reaction using PPRV primers F1b and F2d designed against the PPRV F gene. The protocol given has been validated using a commercial kit, but other reagents could be used after appropriate testing and validation.

iii) Combine 20 µl reaction mix with 5 µl RNA in a 0.5 ml PCR tube. Each assay requires, at a minimum, the sample, negative control, positive control and no-template control (RNase-free water instead of RNA sample).

iv) Transfer the reactions to a thermal cycler and start the following programme:

50°C for 30 minutes	1 cycle	Reverse transcription step
94°C for 2 minutes	1 cycle	Inactivates RT and activates polymerase
94°C for 1 minute 55 °C for 1 minute 72°C for 1 minute	35 cycles	PCR amplification of the cDNA
72°C for 7 minutes	1 cycle	Final extension
4°C (indefinite)	–	–

v) Analyse 10 µl of the reaction product by agarose gel electrophoresis using a 2% agarose gel in either TBE or TAE buffers. If present, PPRV RNA will be amplified to give a DNA fragment of 447 bp. If no DNA product, or a very weak DNA product, is seen, a second round of PCR can be completed to increase the amount of PCR product. This nested PCR can be carried out using primers F1 and F2 and any good quality PCR reagents and enzyme that have been suitably validated.

vi) Combine 24 µl reaction mix with 1 µl of the first stage PCR in a 0.5 ml PCR tube. Each assay requires, at a minimum, the sample, negative control, positive control and no-template control (RNase-free water instead of PCR product). Transfer the reactions to a thermal cycler and start the following programme:

94°C for 3 minutes	1 cycle	Activates polymerase
94°C for 1 minute 55°C for 1 minute 72°C for 1 minute	35 cycles	PCR amplification of the cDNA
72°C for 10 minutes	1 cycle	Final extension
4°C (indefinite)	–	–

vii) Analyse 10 µl of the reaction product by agarose gel electrophoresis using a 2% agarose gel in either TBE or TAE buffers. If present, PPRV PCR product will be amplified to give a DNA fragment of 371 bp.

viii) DNA product remaining from positive samples identified in Section 2.4.2 steps v or vii can be purified and subjected to DNA sequencing.

2.3. Culture and isolation methods

Even when diagnosis has been carried out by rapid techniques, the virus should always be isolated from field samples in tissue culture for further studies.

PPRV may be isolated in primary lamb kidney/lung cells and some cell lines (Vero, B95a). Unfortunately, PPRV isolation using such cells is not always successful on first passage and may require multiple blind passages. Recently, derivatives of cell lines (Vero, CV1) expressing the morbillivirus receptor, the signalling lymphocyte activation molecule (SLAM or CD150), have been developed that enable isolation of field viruses from pathological specimens in less than 1 week, without requirement for blind passages. These include a derivative of the monkey cell line CV1 expressing goat SLAM (Adombi *et al.*, 2011) and derivatives of Vero cells expressing dog SLAM. Monolayer cultures are inoculated with suspect material (swab material, buffy coat or 10% tissue suspensions) and examined daily for evidence of cytopathic effect (CPE). The CPE produced by PPRV can develop within 5 days and consists of cell rounding and aggregation culminating in syncytia formation in lamb kidney cells and cell lines expressing SLAM. In unmodified Vero cells, it is sometimes difficult to see the syncytia. If they exist, they are very small. However, small syncytia are always seen in infected Vero cells stained with haematoxylin and eosin. Syncytia are recognised by a circular arrangement of nuclei giving a 'clock face' appearance. Cover-slip cultures may show CPE earlier than day 5. Some cells may contain intracytoplasmic and intranuclear inclusions, others may be vacuolated. Similar cellular changes may be seen in stained histopathological sections of infected tissues. After 5–6 days, blind passages should always be carried out as CPE may take time to appear.

2.4. Penside (field) test for PPRV antigen

Commercial tests for PPRV antigen are available for use in the field. These tests are based on so-called lateral flow technology. Conjunctival, nasal or oral swabs are taken from suspect animals, the swabs are rinsed with buffer and this buffer is applied to one end of a chromatographic strip. The sample mixes with coloured beads coated with a specific MAb that recognises PPRV antigen. Buffer flow moves the beads along the chromatographic strip. If the sample contains PPRV antigen captured by the swab, this binds to the beads, and the antigen and beads complex is then captured by a line of anti-PPRV MAb part-way along the strip, making a coloured line to indicate a positive result. In the absence of PPRV antigen, no beads are bound by the test line. The tests take 20 minutes and require no additional equipment. Both tests have been validated against PPRV isolates from all four lineages, and show sensitivity similar to IC-ELISA, and 100% specificity in laboratory testing. One test has also been validated in field use in the Côte d'Ivoire, Ethiopia, Pakistan and Uganda (Baron *et al.,* 2014). The tests are designed for use with external swabs and are not suitable for use on blood or tissue samples.

2.5. Agar gel immunodiffusion

Agar gel immunodiffusion (AGID) is a very simple and inexpensive test that can be performed in any laboratory and even in the field. Standard PPRV antigen is prepared from infected mesenteric or bronchial lymph nodes, spleen or lung material and ground up as 1/3 suspensions (w/v) in buffered saline (Durojaiye *et al.*, 1983). These are centrifuged at 500 ***g*** for 10–20 minutes, and the supernatant fluids are stored in aliquots at –20°C. The cotton material from the cotton bud used to collect conjunctival or nasal swabs is removed using a scalpel and inserted into a 1 ml syringe. With 0.2 ml of phosphate buffered saline (PBS), the sample is extracted by repeatedly expelling and filling the 0.2 ml of PBS into an Eppendorf tube using the syringe plunger. The resulting conjunctival/nasal swab extracted sample, like the tissue ground material prepared above, may be stored at –20°C until used. They may be retained for 1–3 years. Negative control antigen is prepared similarly from normal tissues. Standard antiserum is made by hyperimmunising sheep with 1 ml of PPRV with a titre of 10^4 $TCID_{50}$ (50% tissue culture infective dose) per ml given at weekly intervals for 4 weeks. The animals are bled 5–7 days after the last injection (Durojaiye, 1982).

i) Dispense 1% agar in normal saline, containing thiomersal (0.4 g/litre) or sodium azide (1.25 g/litre) as a bacteriostatic agent, into Petri dishes (6 ml/5 cm dish).

ii) Six wells are punched in the agar following a hexagonal pattern with a central well. The wells are 5 mm in diameter and 5 mm apart.

iii) The central well is filled with positive antiserum, three peripheral wells with positive antigen, and one well with negative antigen. The two remaining peripheral wells are filled with test antigen, such that the test and negative control antigens alternate with the positive control antigens.

iv) Usually, 1–3 precipitin lines will develop between the serum and antigens within 18–24 hours at room temperature (Durojaiye *et al.*, 1983). These are intensified by washing the agar with 5% glacial acetic acid for 5 minutes (this procedure should be carried out with all apparently negative tests before recording a negative result). Positive reactions show lines of identity with the positive control antigen.

Results are obtained in one day, but the test is not sensitive enough to detect mild forms of PPR due to the low quantity of viral antigen that is excreted.

2.6. Counter immunoelectrophoresis

Counter immunoelectrophoresis (CIEP) is a more rapid version of the AGID test (Majiyagbe *et al.*, 1984). It is carried out on a horizontal surface using a suitable electrophoresis bath, which consists of two compartments connected through a bridge. The apparatus is connected to a high-voltage source. Agar or agarose (1–2%, [w/v]) dissolved in 0.025 M barbitone acetate buffer is dispensed on to microscope slides in 3-ml volumes. From six to nine pairs of wells are punched in the solidified agar. The reagents are the same as those used for the AGID test. The electrophoresis bath is filled with 0.1 M barbitone acetate buffer. The pairs of wells in the agar are filled with the reactants: sera in the anodal wells and antigen in the cathodal wells. The slide is placed on the connecting bridge and the ends are connected to the buffer in the troughs by wetted porous paper. The apparatus is covered, and a current of 10–12 milliamps per slide is applied for 30–60 minutes. The current is switched off and the slides are viewed by intense light: the presence of 1–3 precipitation lines between pairs of wells is a positive reaction. There should be no reactions between wells containing the negative controls.

3. Serological tests

The demonstration of antibodies in PPRV infected goats and sheep can be used to support a diagnosis based on clinical signs, but such antibodies may also arise from vaccination with any of the current PPRV vaccines. Tests that are routinely used are the virus neutralisation test (VNT) and the competitive ELISA.

3.1. Virus neutralisation

This test is sensitive and specific, but it is time-consuming. The standard neutralisation test is now usually carried out in 96-well microtitre plates although roller-tube cultures may also be used. Vero cells are preferred, but primary lamb kidney cells may also be used.

This test requires the following materials: cell suspensions at 600,000/ml; 96-well cell culture plates; sera to be titrated (inactivated by heating to 56°C for 30 minutes); complete cell culture medium; PPRV diluted to give 1000, 100, 10 and 1 $TCID_{50}$/ml.

i) Dilute the sera 1/5, and then make twofold serial dilutions in cell culture medium.

ii) Mix 100 µl of virus at 1000 $TCID_{50}$/ml (to give 100 $TCID_{50}$ in each well) and 100 µl of a given dilution of serum (using six wells per dilution) in the wells of the cell culture plate.

iii) Arrange a series of control wells for virus and uninfected cells as follows: six wells with 100 $TCID_{50}$ (100 µl) per well; six wells with 10 $TCID_{50}$ (100 µl) per well; six wells with 1 $TCID_{50}$ (100 µl) per well; six wells with 0.1 $TCID_{50}$ (100 µl) per well; and six wells with 200 µl of virus-free culture medium per well. Make the wells containing the virus dilutions up to 200 µl with complete culture medium, and incubate the plates for 1 hour at 37°C.

v) Add 50 µl of cell suspension to each well, pat the sides of the plate lightly to distribute the cells in the well and cover. Incubate the plates at 37°C in the presence of CO_2.

vi) Read the plates after 1 and 2 weeks of incubation. The results should be as follows:

If the virus dilution has been done correctly, all the virus control wells with 100 and 10 $TCID_{50}$/well will show CPE, 50% of the wells will show CPE for the 1 $TCID_{50}$/well dilution, and none should show CPE for the 0.1 $TCID_{50}$/well dilution. The test is only valid if the virus has been suitably diluted.

For the serum titration, there will be no CPE in wells where the virus had been neutralised by serum during the test; any level of CPE means that the virus had not been neutralised by serum. The neutralising titre is the dilution of serum that neutralises virus in half the wells. A neutralising titre of greater than 10 is positive.

3.2. Competitive enzyme-linked immunosorbent assay

Several competitive ELISAs (C-ELISA) have been described, based on the use of MAbs that recognise virus proteins. They are of two types: those where the MAb recognises the N protein and use recombinant N protein produced in baculovirus as the antigen (e.g. Libeau *et al.*, 1995); and those with a viral attachment protein (H) specific MAb and antigen consisting of purified or part-purified PPRV (vaccine strain) (e.g. Anderson & McKay, 1994; Saliki *et al.,* 1993). All the assays work on the principle that antibodies to PPRV in test sera can block the binding of the MAb to the antigen.

Advice on the use and applicability of ELISA methods is available from the WOAH Reference Laboratories for PPR. Some methods are available as commercial kits; these are the only practicable way to carry out this test. Before use, laboratories should seek assurance that the kit has been validated in accordance with the WOAH Validation Standard (see Chapter 1.1.6 *Validation of diagnostic tests for infectious diseases of terrestrial animals*). The only alternative would be for a laboratory to develop and validate all the reagents (monoclonals and antigens) in house.

C. REQUIREMENTS FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1.1. Rationale and intended use of the product

Sheep and goats vaccinated with an attenuated strain of PPR or that recover from PPR develop an active life-long immunity against the disease (Durojaiye, 1982). Several PPR vaccines are available, all of which are cell culture-attenuated strains of natural PPRV (Sen *et al.,* 2010). The two most commonly used vaccine strains (Nigeria/75/1 and Sungri/96) have both been shown in experimental trials to protect animals against PPRV isolates of all lineages (Hodgson *et al.*, 2018); the Nigeria/75/1 vaccine has also been proven to provide such complete cross-lineage protection in field use in a large number of countries.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The history of the vaccine received and stored in the laboratory as master seed should be well known and registered: the origin of the vaccine, the number of passages in cell culture, and the range of the number of passages in cell culture shown to be effective in providing protection against PPR for at least 3 years when administered at the recommended dose. Such a PPR virus vaccine strain should not be excreted by inoculated animals in such a way that it can spread to in-contact animals. It should be proven that the vaccine strain has not reverted to virulence following at least three back passages in sheep and goats (Diallo *et al.,* 1989).

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The seed should be controlled and tested free from bacterial, fungal and mycoplasma contamination. It should be tested free from pestivirus and other potentially contaminating viruses. Only live attenuated PPR virus should be present. The seed should either have passed an innocuity test in animals (rodents, sheep and goats) or have been shown to be free of harmful contaminants by other validated means; it should have demonstrated its efficacy to protect sheep and goats against PPR with the recommended dose.

2.2. Method of manufacture

2.2.1. Requirements for substrates and media

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

i) Cells

All current PPRV vaccine strains are grown in Vero cells. Cells used for the production of PPR vaccine must be free from bacterial, fungal and viral contaminations. The source of these cells should be known and documented.

ii) Serum

The serum used in the cell culture should be free from adventitious virus, in particular pestiviruses. It is recommended to use irradiated serum. The country of origin of the serum must be known (use of serum from countries with high risk of transmissible spongiform encephalopathy [TSE] infections should be avoided).

iii) Culture medium

The traditional culture medium is minimal essential medium (MEM); recent work has shown that Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose or 25 mM fructose gives significantly higher (tenfold) yields of virus (Silva *et al*., 2011). The medium can be supplemented with antibiotics (e.g. penicillin + streptomycin at final concentrations of 100 IU [International Units]/ml and 100 μg/ml, respectively) and an antifungal agent (e.g. nystatin at a final concentration of 50 μg/ml) if required. The medium is enriched with 10% (v/v) fetal calf serum (complete medium) for cell growth. This proportion of serum is reduced to 2% (v/v) for maintenance medium when the cell monolayer is complete.

2.2.2. In-process controls

i) Cells used in cultures must be checked for normal appearance and shown to be free from contaminating mycoplasma and viruses, especially bovine viral diarrhoea virus.

ii) A virus titration must be undertaken on the working and production seed lots and on samples from the final product.

- **Test procedure**

a) Using serum-free medium, make a series of tenfold dilutions (0.5 ml virus + 4.5 ml diluent) down to 10^{-6} of the material to be titrated.

b) Trypsinise Vero cells and suspend in complete culture medium at 300,000/ml. Distribute the cells on a 96-well plate (30,000 cells per well, equivalent to 100 μl of cell suspension).

c) Add 100 μl of diluted virus to the cells (dilutions ranging from 10^{-2} to 10^{-6}). Use one row for each virus dilution; one row of wells serves as a negative control consisting of uninfected cells to which virus-free culture medium (100 μl) is added.

d) Incubate the plate at 37°C in the presence of CO_2.

e) Read the plates (by examining for CPE) 7–10 days after infection. Virus titre is calculated by the Spearman–Kärber method.

iii) The identity of the virus is confirmed at the final batch stage. For this purpose, anti-PPRV serum is used to neutralise the virus in cell culture.

a) Mix the contents of two vaccine bottles with sterile double-distilled water to provide a volume equal to the volume before freeze-drying.

b) Make tenfold dilutions of the reconstituted vaccine in serum-free culture medium (0.5 ml of viral suspension + 4.5 ml of medium).

c) Make two series of mixtures for virus dilutions from each bottle on a 96-well plate as follows:

Series 1:	Dilutions of viral suspension:	–1	–2	–3	–4
	Viral suspension (in µl)	50	50	50	50
	Culture medium (in µl)	50	50	50	50
Series 2:	Dilutions of viral suspension:	–1	–2	–3	–4
	Viral suspension (in µl)	50	50	50	50
	PPR antiserum (in µl)	50	50	50	50

(**Note:** PPR antiserum used for this purpose is prepared in goats and freeze-dried. It is reconstituted with 1 ml of sterile double-distilled water in a dilution of 1/10.)

d) Incubate the mixtures at 37°C for 1 hour

e) Add to each well 100 µl of cells suspended in complete culture medium (30,000 cells/well).

f) Incubate the microplate at 37°C in the presence of CO_2.

g) Read the plate after 7–10 days of incubation.

Normally CPE is present only in the wells containing cells infected with the mixture of virus and culture medium. If it is detected in the wells of Series 2, it will be necessary to identify PPRV by immunofluorescence, using a PPR MAb, or by immunocapture ELISA (using the commercially available kit). If this identification confirms the presence of PPRV, the PPR antiserum used must have been too weak, or the batch must be changed. If immunofluorescence or immunocapture is negative, or if repeating the test with a new PPRV antiserum gives the same result, a viral contaminant must be present, and the material under test must be destroyed.

2.2.3 Manufacturing procedure

A protocol for the production and validation of vaccine from master seeds of the available attenuated PPRV strains is described here. Other protocols for the production of commercial stocks of the vaccine may be used as long as the essential criteria are observed and the final product is appropriately validated.

Essential criteria:

i) The master seed stock should have a known and documented history and be shown to be free of contaminating pathogens.

ii) The virus should be grown at all stages in the same cell line as that used to produce the master seed, since passage in a different cell line is likely to lead to the accumulation of sequence changes in the vaccine, changes which may have effects on the vaccine properties.

iii) The master seed must not be passed more than 10 times for the production of final vaccine batches.

iv) Sterility and potency tests must be carried out on the product and fully documented.

Once the manufacturer has received samples from an institution holding the PPRV vaccine seed – the master seed – it should prepare primary and secondary working seed batches. The production seed batch, from which the final vaccine is produced, is prepared from the secondary working seed. By preparing primary and secondary working seeds, the need to carry out a large

number of passages from the master seed is avoided. In this way, it is possible to comply with the WOAH recommendation of no more than 5–10 passages after the master seed (see chapter 1.1.8 *Principles of veterinary vaccine production*).

i) Primary and secondary working seed batches

When preparing primary and secondary working seed batches it is important to avoid infecting the cells with high doses of virus (high multiplicity of infection [m.o.i.]), as this will lead to the accumulation of defective particles in the viral suspension produced, which will diminish the titre of subsequent products. On the other hand, very low m.o.i. (e.g. 0.0001) will prolong the culture time. The manufacturer is responsible for optimising production work flow for their own cells and culture medium; as a guide, infections at each stage should be at a m.o.i. of 0.001–0.01.

The freeze-dried contents of a flask from the master seed are reconstituted with 2 ml of cell culture medium without serum. This liquid is mixed with Vero cells (if the vaccine is produced on Vero cells), suspended in complete culture medium to provide at least 0.001 $TCID_{50}$ per cell. Cell culture flasks are filled with this virus/cell mixture (around 2×10^7 Vero cells in a 175 cm^2 flask), and are incubated at 37°C. The cultured cells are examined regularly to detect CPE. The medium is renewed every 2 days, reducing the proportion of serum to 2% once the cell monolayer is complete. Virus (the cell culture medium over the cells) is first harvested when there is 40–50% CPE. This viral suspension is stored at –70°C. Successive harvesting is carried out every 2 days until the CPE reaches 70–80%, at which point the culture flasks are frozen at –70°C. In general, at least two further harvestings can be made before final freezing of the culture flasks. All suspensions of virus collected are submitted to two freeze–thaw cycles, then pooled to form a single batch, which serves as the working seed batch. The suspension of virus may be clarified by low speed centrifugation (e.g. 5 minutes at 1250 ***g***) to remove cell debris. The working seed batch is divided into small volumes in bottles and stored at –70°C. The contents of five of these bottles are thawed and titrated (minimum titre required: 10^5 $TCID_{50}$/ml). It is best to freeze-dry this primary working seed in order to store it at –20°C. In this case it will be necessary to titrate the freeze-dried virus (five bottles). A batch made up in this way must pass all tests for sterility.

An aliquot of the primary working seed is then cultured in the same way to prepare the secondary working seed.

ii) Production seed batch

The production seed batch is prepared under the same conditions as for the working seed batches. A large stock of virus is formed, from which the final vaccine will be produced. This batch is distributed into receptacles and stored at –70°C. As previously, five samples are titrated (minimum titre required: 10^6 $TCID_{50}$/ml), and the production seed batch must satisfy tests for sterility.

iii) Vaccine production

This operation is performed on a larger scale, but is otherwise carried out as for the preparation of the production seed. Products of the various harvests, after two freeze–thaw cycles, are brought together to form the final product and stored at –70°C pending the results of titration and tests for sterility. If these results are satisfactory, the vaccine is freeze-dried.

v) Freeze-drying

Several freeze-drying media have been assessed for use with PPRV. Weybridge medium has been used most widely, and is composed of 2.5% (w/v) lactalbumin hydrolysate (LAH), 5% (w/v) sucrose and 1% (w/v) sodium glutamate, in Hank's balanced salt solution (HBSS) pH 7.2. Significantly improved stability at temperatures above 4 ˚C has been reported using a freeze-drying medium containing 5%(w/v) LAH and 10% sucrose in HBSS (Mariner *et al*. 2017; Sarkar *et al*., 2003) or containing 20 mM Tris–HCl pH 7.4, 2 mM EDTA, 0.02% (w/v) Tween 80 and 1 M (34.2% (w/v)) trehalose (Silva *et al*., 2011). The freeze-drying medium is

added to an equal volume of viral suspension (which may have been diluted beforehand to provide the desired number of vaccine doses per bottle). The resulting mixture is kept cool, homogenised, then distributed into bottles and freeze-dried. At the end of a freeze–drying cycle, the probe is adjusted and kept at 35°C for 4 hours. Once this operation has been completed, the bottles are capped under vacuum. Randomly selected samples of this final batch are subjected to tests for innocuity, efficacy and sterility, and residual moisture is estimated by the Karl Fisher method (optimum ≤3.5%). If the tests give unsatisfactory results, the entire batch is destroyed.

2.2.4. Final product batch tests

Determination of the number of samples to be tested will depend on the batch size and the accuracy of the test involved; the principles of Appendix 1.1.2.1 *Epidemiological approaches to sampling: sample size calculations* of Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* apply. Manufacturers must sample sufficient vials to have >95% confidence of detecting contamination at the 0.1% level.

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

The batch must be tested and proven not to be contaminated by bacteria, fungus, mycoplasma or other viruses. These tests are carried out on the vaccine batch before and after freeze-drying. Any product that fails these tests for sterility is destroyed.

The content of at least one container from each filling lot must be checked for identity by culture after neutralisation with specific antiserum.

ii) Safety and efficacy

As stated in Chapter 1.1.8 *Principles of veterinary vaccine production*, the target animal batch safety test (TABST) may be waived by the regulatory authority when a sufficient number of production batches have been produced under the control of a seed lot system and found to comply with the test, thus demonstrating consistency of the manufacturing process. Some regulatory authorities still require safety tests for the release of each batch. Where required, a safety test should be done on the host recipient of the vaccine, currently sheep and goats. Unless specifically required by a local regulatory authority, the safety test in rodents is not considered necessary for live attenuated PPRV vaccines.

Normally the minimum immunising dose is 100× the lowest dose of vaccine virus able to induce a 50% immunising response. For example, in the case of the attenuated Nigeria 75/1 vaccine the required minimum titre per dose has been shown to be $10^{2.5}$ $TCID_{50}$.

For the TABST, five bottles of the PPR vaccine are reconstituted in the normal diluent, mixed and diluted to give a solution of 100 doses/ml. Two goats and two sheep are used, all approximately 1-year old and free from PPR antibodies. The animals are vaccinated subcutaneously with 100 doses per animal. The animals are subjected to daily clinical examination for 3 weeks including recording of rectal temperatures. The vaccine is considered as safe if no abnormal clinical signs are observed in the vaccinated animals.

For the established live attenuated PPRV vaccines, efficacy is determined by the titre of live virus in the vaccine batch (batch potency); challenge with wild type virus is not required. Novel killed, subunit or other vaccines must establish appropriate correlates of protection, or must be batch tested by showing protection against challenge.

iii) Batch potency

For the established live attenuated PPRV vaccines, the potency of each batch can be determined by virus titration in cell culture coupled with confirmation that the virus has not undergone significant mutation during the production process. This confirmation can be provided (a) *in vitro* by carrying out full genome sequencing of the final batch material and

showing an absence of changes in the main immunogenic proteins (N and H) or (b) *in vivo* by inoculating at least six target animals with 1 dose per animal of vaccine and showing that all animals have a neutralising antibody titre of at least 1/10 at 3 weeks post-vaccination.

Potency for novel vaccines will have to be established by other means, depending on the type of vaccine.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target animal safety

The vaccine should be safe for use in all species of target animals, including young and pregnant recipients. Once manufacture has established a consistent safety record, they may apply to their regulatory authority for permission to omit this test.

ii) Reversion-to-virulence for attenuated/live vaccines

Information should be available to indicate that studies have been carried out and have demonstrated that the vaccine strain which has been used has not reverted to virulence after at least three back passages.

iii) Environmental consideration

Information should be available to indicate that studies have been carried out and have demonstrated that the vaccine strain which has been used is not excreted by the inoculated animals.

2.3.2. Efficacy requirements

i) For animal production

Field or other tests should have proven that the attenuated PPR vaccine is efficacious for use in all sheep and goats species, including young and pregnant animals.

ii) For control and eradication

Sheep and goats which have recovered from a PPR infection appear to be protected against a subsequent infection for the rest of their lives. Neutralising antibodies against PPRV were found in sheep and goats up to three years after vaccination with the attenuated PPRV vaccine strain Nigeria 75/1. Other attenuated PPR vaccines that have been developed in India (Sungri/96) have been shown to produce long-lasting immunity too (Saravanan *et al.,* 2010a; Sen et *al*., 2010). All these data indicate that PPR is a disease that can be well controlled, even eradicated, following a large-scale and well planned vaccination campaign such as that done for rinderpest.

2.3.3. Stability

PPR vaccine freeze-dried in the Weybridge medium can be kept for at least 2 years at 2–8°C (although storage at –20°C is better), provided it is stored under vacuum and protected from light.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

No DIVA vaccine and associated test is currently available for field use.

2.3.5. Duration of immunity

The duration of protective immunity should be determined for each vaccine strain in animal trials. For the Nigeria 75/1 and Sungri/96 vaccines, this has been shown to be at least 3 years (Saravanan *et al*., 2010b; Zahur *et al*., 2014). Commercial preparations of these vaccines should therefore offer at least 3 years protection.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Preliminary results on recombinant capripox-based PPR vaccines indicate that they can protect against both capripox and PPR (Berhe *et al.*, 2003; Caufour *et al.,* 2014; Chen *et al*., 2010; Diallo *et al*., 2002). Novel vaccines based on recombinant adenoviruses have also been shown to be effective (Herbert *et al.,* 2014; Holzer *et al.,* 2016; Qin *et al.,* 2012; Rojas *et al.,* 2014; Wang *et al.,* 2013); such vaccines also offer the possibility of providing a DIVA capability. None of these vaccines has yet been validated for field use.

3.2. Special requirements for biotechnological vaccines, if any

None.

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NB: There are WOAH Reference Laboratories for peste des petits ruminants
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for peste des petits ruminants

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.8.10.

SALMONELLOSIS (*S.* ABORTUSOVIS)

See Chapter 3.10.7. Salmonellosis

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CHAPTER 3.8.11.

SCRAPIE

SUMMARY

Description of the disease: *The term scrapie encompasses two different pathological entities: classical and atypical scrapie. Both conditions are chronic, neurodegenerative, fatal diseases of sheep and goats and are caused by similar agents. However, they differ in clinical presentation, pathogenesis, pathology, biochemical properties and epidemiology. This chapter describes tests for both conditions so that they may be differentiated.*

Classical scrapie is characterised by vacuolar changes in the central nervous system (CNS). It has been recognised as a clinical disorder for more than 250 years, and is classified as a transmissible spongiform encephalopathy (TSE), or prion disease, as defined by the accumulation of an abnormal form of a host cell membrane-bound glycoprotein (prion protein or PrP) referred to as PrP^{Sc}, in the CNS. Polymorphisms of the PrP gene are associated with susceptibility to scrapie. Breeding for resistance has been used as an effective tool in the control of ovine classical scrapie. Depending on PrP genotype, PrP^{Sc} can also be detected in lymphoreticular tissues of classical scrapie cases.

The more recently identified condition known as atypical scrapie has some clinical and pathological features similar to classical scrapie but is not considered to be transmitted in field situations. The epidemiology is consistent with a non-contagious condition that occurs sporadically. Consequently, surveillance for classical scrapie will detect occasional cases of atypical scrapie. In sheep, it has also been reported in animals with PrP genotypes that are relatively resistant to classical scrapie.

Classical scrapie is endemic in many countries, where it has often been introduced by importation. For up-to-date information on the occurrence of scrapie, consult the WOAH WAHIS interface[1]*. Classical scrapie may be transmitted from dam to offspring in the period from parturition to weaning, and* in utero. *It can also be transmitted horizontally to unrelated sheep or goats. The infectious material can persist for several years on pastures and in buildings. Fetal membranes are a source of infection, and milk from clinically affected animals can transmit disease. The incubation time between primary infection and clinical disease is usually longer than 1 year and may sometimes exceed the commercial lifespan of the animal. The majority of cases occur between 2 and 5 years of age. Clinical disease develops only if the agent enters the CNS. Atypical scrapie, where it presents clinically, is reported mostly in older animals and epidemiological data suggest that it does not present like an infectious disease but instead it has a sporadic distribution.*

Detection of the agent: *Classical scrapie may be recognised by clinical signs, which are variable but usually start insidiously with behavioural abnormalities that progress to more obvious neurological signs, including pruritus and incoordination. Affected animals may have poor body condition. Atypical scrapie cases may present with ataxia. Diagnosis is confirmed by demonstration of TSE-specific vacuolation or the immuno-detection of PrP^{Sc} in target areas within the brain. Immuno-detection of PrP^{Sc} in brain samples forms the basis of the rapid tests, which are most often used in active surveillance programmes. In experimental studies in sheep and goats, PrP^{Sc} accumulation in the brain is not detectable until several months after challenge, so a negative test result does not necessarily equate to an uninfected animal.*

Detection of PrP^{Sc} in lymphoreticular tissues during the incubation period of classical scrapie in some animals offers a means of preclinical diagnosis of infection and may be useful for surveillance purposes. Testing can also be performed in live animals using biopsied tissue, e.g. rectoanal mucosa-associated lymphoid tissue (RAMALT), third eyelid biopsies and palatine tonsil. It is not, however,

1 https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/

appropriate for detection of atypical scrapie as there is little or no involvement of the lymphoreticular system, or of a proportion of classical scrapie cases, so it can be used only to confirm the presence of infection and cannot be used to prove its absence.

Currently recognised forms of scrapie can be transmitted with variable efficiency to a range of wild type and PrP transgenic laboratory rodents by inoculating them with infected brain tissue, but long incubation times preclude this as a practical diagnostic procedure.

Serological tests: *Scrapie infection is not known to elicit any specific immune response and so there are no diagnostic tests to detect specific antibodies.*

Requirements for vaccines: *There are no vaccines available.*

A. INTRODUCTION

Classical scrapie (also known as la tremblante; Traberkrankheit; Gnubberkrankheit; Prúrigo lumbar) is a naturally occurring progressive, fatal, infectious, neurodegenerative disease of sheep and goats that has been recognised since the eighteenth century, and has been reported in Europe, North America, Asia and Africa. There is no evidence of a causal link between classical or atypical scrapie and human TSEs. It is the archetype of the transmissible spongiform encephalopathies (TSEs). Prion disorders with similar pathology have been found to occur naturally in several species including man (Hornlimann, 2006). They are defined by the consistent accumulation of an abnormal isoform (PrP^{Sc}) of the host-encoded protein (PrP^{C}) in the central nervous system (CNS), and variable PrP^{Sc} detection in other tissues such as in the lymphoreticular system (LRS) and other tissues/body fluids.

Atypical scrapie (also known as Nor98), is also a neurodegenerative disease of sheep and goats first described in Norway in 1998 (Benestad *et al*., 2008). Like the classical disease it is associated with abnormal prion protein accumulations, however unlike classical scrapie it has not been shown to be naturally transmitted under field conditions (EFSA, 2021). Active surveillance using rapid immunochemical methods has provided evidence for its widespread occurrence throughout Europe, with reports of cases also in the Falkland Islands (Epstein *et al*., 2005), North America (Loiacono *et al*., 2009), Australia and New Zealand (Kittelberger *et al.,* 2010). Although the epidemiology is not suggestive of transmission in the field (Benestad *et al*., 2008) and it is not considered transmissible from an animal health perspective, it can be transmitted experimentally (Simmons *et al.,* 2011). Retrospective studies have identified cases from the 1980s, predating active surveillance. Atypical scrapie has been identified in sheep of genotypes considered to be relatively resistant to classical scrapie, and in goats. Co-infection of classical and atypical scrapie is a very rare occurrence with a single case having been reported worldwide (Mazza *et al*., 2010).

Various disease-specific isoforms of the abnormal prion protein (PrP^{Sc}) are now widely regarded to be the causal agents in prion diseases. Disease characterisation is based on a range of host phenotypic parameters, such as clinical signs, histopathological profile and immunopathology, PrP^{Sc} biochemical characteristics such as protease sensitivity and cleavage site and, if necessary, biological parameters in rodent models.

1. Clinical signs

The clinical signs of classical scrapie (Konold & Phelan, 2014) usually start insidiously, often with behavioural changes that are evident only from repeated inspections. These subtle presenting features, which may include apparent confusion, separation from the flock and a staring gaze, progress to a more definite neurological illness, frequently characterised by signs of pruritus and ataxia or incoordination of gait. Either the pruritus or the ataxia usually emerges to dominate the clinical course. Death may occur after a protracted period of only vague neurological signs or may even occur without premonitory signs. These clinical signs, individually, are not disease-specific and clinical suspicion of disease should be confirmed by further testing.

Pruritus is recognised principally by compulsive rubbing or scraping against fixed objects, nibbling at the skin and scratching with a hind foot or horns. This results in extensive loss of wool, particularly over the lateral thorax, flanks, hindquarters and tail head. The persistence of pruritus often results in localised self-inflicted skin lesions. These may occur in areas of wool loss and on the poll, face, ears and limbs. A characteristic 'nibble reflex' can often be elicited by scratching the back, and may also be evoked by the sheep's own scraping movements. Some sheep or goats with scrapie, however, may not present with evident signs of pruritus. Ataxia or incoordination of gait may first become apparent as difficulty in positioning the hind limbs on turning, swaying of the hindquarters and a high stepping or trotting gait of the forelimbs. Stumbling and falling occur, but the animal is generally able to quickly

regain a standing posture. These signs progress to weakness and recumbency. Other signs of classical scrapie may include teeth grinding (bruxism), low head carriage, a fine head or body tremor and, rarely, seizures or visual impairment. In most cases, there is also a loss of bodily condition or weight.

In atypical scrapie the clinical features are dominated by ataxia in the absence of pruritus; circling has also been observed.

Video-clips illustrating the clinical signs of scrapie can be viewed on the webpages of the WOAH Reference Laboratory in the UK[2].

Progression of the clinical disease is very variable, lasting for a week or up to several months, with an inevitably fatal outcome. There is also variation in the clinical signs among individual animals and in different breeds of sheep. These variations may be due to the influence of host genotype and strain of agent. Environmental factors may also have an influence on the disease course. The clinical diagnosis of individual cases of classical scrapie can therefore be difficult. The clinical signs may, especially in the early phase of the disease, resemble those of some other conditions of adult small ruminants, including ectoparasitism, Aujeszky's disease (pseudorabies), rabies, cerebral listeriosis, maedi-visna (ovine progressive pneumonia), pregnancy toxaemia (ketosis), hypomagnesaemia and chemical and plant intoxications.

2. Host genetic factors

Agent strain and host variables determine the expression of disease. In sheep, different PrP genotypes are associated with relative susceptibility to TSEs. Polymorphisms at codons 136 and 171 are of particular significance in determining overall susceptibility of sheep to classical scrapie. Genotypes that confer susceptibility to classical scrapie are associated with resistance to atypical scrapie while polymorphisms at codon 141 and 154 can also affect susceptibility to atypical scrapie.

In goats, PrP genotype also influences susceptibility to disease. However, in this species the PrP sequence is more variable compared with sheep, and polymorphisms may have diverse distribution in goat populations depending on breed and geographical location. Therefore, genetic programmes aiming at increasing resistance to classical scrapie in one region may not be universally applicable. Polymorphisms at codons 146 and 222 affect susceptibility to classical scrapie and can be used to increase genetic resistance in goat herds. Very little is known about the influence of genetics on atypical scrapie in goats, although there is some evidence suggesting that certain codons may be associated with susceptibility to the disease (EFSA 2021).

The mechanisms by which strain and host parameters influence disease phenotype are still incompletely understood. (See EFSA [2014] for recent overview.)

3. Discrimination between classical scrapie and BSE

The ability to distinguish classical scrapie from bovine spongiform encephalopathy (BSE) is of particular importance in small ruminants because of BSE's zoonotic nature and the potential for past exposure of small ruminants through contaminated feed. For this purpose, molecular typing uses differential epitope binding of PrP^{Sc} in immunohistochemistry (IHC) or Western immunoblot. However, if BSE cannot be discriminated from classical scrapie unequivocally by molecular typing, rodent bioassays must be initiated to resolve the issue.

4. Surveillance

Because of the known inadequacies of baseline (passive) surveillance and the absence of active surveillance components, the classical scrapie status of many countries is unknown. Objectively establishing freedom from infection in a national flock requires sustained and substantial levels of active surveillance. Some countries have never recorded classical scrapie against a background of general and/or targeted surveillance, while others have maintained freedom for various periods through rigorous preventative policies and monitoring. Classical scrapie usually occurs in sheep 2–5 years of age. Rarely are cases present in sheep less than 1 year old. In atypical scrapie a significant proportion of cases have been reported in sheep over 5 years of age. Rarely, sheep have been identified with mixed infections of classical and atypical scrapie. In some instances with classical scrapie, the commercial lifespan of the sheep may be too short or exposure has occurred too late in life for the clinical disease to develop. Classical and atypical scrapie have also been described in goats, and classical scrapie in captive moufflon (*Ovis*

2 https://science.vla.gov.uk/tseglobalnet/documents/clinical-signs-tse-sheep-video.pdf

musimon). Most breeds of sheep and goats are affected. Classical scrapie may be transmitted from dam to offspring in the period from parturition to weaning, and *in utero* (Spiropoulos *et al.,* 2014). Infection can also pass horizontally to unrelated animals, even without direct contact (Dexter *et al*., 2009). Fetal membranes are known to be a source of infection, and milk can also transmit disease (Konold *et al*., 2013). Pasture previously grazed by, or buildings previously inhabited by, infected animals also represent a risk (Gough *et al.,* 2015; Hawkins *et al.,* 2015). Animals incubating the disease, and even animals that never develop clinical signs, may still be a source of infection to others.

5. Biosafety

The biohazard for humans from scrapie diagnostic testing appears to be limited, but laboratory manipulations with potentially contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Although Creutzfeldt-Jakob disease (CJD) of humans has been found to occur at no greater frequency in those with occupations providing closest contact with the scrapie agent, the extreme chemical and physical resistance of the scrapie agent and the fact that it is experimentally transmissible by injection to a wide spectrum of mammalian species, including humanised transgenic mice (Cassard *et al.,* 2014) and non-human primates (Comoy *et al.,* 2015) suggests the prudence of preventing human exposure.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of scrapie and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Histopathology	–	–	–	+	–	–
IHC	–	–	++	+++	++	–
Western immunoblot	–	–	++	+++	++	–
Rapid tests	–	–	+++	+	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
IHC = immunohistochemistry.

A disease-specific partially protease-resistant, misfolded isoform (PrP^{Sc}) of a membrane protein PrP^{C}, has a critical importance in the pathogenesis of TSE. According to the prion hypothesis, PrP^{Sc} is the principal or sole component of the infectious agent, and confirmation of the diagnosis is reached by the application of immunohistochemical (IHC) or immunochemical detection of PrP^{Sc} in brain tissue. By definition, specific demonstration of infectivity would rely on experimental transmission, but ethical considerations and the long incubation periods associated with TSEs mean that the criterion of transmissibility is not used for routine diagnosis. However, biological characterisation on transmission is an important experimental component of the definition of any emerging new phenotypic variants of scrapie and for discriminatory approaches to distinguish cases of scrapie from BSE in sheep or goats.

The medulla oblongata at the level of the obex (the earliest consistent neuroanatomical site for morphological vacuolar changes [Wood *et al*., 1997]) is the most consistent and appropriate diagnostic level of CNS for classical scrapie, using either vacuolation or immunodetection of PrP^{Sc} methods. In atypical scrapie, the medulla is affected minimally, while the cerebellum, thalamus and basal ganglia are affected more consistently and overtly (Benestad *et al*., 2008; Moore *et al*., 2008). Therefore, taking practical and logistical sampling considerations into account, the

medulla and cerebellum should both be sampled through the foramen magnum and examined to enable the robust diagnosis and classification of classical and atypical scrapie cases.

Although histopathology can still be used to detect vacuolar lesions in the CNS (Gavier-Widen *et al*., 2005) at the level of the obex for the confirmation of classical scrapie, it will not detect atypical scrapie (Moore *et al.,* 2008). However, PrP^{Sc} detection using IHC examination or immunodetection techniques, performed on the medulla oblongata and cerebellum, have increased diagnostic sensitivity, and the active surveillance of large populations is now undertaken using rapid PrP^{Sc} detection tests (see below). Detectable PrP^{Sc} precedes vacuolation and clinical signs, making the immuno-based tests a more sensitive option. Clinically suspect cases of scrapie could, where suitable samples are available, continue to be investigated initially by histopathological examination for morphological changes. However, any morphological diagnosis must be confirmed by the demonstration of PrP^{Sc} in the CNS.

Some commercially available rapid methods for the detection of PrP^{Sc}, introduced originally for the diagnosis of BSE, are also approved for scrapie diagnosis and others have been specifically developed and approved for use on small ruminant samples. These rapid tests take the form of enzyme-linked immunosorbent assay (ELISA)-based methods, and provide preliminary screening from which samples giving positive or inconclusive results are subject to examination by confirmatory IHC or Western immunoblot methods. All these methods have been shown to be able to detect scrapie in the appropriate samples (EFSA, 2005a; 2012). The analytical sensitivity of these kits is kept under review by the European Commission, and links to information on the performance of currently approved tests can be found on the European Reference Laboratory (EURL) web site[3]. A list of those tests that are currently approved for use by the European Commission can be found in Annex X of Regulation (EC) No. 999/2001 as last amended. Failure to observe either characteristic histological changes or to detect PrP^{Sc} does not confirm the absence of the disease; agreement between the results of multiple diagnostic approaches provides the best assurance of accuracy. In surveillance for the purpose of obtaining evidence of freedom from scrapie in small ruminant populations, it may be necessary to apply multiple diagnostic criteria and to use at least two laboratory methods (histopathological and IHC, or immunoblot) on accurately sampled CNS tissue (minimum medulla and cerebellum) to maintain a high degree of confidence in negative results.

Passive surveillance of classical scrapie, comprising the examination of CNS material from clinically suspect cases, has, in recent years, been complemented in many countries by active surveillance, targeting healthy adult culls and fallen stock (diseased or dead animals, also called risk animals) screened at post-mortem using rapid test methods to detect PrP^{Sc}. In classical scrapie, the opportunity also exists for screening approaches that do not rely solely on examination of the CNS tissue from dead animals to detect exposed animals, but uses the widespread presence of PrP^{Sc} in lymphoreticular tissue in many animals to enable demonstration of infected animals by biopsy of palatine tonsil, nictitating membrane, superficial lymph nodes or, most recently, rectal mucosa lymphoreticular tissue (Gonzalez *et al*., 2006). However it must be noted that not all animals with classical scrapie have detectable lymphoreticular involvement and PrP^{Sc} has not yet been detected in the lymphoreticular tissues of cases of sheep or goats with atypical scrapie (Benestad *et al*., 2008). However, the testing of lymphoreticular tissue offers the opportunity to detect some animals infected with classical scrapie at relatively early stages of incubation, before the CNS is positive.

Due to the complex epidemiology of scrapie, the part of the population that should be targeted for sampling, as well as the tissues to be analysed, differs with the different purposes of the testing. Surveillance for prevalence of the disease could limit tissue examination to the CNS of adult sheep and goats for the reasons given above. However, testing to estimate disease prevalence needs to take into account a number of factors, including the stratification of the sheep-farming industry, dose or level of infection within particular flocks, frequency of disease and relative involvement of the LRS in different genotypes, and the effect of genotype/agent strain combination on incubation period.

The need to distinguish between cases of scrapie and possible BSE in sheep and goats has required the development of diagnostic methods with the potential to discriminate between the agents causing these infections. The conformation of disease-specific PrP produced in BSE-infected sheep is different from that of disease-specific PrP found in natural sheep scrapie. These conformational differences may be detected by immunoblot or IHC techniques using epitope-specific antibodies (summarised in EFSA [2005b]). Within the European Union, the strategy for this distinction comprises examination of source CNS material after initial detection through active or passive surveillance (initial screening) in a primary, secondary and tertiary phase procedure involving a Western immunoblot method capable of such discrimination. This is followed by peer review and further investigation by

3 https://www.izsplv.it/it/istituto/212-centri-eccellenza/laboratori-internazionali-riferimento/422-eurl_tses.html

biochemical and IHC methods of any cases in which primary discrimination was equivocal and, finally, if classification still cannot exclude BSE, mouse transmission to a standardised panel of transgenic mice as described in the https://science.vla.gov.uk/tseglobalnet/documents/tse-oie-rl-handbook.pdf. Interpretation of the *in-vitro* methods (Western immunoblot or ELISA) is reliant on differences between BSE and scrapie in the N-terminal cleavage site for Proteinase-K digestion of PrP^{Sc}. The *in-situ* IHC approach relies on distribution and epitope-specific labelling patterns of PrP^{Sc} in brain and lymphoreticular tissues. Newer *in-vitro* methods, such as quaking-induced conversion (QuIC) (Orru *et al.,* 2012), and protein misfolding cyclic amplification (PMCA) (Castilla *et al.,* 2006) are increasingly being used to increase diagnostic sensitivity, although none have been formally approved for statutory purposes at present. These methods use normal PrP as a substrate, and multiple rounds of protein aggregation to achieve amplification of even very small amounts of PrP^{Sc}, PMCA in particular also demonstrates some potential for agent strain discrimination (Gough *et al.,* 2014).

Quality control (QC), quality assurance (QA) and appropriate positive and negative control samples are essential parts of testing procedures and advice and control materials can be requested from the WOAH Reference Laboratories.

1. Detection of the agent

1.1. Specimen selection and preparation

Concerns regarding BSE in small ruminant populations and the recognition of atypical scrapie have influenced the strategies for sampling and diagnosis. Although comprehensive sampling and multiple testing methods would provide the most robust contingencies for these and possible future uncertainties in the diagnosis of prion diseases of small ruminants, operational factors also determine what is practical and economical. The relative implementation of passive and active surveillance programmes, and the diagnostic methods applied, further influence sampling strategy. Selection and recommendation of methods is therefore under constant review.

For routine diagnosis, the sampled CNS material is either stored fresh or frozen for subsequent biochemical tests or is fixed for histological preparations. Where programmes are in place to identify possible infections with BSE in small ruminant populations, all sampling should be conducted aseptically, using new sterile disposable instruments, or instruments sterilised under conditions specified for the decontamination of prions (see Chapter 3.4.5 *Bovine spongiform encephalopathy*). Cross contamination at necropsy/sampling should be avoided. Thus, in the following procedures where fresh tissue is sampled for biochemical methods, an aliquot should be reserved for transmission studies. Although in many instances disease can be confirmed on autolysed or suboptimally preserved material, such samples can provide only limited evidence of the absence of scrapie.

1.1.1. Suspect clinical cases

Sheep in which clinical classical or atypical scrapie is suspected (detected by passive surveillance) should be killed by intravenous injection of barbiturate and the whole brain removed by standard necropsy procedures as soon after death as possible. Whole brain removal is advisable to allow pathological examination for differentiation among possible different manifestations of prion disease and the differential diagnosis of non-prion associated brain disorders. Methods of sampling the brain tissue for application of PrP-detection techniques requiring fresh tissue and for histological techniques are based on knowledge of the diagnostic sensitivities of each of the tests for different brain areas and the compromise that precisely the same area cannot be used for both fresh/frozen and fixed tissue approaches. The following protocol is recommended but may be subject to modification to satisfy any particular portfolio of tests. Further information can be obtained from WOAH Reference Laboratories (consult the WOAH Web site[4] for the most up-to-date list).

Initially, a coronal block of medulla oblongata inclusive of the obex (see chapter 3.4.5, Figure 1) is taken for fixation into at least 10 times its volume of 10% formol saline and held for 3–5 days prior to processing for histopathological and IHC examinations. Care should be taken to ensure that this sample is not frozen. For the detection of PrP^{Sc}, fresh tissue samples are taken for immediate testing or stored frozen (–20°C or below) prior to extraction of protein. Specimens should, if

4 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

possible, provide 5 g of tissue. This should be taken initially from the caudal medulla and, if necessary supplemented with brainstem immediately rostral to the medulla – obex sample. Subsampling of this tissue to accommodate multiple biochemical methods can be achieved by hemisecting in the median plane or by transverse sectioning. Possible variation in sampling for rapid test requirements at the level of the obex is dealt with below in the section on sampling for rapid tests. For optimal detection of atypical scrapie, the cerebellum should also be sampled; part of it should be kept fresh/frozen or fixed as described above. Medulla and cerebellar samples are processed identically.

Where the whole brain is available, after the initial sampling of the medulla and the cerebellum, the remaining brain should be hemisectioned. One half should be kept fresh/frozen whilst the other half should be fixed in 10 times its volume of 10% formol saline for at least 7–10 days. This would allow for testing additional brain areas using various methods, including bioassays in rodents, to characterise fully the TSE phenotype of challenging cases.

Requirements for pathological characterisation or differential diagnosis from other CNS pathological conditions can be fulfilled by taking additional sections of all major brain regions from the fixed hemisected brain for histological or IHC examination.

1.1.2. Sampling for active surveillance using rapid tests

For the application of rapid tests, methods for removal of the brainstem via the foramen magnum using proprietary spoon-shaped instruments, similar to those employed in cattle for sampling for BSE diagnosis (see chapter 3.4.5, Figure 2) have been devised for sheep. Although not ideal, the approach can also be used for clinical suspect cases. The minimum specimen tissue required is the brainstem at the level of the obex. To detect atypical scrapie the cerebellum should also be sampled with a spoon via the foramen magnum following removal of the brainstem. The brainstem portion is either hemisectioned in the median plane to provide half (fresh/frozen) for a rapid test and half (fixed) for histopathology. Alternatively a complete coronal slice inclusive of the obex is fixed and a similar adjacent caudal medulla slice taken for the rapid test. The complete coronal slice has been recommended in the past to establish the symmetry of morphological changes, but with the use of rapid molecular techniques there is competition between tests for the optimal diagnostic sites at the obex. Some rapid test kits use a core sampling approach to obtain an appropriate mass of material from the obex region. While hemisectioning of the obex region of the brainstem will result in loss of the ability to assess vacuolar lesion symmetry, the greater specificity provided by IHC to detect PrP^{Sc} offsets this disadvantage. However if this, or a core sampling approach, is adopted, it is critical to ensure that the contralateral target site is not compromised. The dorsal nucleus of the vagus nerve (the optimal target area for cases of classical scrapie) is a narrow column that lies close to the midline (see chapter 3.4.5, Figure 3). The options are also dependent on the specific sampling instruments provided by the test kit manufacturer.

For all sampling methods it is vital that operators are trained and that the training includes instruction in the gross and cross-sectional neuroanatomy of the brainstem and the precise location of the target areas for disease-specific PrP^{Sc} accumulation.

For differentiation of classical and atypical cases, portions of cerebellum are required fixed and fresh/frozen.

1.2. Histological examination

Morphological changes in the CNS are those of a spongiform encephalopathy comprising principally vacuolation of neuronal cell bodies and the surrounding neuropil, accompanied by a variable and usually less conspicuous gliosis (particularly an astrocytic reaction). Typically, the lesions have a bilaterally symmetrical distribution. There is considerable variation in the distribution pattern of vacuolation and other changes. In classical scrapie, lesions are usually most apparent in the brainstem and frequently affect the dorsal nucleus of the vagus nerve. Care must be taken if interpreting histopathology alone, as some incidental vacuolation of neurons may also be present in the brains of apparently healthy sheep, albeit at a low frequency (Hornlimann, 2006). There is no direct correlation between the severity of clinical signs and pathological changes. A clinical diagnosis of suspected scrapie cannot be refuted by a failure to find significant vacuolar changes in the brain, and the examination should be supported by tests

to detect the accumulation of disease-specific forms of PrP. This is particularly relevant for atypical scrapie, in which there is no vacuolation in the brainstem. In these cases, vacuolation, if it occurs at all, is generally restricted to the molecular layer of the cerebellar cortex, the cerebral cortex and the basal ganglia.

Despite this variability the histological examination of sections of medulla oblongata at the obex may be sufficient, in most cases, to confirm a diagnosis of clinically suspect classical scrapie (Gavier-Widen *et al*., 2005; Wood *et al*., 1997). The absence of lesions can be established with greatest confidence by examining a number of areas representative of the whole brain.

Although histological examination should not be used as a primary screening or confirmatory test in scrapie surveillance programmes, it can be used to identify scrapie cases by examining haematoxylin and eosin sections that may be generated during other surveillance schemes. Histological examination is still a valuable research tool particularly for recording distribution and intensity of vacuolar lesions in the CNS and for reaching a differential diagnosis in case of a scrapie negative result.

1.3. Detection of disease-specific forms of PrP

Methods for the demonstration of accumulation of disease-specific forms of PrP in specified target areas now provide the principal approach to the diagnosis of both classical and atypical scrapie (Gavier-Widen *et al*., 2005). In suspect clinical cases use of Western immunoblot, IHC or, wherever possible, both is advocated to confirm the diagnosis. IHC on tissue sections to demonstrate accumulation of PrP^{Sc} should be carried out in parallel with routine histology in suspected cases. Combined use of IHC and Western blot is also recommended to provide additional information, particularly where histological lesions are mild in severity and considered equivocal. In active surveillance programmes, the primary diagnosis will usually be accomplished using rapid test methods and, in the case of positive or inconclusive results, confirmatory methods (Western immunoblot, IHC or, ideally, both) should also be applied. A wide range of antisera and monoclonal antibodies for PrP detection by immunochemical methods are now in use and some are commercially available. Advice on test methods and reagents is available from the WOAH Reference Laboratories for scrapie (consult the WOAH Web site for the most up-to-date list).

1.3.1. Immunohistochemical methods

Disease-specific accumulation of PrP^{Sc} in scrapie-affected brain is demonstrated by IHC on routinely formalin-fixed material by the application of a variety of epitope demasking techniques and the use of appropriate antibodies against PrP. Recognition of morphological disease-specific immunolabelling configurations, their cellular associations and neuroanatomical distribution patterns provide the basis for a confirmatory diagnosis in classical (Ryder *et al*., 2001) and in atypical (Benestad *et al*., 2008) scrapie. A protocol and a list of antibodies proven to be of use for IHC can be accessed at the UK WOAH Reference Laboratory web site[5]. In recognition of the distribution of generic skills in national reference laboratories, and the power of the IHC approach, variation in methodology is possible from laboratory to laboratory, subject to appropriate proficiency testing and quality assurance.

If histopathological examination and IHC results cannot be achieved, e.g. owing to the poor state of the sample, (i.e. severely autolysed cases), then Western immunoblot and the rapid test methods are the remaining test options available. Similarly, these methods can also be applied in circumstances where, sometimes in error at necropsy, CNS material intended for fixation and histological examination has been frozen. IHC methods can still be applied to previously frozen samples if they are subsequently fixed, but the ability to identify anatomical sites may be compromised, meaning that any 'negative' result must be qualified. With modification, the method for Western immunoblot detection may also be applied successfully to formalin-fixed tissue (Kunkle *et al*., 2008).

1.3.2. Western immunoblot methods

All Western immunoblot techniques rely on detergent extraction followed by treatment with proteinase K enzyme to digest any normal host protein (PrP^{c}). This leaves only PrP^{res} (the truncated, partially protease-resistant form of the abnormal prion protein [PrP^{Sc}]) to be bound by

5 https://science.vla.gov.uk/tseglobalnet/confirmatory-diagnosis.html

a specific antibody, which provides a detection signal in positive brain samples. A diagnosis based on the detection of PrP^{res} by Western immunoblot for classical scrapie cases requires that immunolabelled bands corresponding to proteins within a range of molecular mass from 17 kDa (unglycosylated PrP^{res}) to 27 kD (diglycosylated PrP^{res}) be present in the proteinase-K-treated scrapie sample lanes only, and that control sample lanes provide appropriate comparisons. Several sensitive Western immunoblot methods for the detection of ovine classical scrapie PrP^{res} have been published (Arsac *et al*., 2007; Langeveld *et al.,* 2014; Stack, 2004; Stack *et al.,* 2006).

For atypical or Nor98 scrapie cases, multiple bands are visualised by Western immunoblot ranging from approximately 11 to 31 kD. As atypical scrapie PrP^{Sc} is less resistant to proteinase K digestion than classical scrapie PrP^{Sc}, the techniques optimised to detect atypical scrapie employ a reduced concentration of this enzyme in the procedure (Arsac *et al.,* 2007; Benestad *et al*., 2008).

The original technique used for diagnosis of BSE, which has been referred to as 'the WOAH Western immunoblot technique' (Stack *et al.,* 2006) relies on detergent extraction of large amounts of fresh brain material (nominally 2–4 g) followed by ultracentrifugation to concentrate the PrP and finally the proteinase K treatment is applied. This technique can also be used to detect classical and atypical scrapie samples.

Detailed protocols for Western immunoblot are available from WOAH Reference Laboratories for scrapie (consult the WOAH Web site for the most up-to-date list).

1.3.3. Rapid test methods

Rapid immunodiagnostic tests for the detection of PrP^{Sc} in small ruminant brain tissue have been developed and have been evaluated for diagnostic use (EFSA, 2005a; 2012), and these are all commercially available. Reference should be made to instructions provided by commercial manufacturers, which will have been subject to approval before use, and subsequent quality assurance. Deviation from test methods provided by commercial manufacturers is not normally permitted, and not recommended without assessment and documentation (see Validation Recommendation Chapter 2.5.8 *Comparability of assays after changes in a validated test method*).

The rapid tests rely on the optimisation of the reagents used for extraction and digestion and specific antibodies for detection. The tests require fresh brain tissue, which to maximise diagnostic sensitivity for classical scrapie, should be brainstem taken at the obex or just caudal to the obex. To ensure maximum diagnostic sensitivity for atypical scrapie, cerebellum should also be tested. Most rapid tests use less than 0.5 g of material and many sampling tools are designed to sub-sample precise amounts. However, to allow for possible additional testing at least 1 g of initial sample is advised. If enough tissue is available, some laboratories use the Western immunoblot technique (see Section B.1.3.2 above) to confirm any weak-positive samples that are initially detected using a rapid test. The increased amount or concentration of PrP^{res} extracted by ultracentrifugation from the larger aliquot of brain tissue can give improved sensitivity.

Prospects for more sensitive diagnostic tests for scrapie and other TSEs are mainly directed at present on the refinement of existing methods and the development of new approaches to the detection of disease-specific forms of PrP. Achievement of the consistent performance of rapid test methods for the primary diagnostic approach is paramount Overall diagnostic sensitivity is strongly influenced by the accuracy of sampling. In addition, some ELISA-based rapid tests appear to have limited sensitivity when applied to goat samples (Corbiere *et al.,* 2013; Simmons *et al.,* 2020). These tests have not been validated for this species but it has been assumed that they are suitable for detecting scrapie in caprine samples because they have been used successfully for this purpose in ovine samples. There is evidence that goat PrP polymorphisms may also affect the performance of other immunoassays (Western immunoblot, IHC) in an antibody-dependent manner (Madsen-Bouterse *et al.,* 2015; Mazza *et al.,* 2012).

1.4. Other diagnostic tests

As for BSE of cattle (see chapter 3.4.5) tests that can be applied effectively to the live animal to detect scrapie cases in the early stage of incubation remain elusive, despite several avenues of research. The pursuit of non-prion protein biomarkers, including possibly through metabolomic or proteomic approaches, may offer prospects but there are constraints including accessibility of tissue to be tested and specificity of the test method. Two methods using *in-vitro* protein amplification have been developed: protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC). They are proving to be very sensitive for the detection of some prion diseases (Castilla *et al.,* 2006; Orru *et al.,* 2012), but have not yet been formally evaluated for application within statutory surveillance systems, although some have been successfully piloted for surveillance applications in humans (Lacroux *et al.,* 2014; Orru *et al.*, 2014).

2. Serological tests

An antibody response to the scrapie agent has not been detected and so serological tests are not feasible.

3. Genetic screening for resistance

Scrapie control and elimination strategies based on genetic selection for resistance to classical scrapie in sheep have been deployed successfully in some countries. Selection is made on determination of the common polymorphisms of the sheep *PrP* gene. As an aid to the control of classical scrapie: breeding stock, particularly rams, of appropriate *PrP* genotype can be selected to produce progeny with reduced risk of developing disease (recently reviewed in EFSA 2014). Such genotyping services are available on a commercial basis in North America and in several countries in Europe. The test is performed using DNA extracted from white blood cells obtained from ethylene diamine tetra-acetic acid (EDTA)-treated blood samples. (Other tissue such as skin [e.g. ear punches] can also be used, as can other tissues such as brain, for screening cull population samples.) Selection of breeding stock can be based on the most scrapie-resistant animals, i.e. animals of genotypes which encode alanine on both alleles at codon 136, arginine on both alleles at codon 154 and arginine on both alleles at codon 171 (so called ARR/ARR animals), thereby reducing the incidence of classical scrapie in individual flocks. However, these animals are not always common in flocks, and in some breeds the genotype is actually absent.

A breeding programme selecting ARR/ARR sheep will not however ensure resistance to atypical scrapie. Decisions on the appropriateness of such programmes must take into account a thorough evaluation of the current national/regional/local scrapie situation, the availability of replacement resistant sheep, the sheep importation policy, availability of testing facilities and the desirability and support of the sheep industry; especially the willingness of sheep breeders to commit themselves to the programme for a long period of time.

Unlike in sheep, in goats none of the PrP polymorphisms that are associated with genetic resistance to classical scrapie are distributed universally in relative abundance. Consequently application of scrapie control and elimination programmes in this species has been successful only in limited cases were appropriate polymorphisms have been identified to allow selection for genetic resistance to classical scrapie in goats (EFSA, 2017; Georgiadou *et al.* 2017). While the possibility of applying a breeding programme for resistance in goats may not be feasible due to the low abundance of resistant polymorphisms, screening programmes at the local level can help to identify resistant goats to restock affected herds after the application of genetically-based selective culling, as it has been applied for decades in sheep.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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NB: There are WOAH Reference Laboratories for scrapie
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for scrapie

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.8.12.

SHEEP POX AND GOAT POX

SUMMARY

Sheep pox and goat pox are viral diseases of sheep and goats characterised by fever, generalised papules or nodules, vesicles (rarely), internal lesions (particularly in the lungs), and death. Both diseases are caused by strains of capripoxvirus, all of which can infect sheep and goats. Although most of the strains examined cause more severe clinical disease in either sheep or goats, some strains have been isolated that are equally pathogenic in both species.

Sheeppox virus (SPPV) and goatpox virus (GTPV) are the causative agents of sheep pox and goat pox, and with lumpy skin disease virus make up the genus Capripoxvirus *in the family* Poxviridae*. Sheep pox and goat pox are endemic in Africa north of the Equator, the Middle East and Asia, while some parts of Europe have experienced outbreaks recently. Countries that reported outbreaks of the disease between 2010 and 2015 include Bulgaria, Chinese Taipei, Israel, Kazakhstan, Kyrgyzstan, Mongolia, Morocco, Greece and Russia, with Greece, Israel and Russia having experienced recurring incidences.*

Identification of the agent: *Laboratory confirmation of capripox is most rapid using the polymerase chain reaction (PCR) method in combination with a clinical history consistent with generalised capripox infection. Isolation of the virus is possible as capripoxviruses will grow on tissue culture of ovine, caprine or bovine origin, although field isolates may require up to 14 days to grow or require one or more additional tissue culture passage(s). The virus causes intracytoplasmic inclusions that can be clearly seen using haematoxylin and eosin staining. The antigen can also be detected in tissue culture using specific sera and immunoperoxidase or immunofluorescence techniques. Capripoxvirus antigen and inclusion bodies may be seen in stained cryostat or paraffin sections of biopsy or post-mortem lesion material.*

An antigen-detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus has been developed.

Serological tests: *The virus neutralisation test is the most specific serological test. The indirect immunofluorescence test is less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of capripoxvirus with test sera is both sensitive and specific, but is expensive and difficult to carry out. The use of this antigen, or other appropriate antigens expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test in the future.*

Requirements for vaccines: *Live and inactivated vaccines have been used for the control of capripox. All strains of capripoxvirus so far examined share a major neutralisation site and some will cross protect. Inactivated vaccines give, at best, only short-term immunity.*

A. INTRODUCTION

The *Capripoxvirus* genus, in the family *Poxviridae,* consists of three species – lumpy skin disease virus, which causes disease in cattle only (see Chapter 3.4.12), and sheeppox virus (SPPV) and goatpox virus (GPPV), which cause sheep pox and goat pox, respectively. Sheep pox and goat pox are characterised disseminated cutanous nodules and up to 100% mortality in fully susceptible breeds of sheep and goats. In indigenous animals,

generalised disease and mortality are less common, although they are seen where disease has been absent from an area or village for a period of time, when intensive husbandry methods are introduced, or in association with other disease agents, such as peste des petits ruminants virus or foot and mouth disease virus. Sheep pox and goat pox are major constraints to the introduction of exotic breeds of sheep and goats to endemic areas, and to the development of intensive livestock production.

Strains of SPPV and GTPV can pass between sheep and goats, although most cause more severe clinical disease in only one species. SPPV and GTPV are transboundary diseases that regularly spread into adjacent, non-endemic areas. Sheep pox and goat pox are endemic in Africa north of the Equator and parts of the Middle East and Asia (see WOAH WAHIS for most up-to-date distribution (https://wahis.woah.org/#/home). Outbreaks have been reported in non-endemic countries of Asia, Europe and the Middle East.

The incubation period of sheep pox and goat pox is between 8 and 13 days following contact between infected and susceptible animals. It may be as short as 4 days following experimental infection by intradermal inoculation or mechanical transmission by insects. Some breeds of European sheep, such as Soay, may die of acute infection before the development of skin lesions. In other breeds there is an initial rise in rectal temperature to above 40°C, followed in 2–5 days by the development of, at first, macules – small circumscribed areas of hyperaemia, which are most obvious on unpigmented skin – and then of papules – hard swellings of between 0.5 and 1 cm in diameter – which may cover the body or be restricted to the groin, axilla and perineum. Papules may be covered by fluid-filled vesicles, but this is rare. Some researchers have distinguished between a vesicular and nodular form of sheep pox and goat pox (Zro *et al*., 2014b).

Within 24 hours of the appearance of generalised papules, affected animals develop rhinitis, conjunctivitis and enlargement of all the superficial lymph nodes, in particular the prescapular lymph nodes. Papules on the eyelids cause blepharitis of varying severity. As the papules on the mucous membranes of the eyes and nose ulcerate, so the discharge becomes mucopurulent, and the mucosae of the mouth, anus, and prepuce or vagina become necrotic. Breathing may become laboured and noisy due to pressure on the upper respiratory tract from the swollen retropharyngeal lymph nodes, due to the developing lung lesions.

If the affected animal does not die in this acute phase of the disease, the papules start to become necrotic from ischaemic necrosis following thrombi formation in the blood vessels at the base of the papule. In the following 5–10 days the papules form scabs, which persist for up to 6 weeks, leaving small scars. The skin lesions are susceptible to fly strike, and secondary pneumonia is common. Anorexia is not usual unless the mouth lesions physically interfere with feeding. Abortion is rare.

On post-mortem examination of the acutely infected animal, the skin lesions are often less obvious than on the live animal. The mucous membranes appear necrotic and all the body lymph nodes are enlarged and oedematous. Papules, which may be ulcerated, can usually be found on the abomasal mucosa, and sometimes on the wall of the rumen and large intestine, on the tongue, hard and soft palate, trachea and oesophagus. Pale areas of approximately 2 cm in diameter may occasionally be seen on the surface of the kidney and liver, and have been reported to be present in the testicles. Numerous hard lesions of up to 5 cm in diameter are commonly observed throughout the lungs, but particularly in the diaphragmatic lobes.

The clinical signs and post-mortem lesions vary considerably with breed of host and strain of capripoxvirus. Indigenous breeds are less susceptible and frequently show only a few lesions, which could be confused with insect bites or contagious pustular dermatitis. However, lambs that have lost their maternally derived immunity, animals that have been kept isolated and animals brought into endemic areas from isolated villages, particularly if they have been subjected to the stress of moving long distances and mixing with other sheep and goats, and their pathogens, can often be seen with generalised and sometimes fatal capripox. Invariably there is high mortality in unprotected imported breeds of sheep and goats following capripoxvirus infection. Capripox is not infectious to humans.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of sheeppox and goatpox and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	+	++	+	+++	+	–
Antigen detection	++	++	++	++	++	–
PCR	++	+++	++	+++	++	–
Detection of immune response						
VN	++	++	++	++	++	++
IFAT	+	+	+	+	+	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; VN = virus neutralisation; IFAT = indirect fluorescent antibody test.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Specimen collection and submission

Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin papules, lung lesions or lymph nodes. Samples for virus isolation and antigen-detection enzyme-linked immunosorbent assay (ELISA) should be collected within the first week of the occurrence of clinical signs, before the development of neutralising antibodies. Samples for genome detection by polymerase chain reaction (PCR) may be collected before or after the development of neutralising antibody responses. Buffy coat from blood collected into EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of capripox infection (before generalisation of lesions or within 4 days of generalisation), can also be used for virus isolation. Samples for histology should include tissue from the surrounding area and should be placed immediately following collection into ten times the sample volume of 10% formalin or neutral buffered 10% formal saline.

Tissues in formalin have no special transportation requirements. Blood samples, for virus isolation from the buffy coat, should be collected in tubes containing anticoagulant, placed immediately on ice and processed as soon as possible. In practice, the blood samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues and dry scabs for virus isolation, antigen detection and genome detection should preferably be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation/detection.

1.2. Virus isolation

Lesion material for virus isolation and antigen detection is homogenised. The following is an example of one technique for homogenisation: The tissue is minced using sterile scissors and forceps, and then macerated in a steel ball bearing mixer mill or ground with a sterile pestle in a mortar with sterile sand

and an equal volume of sterile phosphate buffered saline (PBS) or serum-free Modified Eagle's Medium (MEM) containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (2.5 μg/ml) and neomycin (200 IU/ml). The homogenised suspension is freeze–thawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 ***g*** for 10 minutes. In cases where bacterial contamination of the sample is expected (such as when virus is isolated from skin samples), the supernatant can be filtered through a 0.45 μm pore size filter after the centrifugation step, however, the amount of virus in the supernatant might be reduced. Buffy coats may be prepared from 5–8 ml unclotted blood by centrifugation at 600 ***g*** for 15 minutes; the buffy coat is carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 ***g*** for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml of growth medium, such as Glasgow's modified Eagle's medium (GMEM). After centrifugation at 600 ***g*** for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample using a density gradient.

Capripoxvirus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary cultures of lamb testis (LT) or lamb kidney (LK) cells are considered to be the most susceptible, particularly those derived from a wool sheep breed. The following is an example of an isolation technique: either 1 ml of buffy coat cell suspension or 1 ml of clarified biopsy preparation supernatant is inoculated on to a 25 cm^2 tissue culture flask of 90% confluent LT or LK cells, and the supernatant is allowed to adsorb for 1 hour at 37°C. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing LT or LK cells and a flying cover-slip, or tissue culture microscope slides, are also infected.

The flasks should be examined daily for 7–14 days for evidence of cytopathic effect (CPE). Contaminated flasks should be discarded. Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 4 days after infection; over the following 4–6 days these expand to involve the whole cell sheet. If no CPE is apparent by day 7, the culture should be freeze–thawed three times, and clarified supernatant inoculated on to fresh LT or LK culture. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are indicative of poxvirus infection. Syncytia formation is not a feature of capripoxvirus infection. If the CPE is due to capripoxvirus infection of the cell culture, it can be prevented or delayed by inclusion of specific anti-capripoxvirus serum in the medium; this provides a presumptive identification of the agent. Some strains of capripoxvirus have been adapted to grow on African green monkey kidney (Vero) cells, but these are not recommended for primary isolation.

1.3. Electron microscopy

The characteristic poxvirus virion can be visualised using a negative-staining preparation technique followed by examination with an electron microscope. There are many different negative-staining protocols, an example is given below:

Material from the original tissue suspension is prepared for transmission electron microscope examination, prior to centrifugation, by floating a 400-mesh hexagon electron microscope grid, with piloform-carbon substrate activated by glow discharge in pentylamine vapour, on to a drop of the suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The capripox virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from *Vaccinia* virus, no orthopoxvirus causes lesions in sheep and goats. However, capripoxvirus is distinguishable from the virions of parapoxvirus, that cause contagious pustular dermatitis, as they are smaller, oval in

shape, and each is covered in a single continuous tubular element, which appears as striations over the virion.

1.4. Histopathology

Material for histopathology should be prepared by standard techniques. Following preparation, staining with haematoxylin and eosin (H&E), and mounting of the formalin-fixed biopsy material, a number of sections should be examined by light microscopy. On histological examination, the most striking aspects of acute-stage skin lesions are a massive cellular infiltrate, vasculitis and oedema. Early lesions are characterised by marked perivascular cuffing. Initially infiltration is by macrophages, neutrophils and occasionally eosinophils, and as the lesion progresses, by more macrophages, lymphocytes and plasma cells. A characteristic feature of all capripoxvirus infections is the presence of variable numbers of 'sheep pox cells' in the dermis. These sheep pox cells can also occur in other organs where microscopic lesions of sheep and goat pox are present. These cells are large, stellate cells with eosinophilic, poorly defined intracytoplasmic inclusions and vacuolated nuclei. Vasculitis is accompanied by thrombosis and infarction, causing oedema and necrosis. Epidermal changes consist of acanthosis, parakeratosis and hyperkeratosis. Changes in other organs are similar, with a predominant cellular infiltration and vasculitis. Lesions in the upper respiratory tract are characterised by ulceration.

1.5. Immunological methods

1.5.1. Fluorescent antibody tests

Capripoxvirus antigen can also be identified on infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test using immune sheep or goat sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent sheep or goats or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control because cross-reactions, due to antibodies to cell culture antigens, can cause problems. The fluorescent antibody tissue section technique has also been used on cryostat-prepared slides.

1.6. Nucleic acid recognition methods

Amplification methods for detection of the viral DNA genome are specific to the genus *Capripoxvirus* and sensitive for detection throughout the course of disease including before and after the emergence of antibody responses. These methods include conventional PCR, real-time PCR, and most recently loop-mediated isothermal amplification. Nucleic acid recognition methods can be used to detect the capripoxvirus genome in biopsy, swab or tissue culture samples.

1.6.1. Conventional PCR methods

Several conventional PCR methods have been reported with varying specificity for capripox viruses in general, sheeppox, or goatpox virus (Heine *et al.,* 1999; Ireland & Binepal, 1998; Zro *et al.,* 2014a). Conventional PCR methods are particularly useful for obtaining sufficient genetic material necessary for species identification by subsequent sequence and phylogenetic analysis (Le Goff *et al.,* 2009).

1.6.2. Real-time PCR methods

Several highly sensitive and specific fluorescent detection-based real-time PCR methods have been developed and validated (Balinsky *et al*., 2008; Bowden *et al.,* 2008; Das *et al.,* 2012; Stubs *et al.,* 2012). Each test detects a small conserved genetic locus within the capripoxvirus genome, but these methods do not discriminate between sheeppox virus, goatpox virus or lumpy skin disease virus. Real-time PCR methods for direct capripox genotyping without the need for gene sequencing have been described (Gelaye *et al.,* 2013; Lamien *et al.,* 2011).

1.6.3. Isothermal genome amplification

Molecular tests using loop-mediated isothermal amplification (LAMP) to detect capripoxvirus genomes are reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and at lower cost (Das *et al.,* 2012; Murray *et al.,* 2013). Field validation of the Das *et al.,* 2012 LAMP method has been further reported by (Omoga *et al.,* 2016) and a combination of this universal capripoxvirus test with two additional LAMP assays was reported to show utility in discriminating between goatpox and sheeppox viruses (Zhao *et al.,* 2014).

2. Serological tests

2.1. Virus neutralisation

A test serum can either be titrated against a constant titre of capripoxvirus (100 $TCID_{50}$ [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the use of 100 $TCID_{50}$, the neutralisation index is the preferred method, although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. The use of Vero cells in the virus neutralisation test has been reported to give more consistent results (Kitching & Taylor, 1985).

2.1.1. Test procedure

i) Test sera including a negative and a positive control are diluted 1/5 in Eagle's/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for 30 minutes.

ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES without serum is placed in columns 11 and 12 and to all wells of row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over $\log_{10}$ 6 $TCID_{50}$ per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log dilution series of $\log_{10}$ 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 $TCID_{50}$ per ml (equivalent to $\log_{10}$ 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2 $TCID_{50}$ per 50 µl).

iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

v) The plates are covered and incubated for 1 hour at 37°C.

vi) LT cells are prepared from pregrown monolayers as a suspension of 10^5 cells/ml in Eagle's medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum toxicity controls.

vii) The microtitre plates are covered and incubated at 37°C for 9 days.

viii) Using an inverted microscope, the monolayers are examined daily starting at day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated according to the Kärber method. If left longer, there is invariably a 'breakthrough' of virus in which virus that was at first neutralised appears to disassociate from the antibody.

ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because immunity to capripox is predominantly cell mediated, a

negative result, particularly following vaccination in which the response is necessarily mild, does not imply that the animal from which the serum was taken is not protected.

A constant-virus/varying-serum method has been described using serum dilutions in the range 1/5 to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity to capripoxvirus than LT cells, the problem of virus 'breakthrough' is overcome.

2.2. Indirect fluorescent antibody test

Capripoxvirus-infected tissue culture grown on flying cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/5, and positives are identified using an anti-sheep gamma-globulin conjugated with fluorescein isothiocyanate (Davies & Otema, 1978). Cross-reactions can occur with orf, bovine papular stomatitis virus and perhaps other poxviruses.

2.3. Western blot analysis

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out (Chand *et al.*, 1994).

2.4. Enzyme-linked immunosorbent assay

No validated ELISA is available for the serological diagnosis of SPP or GTP.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

A variety of attenuated live and inactivated capripoxvirus vaccines has been used to provide protection against sheeppox and goatpox. All strains of capripoxvirus of ovine, caprine or bovine origin examined so far share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain (Capstick, 1961). Consequently, it is possible to use a single strain of capripoxvirus to protect both sheep and goats against all field strains of virus, regardless of whether their origin was in Asia or Africa (Kitching *et al.*, 1986; Kitching & Taylor, 1985). However, field evidence suggests some strains are quite host-specific and are used only in sheep against SPPV and only in goat against GTPV.

A number of strains of capripoxvirus have had widespread use as live vaccines (Davies & Mbugwa, 1985), for example the Romanian and RM-65 strains used mainly in sheep and the Mysore and Gorgan strains used in goats. The real identity of the commonly used Kenyan sheep and goat pox vaccine virus (KSGP) 0240 was recently shown to be actually LSDV (Tuppurainen *et al.,* 2014). Virus strain identity and attenuation properties must be ascertained and taken into consideration when selecting vaccine strains for use in cattle, sheep and goats. The protective dose depends on the vaccine strain used. Immunity in sheep and goats against capripox following vaccination with the 0240 strain lasts over a year and the Romanian strain gave protection for at least 30 months.

Killed vaccines produced from tissue culture contain only the intracellular mature virion form of the virus, and lack the less robust but biologically crucial extracellular enveloped virion form. As a result, the vaccine does not stimulate immunity against the extracellular enveloped virion, resulting in poor protection. Killed capripox vaccines provide, at best, only temporary protection.

2. Outline of production and minimum requirements for conventional vaccines

General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*. The documentation should include the standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batches and the final product.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its origin and tissue culture or animal passage. It must be safe to use in all breeds of sheep and goats for which it is intended, including pregnant and young animals. It must be non-transmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared and stored in order to provide a consistent working seed for regular vaccine production.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi and/or mycoplasmas. The general procedures for sterility or purity tests are described in chapter 1.1.9. The master seed must also be safe and produce no clinical reaction in all breeds of sheep or goats when given by the recommended route and stimulate complete immunity to capripox in all breeds of sheep and goats for at least 1 year. The necessary safety and potency tests are described in Section C.2.2.4 *Final product batch tests*.

2.2. Method of manufacture

The method of manufacture should be documented as the Outline of Production.

2.2.1. Procedure

Vaccine seed should be lyophilised and stored in 2 ml vials at –20°C. It may be stored wet at –20°C, but when wet, is more stable at –70°C or lower. The virus should be cultured in primary or secondary LT or LK cells of wool sheep origin for maximum yield. Vero cells may also be used with suitably adapted strains.

Vaccine batches are produced on fresh monolayers of secondary LT or primary LK cells. A vial of seed virus is reconstituted with GMEM or another appropriate medium and inoculated on to an LT or LK monolayer that has been previously washed with warm PBS, and allowed to adsorb for 15 minutes at 37°C before being overlaid with additional GMEM. After 4–6 days, there will be extensive (80–90%) CPE. The culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in medium pH. The culture is freeze–thawed three times, the suspension removed and centrifuged at 600 ***g*** for 20 minutes. A second passage may be required to produce sufficient virus for a production batch. Live vaccine may be produced on roller bottles.

The procedure is repeated and the harvests from individually numbered flasks are each mixed separately with an equal volume of sterile and chilled 5% lactalbumin hydrolysate and 10% sucrose, and transferred to individually numbered bottles for storage at –20°C. Prior to storage, 0.2 ml is removed from each bottle for sterility control. An additional 0.2 ml is removed for virus titration; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used. A written record of all the procedures must be kept for all vaccine batches.

Inactivated vaccines are produced, usually from unattenuated field strains of capripoxvirus, grown in tissue culture as described above, inactivated with 0.03% formaldehyde, and mixed

with an equal volume of alhydrogel as adjuvant. Formaldehyde is no longer considered to be a suitable inactivant for certain viral vaccines because its mode of action cannot be guaranteed to be totally effective in inactivating all the live virus. This has not been fully investigated for capripoxvirus.

2.2.2. Requirements for substrate and media

The specification and source of all ingredients used in the manufacturing procedure should be documented and the freedom from extraneous agents: bacteria, fungi, mycoplasma and any other viruses should be tested. The detailed testing procedure is described in the chapter 1.1.9. The use of antibiotics must meet the requirements of the licensing authority.

2.2.3. In-process controls

i) Cells

Cells should be obtained from the testis or kidney of a healthy young lamb from a scrapie-free flock of a wool sheep breed. During cultivation, cells must be observed for any evidence of CPE, and for normal morphology (predominantly fibroblastic). They can usually be passaged successfully up to ten times. When used for vaccine production, uninfected control cultures should be grown in parallel and maintained for at least three additional passages for further observation. They should be checked for the presence of noncytopathic strains of bovine virus diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells should be prepared and screened prior to vaccine production and stocked in 1–2 ml aliquots containing 2×10^7 cells/ml in sterile 10% DMSO (dimethyl sulphoxide) and 90% FBS (fetal bovine serum) solution stored in liquid nitrogen.

ii) Serum

Bovine serum used in the growth or maintenance medium must be free from transmissible spongiform encephalopathies (TSEs) and antibody to capripoxvirus, and tested for contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

iii) Medium

Medium must be tested free from contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

iv) Virus

Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. Vaccine samples must be examined for the presence of adventitious viruses including cytopathic and noncytopathic strains of pestivirus, and should be mixed with a high-titre capripoxvirus-immune serum that has tested negative for antibody to pestivirus to prevent the vaccine virus itself interfering with the test. The vaccine bulk can be held at –20°C or below until all sterility tests and titrations have been completed, at which time it should be freeze-dried in 1 ml aliquots in vials sufficient for 100 doses. The vaccine harvest diluted with lactalbumin hydrolysate and sucrose should have a minimum titre $\log_{10}$ 4.5 $TCID_{50}$ per ml after freeze-drying, equivalent to a field dose of $\log_{10}$ 2.5 $TCID_{50}$. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

The safety studies should be demonstrated by statistically valid vaccination studies using seronegative young sheep and goats of known susceptibility to capripox virus. The procedure described is suitable for vaccine strains such as 0240 that are equally immunogenic in both sheep and goats. The choice of target animal should be adapted for strains with a more restricted host preference.

iii) Potency

Potency tests must be undertaken if the minimum immunising dose of the virus strain is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of wool or hair. Log_{10} dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals should develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The macule/papule is measured at between 8 and 10 days post-challenge. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference of log_{10} titre > 2.5 is taken as evidence of protection.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

The vaccine must be safe to use in all breeds of sheep and goats for which it is intended, including young and pregnant animals. It must also be non-transmissible, remain attenuated after further tissue culture passage.

Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.

The safety of the vaccine in non-target animals must have been demonstrated using mice and guinea-pigs as described in Section C.2.2.4. There should be no evidence of pathology caused by the vaccine.

ii) Reversion-to-virulence for attenuated/live vaccines

The selected final vaccine should not revert to virulence during a further passages in target animals.

iii) Environmental consideration

Attenuated vaccine should not be able to perpetuate autonomously in cattle, sheep or goat populations. Vaccines using the 0240 strain should not be used in *Bos taurus* breeds. Strains of capripoxvirus are not a hazard to human health. There are no precautions other than those described above for sterility and freedom from adventitious agents.

2.3.2. Efficacy requirements

i) For animal production

The efficacy of the vaccine must be demonstrated in vaccination challenge experiment under laboratory conditions. As described in Section C.2.2.4.

Once the potency of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to

repeat this on the final product of each batch, provided the titre of virus present has been ascertained.

ii) For control and eradication

Vaccination is the only effective way to control the sheep pox and goat pox outbreaks in endemic countries. Unfortunately, currently no marker vaccines allowing the differentiation of infected from vaccinated animals are available.

Immunity to virulent field virus following vaccination of sheep or goats with the 0240 strain lasts over 1 year, and protection against generalised infection following intradermal challenge lasts at least 3 years and is effective lifelong. The duration of immunity produced by other vaccine strains should be ascertained in both sheep and goats by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus confusing the results. The inactivated vaccines provide immunity for less than 1 year, and for the reasons given at the beginning of this section, may not give immunity to the form of capripoxvirus usually associated with natural transmission.

2.3.3. Stability

All vaccines are initially given a shelf-life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life to determine the vaccine variability.

Properly freeze-dried preparations of capripox vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported. The inactivated vaccines must be stored at 4°C, and their shelf- life is usually given as 1 year.

No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Currently, no recombinant vaccines for capripoxviruses are commercially available. However, a new generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens such as peste des petits ruminants (PPR) virus (Berhe *et al.*, 2003; Tuppurainen *et al*., 2014).

3.2. Special requirements for biotechological vaccines, if any

Not applicable.

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NB: There are WOAH Reference Laboratories for sheep pox and goat pox (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for sheep pox and goat pox

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 3.8.13.

THEILERIOSIS IN SHEEP AND GOATS (INFECTION WITH *THEILERIA LESTOGUARDI*, *T. LUWENSHUNI* AND *T. UILENBERGI*)

SUMMARY

Description and importance of the disease: *Theileriosis affects small ruminants in tropical and subtropical regions of Europe, Africa, the Middle East, Far East and Asia. The disease causes clinical signs in domestic ruminants, such as decreased weight and milk production and increased mortality, resulting in significant economic losses. The aetiologic agent is a protozoan transmitted by ixodid ticks, belonging to the* Haemaphysalis, Hyalomma *and* Rhipicephalus *genera. Both internal factors* (*genetics, breed, lambing and lactation*) *and external factors* (*nutrition, other concurrent infections*) *influence susceptibility to infection. Although sheep and goat breeding is one of the most important economic resources in some regions of the world, parasitic protozoa are not well characterised in these species. Among* Theileria *species affecting small ruminants,* Theileria lestoquardi, T. uilenbergi *and* T. luwenshuni *are considered highly pathogenic, while other* Theileria *species are scarcely or not pathogenic.* Theileria lestoquardi *is the causative agent of malignant ovine theileriosis. The common acute form is characterised by fever, cessation of rumination, swelling of superficial lymph nodes, cardiovascular problems, diarrhoea, jaundice and haemorrhages, and mortality rates of 46–100%. Subacute and chronic forms may also occur, showing intermittent fever, inappetence, anaemia, jaundice and emaciation. Suitable approaches to reducing the risk of theileriosis include the use of chemical repellents and pesticides, habitat management, personal and environment-based preventive and control measures, genetic selection of hosts more resistant to ticks, and vaccines.*

Detection of the agent: *Laboratory diagnosis of acute cases is carried out by the examination of Giemsa-stained smears of peripheral blood or aspirated lymph nodes. However, the technique is not useful for detecting the carrier state nor does it allow species differentiation. The use of molecular biology has provided a useful contribution for* Theileria *species identification and classification in both vertebrate and invertebrate hosts, and for genetic characterisation. The small subunit ribosomal RNA* (*18S rRNA*) *gene is the marker used for* Theileria *spp. characterisation due to its highly conserved sequence interspersed with variable regions differing among species. Several molecular methods targeting different markers have been developed, including polymerase chain reaction* (*PCR*), *real-time PCR, reverse line blot hybridisation* (*RLB*), *loop-mediated isothermal amplification of DNA* (*LAMP*), *and sequencing of the amplified amplicons, allowing not only* Theileria *species identification, but also, in the case of real-time PCR, the simultaneous detection and quantification of pathogens from hosts and tick vectors.*

Serological tests: *Different serological methods have been developed, the most relevant of which are the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay. These methods are easy to perform and assist with diagnosis; however there are problems with cross-reactions and sometimes their sensitivity is low.*

Requirements for vaccines: *A live attenuated cell culture vaccine for* T. lestoquardi (T. hirci) *has been developed through progressive attenuation of schizonts propagated in a lymphoid cell culture. It has been successfully used in the Middle East. Tick vaccines are considered useful integrated tick control methods allowing alternative strategies to reduce acaricide use.*

A. INTRODUCTION

Theileriosis in small ruminants is caused by protozoan parasites of the *Theileria* genus (phylum Apicomplexa,order Piroplasmida), in particular by the species *Theileria lestoquardi* (aetiologic agent of malignant ovine theileriosis [MOT]), *T. ovis* (agent of benign theileriosis), *T. recondita* (causing mild ovine theileriosis), *T. separata*, *T. luwenshuni* (or *Theileria* sp. China 1), *T. uilenbergi* (or *Theileria* sp. China 2), Candidatus *Theileria* sp. and *Theileria* sp. OT3 (Stuen, 2020). Among these species, *T. lestoquardi*, *T. luwenshuni* and *T. uilenbergi* are pathogenic in sheep and goats, *T. ovis*, *T. recondita* and *T. separata* are considered scarcely or not pathogenic (Torina & Caracappa, 2012), even if benign *Theileria* species infections can cause significant production losses in imported, immunocompromised or stressed animals (Stuen, 2020). Experimental infections of sheep and goats with *T. annulata*, the causative agent of tropical theileriosis in cattle, have been reported to induce mild signs with no development of piroplasms. A further indication of the close relationship between *T. annulata* and *T. lestoquardi* is the cross-immunity developed by these two pathogens in sheep (Leemans *et al.,* 1999). *Theileria uilenbergi* and *T. luwenshuni* are pathogenic ovine piroplasms described in north-western China (People's Rep. of). As these pathogens also affect cervids, the disease has been termed cervine theileriosis, and *Theileria* parasites with similar sequences but with a low pathogenicity have been detected in sheep in northern Spain, Turkey and Italy (Torina *et al.,* 2012). Reports of *Theileria luwenshuni* in clinically affected sheep have occurred in the United Kingdom (Phipps *et al.,* 2016). The morbidity rate of *T. uilenbergi* and *T. luwenshuni* infections in sheep and goats varies between 18.8% and 65%, the mortality between 17.8 % and 75.4%.

Theileria species are mainly transmitted by ixodid ticks where sexual life cycle and sporogony of these pathogens occur. When the blood meal takes place, sporozoites are transmitted through the tick's saliva to the vertebrate host where they infect nucleated blood cells and may transform into schizonts. *Theileria* can be grouped into 'schizont-transforming' and 'schizont-non-transforming' species. Transforming *Theileria* species are able to induce uncontrolled proliferation in the infected cells (leukocytes), and schizont-infected cells are often found in the circulating blood. *Theileria* parasites develop within the cytoplasm of host leukocytes where the endosomal cell membrane dissolves, hiding the parasite from antibodies. Following the lysis of the infected leukocytes, the released merozoites infect host erythrocytes (RBCs), developing into piroplasms (Lempereur *et al.,* 2017). *Theileria lestoquardi* causes malignant theileriosis in goats and sheep with indefinite proliferation of schizonts, and appears to transform mainly major histocompatibility complex class II-positive cells. The subsequent switch in cytokine production may induce fever, anaemia, muscle wasting and necrosis. Innate immunity takes part in the initial response against transforming *Theileria* species, with the involvement of natural killer (NK) cells and several plasma proteins, such as the proteins of the complement system. In non-transforming *Theileria* species a further multiplication of the piroplasms (merogony) occurs in the RBCs. Non-transforming *Theileria* species are usually considered benign even if they are able to induce anaemia following the piroplasm stage.

Theileria lestoquardi infects the host's monocytes/macrophages and B cells, and recovered animals are resistant to further infections. Indigenous sheep and goats may acquire immunity at an early age. The main clinical signs of MOT include: generalised enlargement of the superficial lymph nodes, high fever, listlessness, anorexia, emaciation, intermittent diarrhoea or constipation and loss of condition (Leemans *et al.,* 1999). Anaemia due to erythrocyte destruction has been reported in infected sheep as well as a marked fall in white blood cells resulting in leukopaenia, a fall in blood packed cell volume and haemoglobin. Serious cardiovascular problems in sheep have been reported following *T. lestoquardi* infection. Sheep are indeed a very receptive host for *T. lestoquardi*, with infections usually ranging from subacute to acute theileriosis even in indigenous sheep. Goats are more resistant to the infection than sheep despite the natural resistance or tolerance shown by indigenous sheep in *T. lestoquardi* endemic areas. These differences may be related to the parasite epidemiology, the seasonal activity of vector ticks, the severity of host infestation with ticks, behavioral differences between sheep and goats, as well as the small ruminant population in the investigated region (Inci *et al.,* 2010). Analysis of risk factors reported a higher prevalence of *T. lestoquardi* in older sheep, with animals older than 2 years of age showing the highest prevalence of infection. Acute *T. lestoquardi* infection has a morbility of 30–40% and mortality of 80–100%, with the outcome of the disease influenced by the infection dose (Stuen, 2020). Transplacental transmission has been reported in both sheep and goats.

Fever is usually the first clinical sign of *T. uilenbergi* and *T. luwenshuni* infections, with body temperature reaching 41.0–42.3°C and a continual or intermittent fever persisting for 12–20 days. Other late signs include: inappetence, cessation of rumination, rapid heartbeat, dyspnoea, weakness, listlessness and swelling of the superficial lymph nodes. Marked anaemia and icterus arise in a few days. A single erythrocyte can contain 1–7 piroplasms, which may appear round, oval or pyriform. In sheep, the parasitemia ranges from 3.2% to 3.7%. *Theileria uilenberg* and *T. luwenshuni* merozoites, in addition to schizonts, are involved in the pathogenesis. Some studies reported a higher incidence as well as a higher death rate in young animals than in adults. Incidence and lethality of 28.3% and 75.3%, respectively, have been reported with most cases involving lambs and 1–2 year-old animals.

Theileria species infecting small ruminants are transmitted by ixodid ticks, with different tick genera acting as vectors of the same *Theileria* pathogen in different geographical regions. However, while for some tick species the vector role has been confirmed, for others only pathogen detection in the arthropod has been reported, with no confirmation of the carrier's role. A transstadial mode of transmission has been observed, while no reports of transovarial transmission have been reported. Tick species involved in *T. lestoquardi* transmission are *Hyalomma anatolicum anatolicum*, *H. detritum, H. impeltatum*, *H. excavatum*, *Rhipicephalus turanicus, Rh. sanguineus*. *Theileria ovis* was detected in *Rh. bursa, Rh. sanguineus*, *Rh. evertsi evertsi*, *H. impeltatum, H. anatolicum anatolicum*, and *H. marginatum*. *Theileria luwenshuni* and *T. uilenbergi* have been detected in *H. qinghaiensis* and *H. longicornis*. Both field nymphs and adults are able to efficiently transmit the pathogens. *Theileria recondita* is transmitted by *Haemaphysalis punctata*, while *Theileria* sp. OT3 was detected in unfed *Hyalomma detritum* and *Haemaphysalis punctata* (Aktas *et al.,* 2006; Kumar *et al.,* 2020; Li *et al.,* 2009).

A relationship among susceptibility to the infection, internal characteristics (genetics, breed, lactation, parturition) and external determinants (nutrition, concomitant infections) has been reported (Stuen, 2020). Some of the main factors influencing the infection process include host range and number, midgut infection and escape barrier, innate immunity, pathogen characteristics, interplay between tick microbiome and pathogen, cross-immunity interference and abiotic factors. Investigation of molecular drivers affecting vector competence may lead to the identification of novel antigens derived from ticks or pathogens useful for implementing novel control and prevention strategies for tick-borne diseases (de la Fuente *et al.,* 2017)

Chemotherapeutic agents such as parvaquone, buparvaquone and imidocarb have been used for theileriosis treatment. Buparvaquone is effective in clearing *Theileria* parasites from sheep and goats, but it does not completely allow pathogen eradication from the host, leading to the establishment of a carrier status. Acaricides, which reduce tick infestations, have been used to control ovine theileriosis. Vaccines however, remain the most effective approach to the prevention and control of tick borne diseases (TBDs) due to the establishment of tick resistance to acaricides, the short-lasting effect of these chemicals and safety issues. The use of vaccines against ticks as components of integrated tick control methods would reduce the use of acaricides. Among tick vaccines, the ones based on the subolesin antigen act by decreasing both tick infestation and pathogen infection/transmission, thus reducing both the tick population and their vector capacity (Torina *et al*., 2014). Anti-subolesin antibodies elicited by the vaccine could enter into tick cells and interact with cytosolic subolesin, preventing its translocation to the nucleus and thus its action of regulatory protein. Vaccines based on attenuated schizont-infected cell lines has been widely used for *T. lestoquardi* (Ahmed *et al.*, 2013) and *T. lestoquardi* sporozoite proteins are under evaluation to be included in sub-unit vaccine trials.

B. DIAGNOSTIC TECHNIQUES

Diagnosis of acute cases, in correlation with clinical data, is based on the examination of smears of peripheral blood or aspirated lymph node stained with Giemsa, which allows the detection of schizonts in white blood cells or piroplasms in erythrocytes.

Table 1. Test methods available for the diagnosis of theileriosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Microscopic examination	–	–	–	+++	–	–
PCR	+	++	+++	+++	+	–
Nested PCR	+	+++	+++	+++	+	–
RLB	+	+++	+++	+++	+	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
IFAT	+	++[(b)]	++	–	++	++
ELISA	+++	++[(b)]	++	–	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; RLB = reverse line blot hybridisation;
IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent detection methods applied on the same clinical sample is recommended.
[(b)]For the purpose of animal movement, serology should not be used alone but always combined with an agent detection test.

The identification of these pathogens by microscopy is difficult, particularly when mixed infections occur. Moreover, expertise in microscopic detection of piroplasm is required for subclinical or chronic infections as parasitaemia is often extremely low and may otherwise be missed. Indirect immunofluorescence and enzyme-linked immunosorbent assays (ELISA) are commonly used serological tests for the detection of specific antibodies as they are easy to perform and useful in diagnosis, but there are problems with cross-reactivity. Techniques based on molecular biology have been developed, such as the polymerase chain reaction (PCR), PCR-RFLP (restriction fragment length polymorphism) methods, nested-PCR, real-time PCR, reverse line blot hybridisation (RLB), loop-mediated isothermal amplification of DNA (LAMP), pan-FRET (fluorescence resonance energy transfer) based assays and the sequencing of several genes, allowing the identification at the species level and the phylogenetic analysis of the pathogens.

1. Detection of the agent

1.1. Microscopic examination

Microscopic examination of thick and thin blood films is the traditional method for identifying the agent. If it is possible to make fresh films from capillary blood, or from venous blood with anticoagulant such as lithium heparin or ethylene diamine tetra-acetic acid (EDTA); samples can be kept at 4°C, until delivery to the laboratory. Macroschizonts and microschizonts cells may be found in impression smears from liver, spleen, lung, kidney, and lymph nodes. Lymph node smears are particularly useful for demonstrating schizonts.

Thin blood smears are air-dried, fixed with methanol for 10–60 seconds, stained with 10% Giemsa for 15–30 minutes, and examined at ×800–1000 magnification under oil immersion. Other stains, such as Romanowsky or Wright's, can also be used.

In erythrocytes, *Theileria* merozoites are predominantly rod shaped, up to 2.0 μm long and 1.0 μm wide. Round, oval, and ringshaped forms also occur. Multiple parasites per erythrocyte are common. In the cytoplasm of lymphocytes, two types of schizonts (Koch's blue bodies) can be found: macroschizonts and microschizonts, both about 8.0 μm, containing up to 8 and 36 small nuclei, respectively (Lempereur *et al.,* 2017). This technique is good for detection of acute infections, but not for detection of carriers, where the parasitaemia is usually very low. Parasite identification and differentiation can be improved by using a fluorescent dye, such as acridine orange, instead of Giemsa. However, negative results from microscopic examination of blood films do not exclude latent infection. Sensitivity may be improved by using 'thick' blood smears, placing a small droplet of blood (~50 μl) on to a clean glass slide and spreading this over a small area using a circular motion with the corner of another slide. This droplet is air-dried, heat-fixed at 80°C for 5 minutes, and stained in 10% Giemsa. RBCs are lysed and parasites concentrated, however, identification of the parasite outside the host cell and in the presence of the many artefacts produced by this method is challenging.

Theileria species can be detected in whole tick salivary glands (SG) using the Feulgen technique. Unfed or 4–5 day engorged ticks are embedded in a small Petri dish in a 1 cm-diameter circle of melted paraffin,

with the tick's dorsal surface facing up. To lift the scutum, an incision is made with a scalpel blade around the margin of the body, starting and ending at the base of the capitulum. This facilitates removal of the gut and exposure of the SG. Trachea fragments are removed, and the whole SG are immersed in physiological saline solution. SG are then fixed for 15–30 minutes in Carnoy's fluid followed by dehydration overnight in absolute ethanol. Using a small brush, samples are washed in a small Petri dish, stained for 2 hours with Feulgen's reagent, washed again, dehydrated, and cleared with xylol. Infected acini that appear as Feulgen-positive bodies (DNA red-purple and cytoplasm green) can be quantified by immersion in xylol or methyl salicylate in a Petri dish or after slide mounting in Canada balm or DPX using a stereomicroscope at magnifications of ×500 or higher (Lempereur *et al.,* 2017).

1.2. Molecular methods

Molecular biology is very useful for the identification and classification of *Theileria* species in vertebrate and invertebrate hosts; these techniques are usually more sensitive than microscopy. Moreover, molecular techniques may allow pathogen identification to the genus or species level or may be used for phylogenetic analysis.

Despite advances in molecular-based methodologies for the diagnosis of theileriosis, the use of blood smears is often maintained due to the higher costs associated with molecular techniques. An inexpensive method for extracting *T. orientalis* DNA from bovine blood has been developed to reduce the cost of DNA extraction in molecular assays (Bogema *et al.,* 2015). The method is based on mild hypotonic erythrocyte lysis, followed by centrifugation to remove contaminating PCR inhibitors, and by a detergent-proteinase K treatment (DPK method). 100 µl of blood sample is mixed with 900 µl TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). After centrifugation for 10 seconds at 16,000 ***g***, the supernatant is discarded, the pellet resuspended in 1 ml TE buffer and centrifuged again for 10 seconds at 16,000 ***g***. This step is repeated one additional time. Each pellet is resuspended in 200 µl DPK digest reagent (50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 100 g/ml proteinase K and 0.5% Tween 20) and incubated for 30 minutes at 60°C followed by 10 minutes at 100°C.

Many molecular targets and assays for the detection of ovine and caprine *Theileria* species are the same as those used for cattle *Theileria* agents. The main molecular diagnostic methods specifically developed for detection and identification of small ruminant *Theileria* species are reported below.

The most commonly used marker in the characterisation of *Theileria* spp. is the small subunit ribosomal RNA (18S rRNA) gene (Liu *et al.,* 2010; Sparagano *et al.,* 2006). This gene is highly conserved among the organisms, but it also includes some variable regions differing among species. Primers can therefore be designed in the conserved regions allowing the amplification of gene fragments from all the related species, while species-specific probes can be designed that are complementary to the variable regions to differentiate the species (Mans *et al.,* 2015)

Other genetic markers used in the characterisation and phylogeny of *Theileria* species are: 28S rRNA, 5.8S rRNA and S5 rRNA genes (Mans *et al.,* 2015; Mohammadi *et al*., 2017), internal transcribed spacers (ITS) (Aktas *et al.,* 2007), micro- and minisatellite markers, and 30 kD merozoite surface protein (Kirvar *et al.,* 1998).

Different techniques have been developed, such as classical PCR (Altay *et al.,* 2008; Kirvar *et al.,*1998; Mohammadi *et al.,* 2017; Nagore *et al.,* 2004; Yin *et al.,* 2008), multiplex PCR (Zhang *et al.,* 2014), semi-nested PCR (Mohammadi *et al.,* 2017), nested PCR (Altay *et al.,* 2005;), PCR-RFLP (Heidarpour Bami *et al.,* 2009), LAMP (Salih *et al.,* 2012), pan-*Theileria* real-time FRET-PCR (Yang *et al.,* 2014), a multiplex DNA bead-based suspension array (Ros-Garcia *et al.,* 2013) and RLB (Gubbels *et al.,* 1999; Nagore *et al.,* 2004; Schnittger *el al.,* 2004).

Biomolecular methods have partially solved the problems of pathogen detection. *Theileria* species may remain in a latent state in infected animals, manifesting a very low parasitic charge, which is sometimes not enough to stimulate the immune system. Therefore, this latent state cannot be detected even with serological tests unless the animals are in endemic areas and thus subject to continual antigenic stimulation for reinfection by the vector ticks. Identification of carrier animals is very important for animal movement from endemic to free territories.

Another problem for tick-borne diseases is the possibility of co-infections caused by pathogens belonging to the same genus. To overcome this problem, researchers aimed to develop very sensitive and highly specific assays, even if they are sometimes complex and require specialised laboratories. One of these methods is the RLB, based on the hybridisation of PCR products to specific probes immobilised into a nitrocellulose membrane. For detection of *Theileria* spp. DNA, the hypervariable V4 region of the 18S rRNA gene is amplified by PCR (Nagore *et al.,* 2004) using the forward RLB-F (5'-GAC-ACA-GGG-AGG-TAG-TGA-CAA-G-3') and reverse RLB-R (biotin-5'-CTA-AGA-ATT-TCA-CCT-CTG-ACA-GT-3') primers (Georges *et al.,* 2001; Nagore *et al.,* 2004). Some protocol variants include an initial nested PCR to increase the sensitivity of the test (Schnittger *et al.,* 2004). PCR reactions can be performed in a 50 or 100 µl volume, including 1×PCR buffer, 1.5 mM $MgCl_2$, 200 mM each deoxynucleoside triphosphate, 1.125 or 2.5 U of Taq polymerase, respectively, 100 pmol of each primer, and 50–100 ng of purified DNA sample. To minimise nonspecific amplification, a touchdown PCR programme can be used. This protocol includes 3 minutes at 94°C, two cycles of 20 seconds at 94°C, 30 seconds at 67°C, and 30 seconds at 72°C, and then two cycles with conditions identical to the previous cycles but with an annealing temperature of 65°C. During subsequent two cycle sets, the annealing temperature is lowered by 2°C until it reaches 57°C. Then, an additional 40 cycles each consisting of 20 seconds at 94°C, 30 seconds at 57°C, and 20 seconds at 72°C are performed. The PCR ends with an extra incubation for 7 minutes at 72°C.

PCR products are used for hybridisation with specific oligonucleotide probes containing an N-terminal N-(trifluoracetamidohexyl-cyanoethyl,N,N-diisopropyl phosphoramidite [TFA])-C6 amino linker for nitrocellulose membrane binding. Designed *Theileria* spp. oligonucleotides are given in the table below.

Probe specificity	18S probe sequence (5' → 3')	Reference
Theileria/Babesia catch-all	TAA-TGG-TTA-ATA-GGA-(A/G)C(A/G)-GTT-G	Gubbels *et al.,* 1999
Theileria spp.	TGA-TGG-GAA-TTT-AAA-CC(CT)-CTT-CCA	Nagore *et al.,* 2004
Theileria ovis	TTT-TGC-TCC-TTT-ACG-AGT-CTT-TGC	Nagore *et al.,* 2004
Theileria lestoquardi	ATT-GCT-TGT-GTC-CCT-CCG	Schnittger *et al.,* 2004
Theileria uilenbergi	TGC-ATT-TTC-CGA-GTG-TTA-CT	Schnittger *et al.,* 2004
Theileria uilenbergi	TGC-ATT-TTC-CGA-GTG-TTA-CT	Niu *et al.,* 2009
Theileria luwenshuni	ATC-TTC-TTT-TTG-ATG-AGT-TG	Niu *et al.,* 2009
Theileria luwenshuni	TCG-GAT-GAT-ACT-TGT-ATT-ATC	Schnittger *et al.,* 2004
Theileria annulata	CCT-CTG-GGG-TCT-GTG-CA	Gubbels *et al.,* 1999
Theileria sp. OT1	ATC-TTC-TTT-TTG-ATG-AGT-TGG-TGT	Nagore *et al.,* 2004
Theileria sp. OT3	ATT-TTC-TCT-TTT-TAT-ATG-AGT-TTT	Nagore *et al.,* 2004
Theileria sp. MK	CAT-TGT-TTC-TTC-TCA-TGT-C	Altay *et al.,* 2008

1.2.1. Reverse line blot hybridisation

The RLB protocol has been described (Georges *et al.*, 2001; Gubbels *et al.,* 1999; Niu *et al.,* 2009) and is summarised here. Membrane preparation includes the activation of the nitrocellulose membrane by 10 minutes incubation in 10 ml of 16% (wt/vol) 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDAC) at room temperature. The membrane is then washed in distilled water and placed in a miniblotter. *Theileria*-specific oligonucleotides are diluted to 200–1600 pmol/150 ml in 500 mM $NaHCO_3$ (pH 8.4) and loaded into the miniblotter slots. An incubation step for 1 minute at room temperature is carried out to covalently link the membrane with the amino linker. After aspiration of solutions, the membrane is first inactivated by incubation in 100 ml of a 100 mM

NaOH solution for 10 minutes at room temperature, then washed in 125 ml of 2× SSPE–0.1% sodium dodecyl sulfate (SDS) for 5 minutes at 60°C.

Before hybridisation with PCR products, the membrane is first washed for 5 minutes at 42°C with 125 ml of 2× SSPE–0.1% SDS and placed perpendicular to its previous orientation into the miniblotter. 40 µl of PCR products are diluted with 2× SSPE, 0.1% SDS to a final volume of 150 µl, heated to 95°C for 5 minutes and then cooled on ice. Denatured PCR samples are applied into the slots and incubated for 60 minutes at 42°C, then aspirated and the membrane is washed at 42°C for 10 minutes in 2×·SSPE, 0.1% SDS.

The membrane is subsequently incubated for 30 minutes at 42°C in 10 ml of 1/4000 diluted peroxidase-labelled streptavidin in 2× SSPE–0.5% SDS, and washed twice in 125 ml of 2× SSPE–0.5% SDS for 10 minutes at 42°C with shaking. After two rinses in 125 ml of 2× SSPE at room temperature, the membrane is incubated for 1 minute in 10 ml of ECL detection solution followed by exposure to a chemo-luminescent detection film. The film is then developed in an X-ray film developer and fixed. A black spot in the sample–probe cross in the hyperfilm indicates a positive signal for that pathogen.

After use, all PCR products are stripped from the membrane by two washes in 1% SDS for 30 minutes each time at 80°C. The membrane is rinsed in 20 mM EDTA (pH 8.0) and stored in fresh EDTA solution at 4°C; it can be reused about 20 times (Schnittger *et al.*, 2004).

The RLB finds wide application for its high sensitivity and the ability to identify co-infections. Exceptions may occur when the sensitivity of the assay is affected by the presence of mixed infections. Sensitivity of the RLB is severely affected by the use of universal primers and these are depleted by the predominant species present, thereby suppressing the signal from less abundant templates (Mans *et al.,* 2015).

1.2.2. Polymerase chain reaction

In cases of mixed infections, species-specific PCRs can be useful in investigations of field samples. Some of the most used PCRs for detection of *Theileria* pathogens in small ruminants are reported below.

A PCR and a nested PCR targeting the *ssu rRNA* of *T.ovis* have been developed (Aktas *et al.,* 2006). The first method (Aktas *et al.,* 2006) is based on the amplification of a 520 bp fragment of the *ssu rRNA* gene. Amplification conditions consist in a 50 µl final volume containing: 5 µl of 10× PCR buffer (100 mM Tris–HCl [pH 9], 500 mM KCl, 1% Triton X-100), 250 µM of each of the four deoxynucleotide triphosphates, 2U Taq DNA polymerase, 10 pg of each primer, 5 µl of DNA. The thermal profile includes an initial denaturation at 96°C for 3 minutes, 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes and final extension of 72°C for 10 minutes. The second method (Altay *et al.,* 2005) add a second amplification step using inner primers to the previously described steps, amplifying a 398 bp smaller fragment. The nested PCR is carried out in a total reaction volume of 50 µl containing 5 µl of 10× PCR buffer (100 mM Tris–HCl [pH 9], 500 mM KCl, 1% Triton X-100), 250 µM of each of the four deoxynucleotide triphosphates, 2 U Taq DNA polymerase, and 10 pg of each primer. In the first round of amplification, 5 µl of the DNA suspension is added as template, while for the nested PCR, 5 µl of a 1:20 dilution of the primary product is used. The amplification protocol includes an initial denaturation step at 96°C for 3 minutes, followed by 30 cycles at 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 2 minutes and a final extension of 72°C for 10 minutes.

For detection of *T. lestoquardi*, a specific PCR has been developed (Kirvar *et al.,* 1998), amplifying a fragment of 785 bp of the 30 kDa merozoite surface gene (*Tlms*). The amplification is carried out in a final volume of 50 µl containing: 5 µl of 10× PCR buffer (2.5 mM $MgCl_2$, 20 mM Tris-HCl pH 8.55, 16 mM $[NH4]_2SO_4$, 150 µg/ml bovine serum albumin), dNTP mixture 0.2 mM each, primers 0.5 µM each, 0.025 U/µl Taq polymerase and 5 µl DNA. The amplification protocol includes an initial denaturation at 94°C for 3 minutes followed by 40 cycles at 94°C denaturation, 65°C annealing and 72°C extension and a final extension step of 72°C for 5 minutes. A semi-nested PCR has been also developed for detection of *T. lestoquardi* (Mohammadi *et al.,* 2017), amplifying a 235 bp fragment of the *SSU-rRNA* gene of *T. lestoquardi*. The semi-nested PCR is carried out in

50 µl total reaction volume containing 5 µl of 10× PCR buffer, 2 mM $MgCl_2$, 250 µM of each of the four deoxynucleotide triphosphate, 1.25 U Taq DNA, 50 pmol of each primer, and 10 ng of first PCR product. Cycling condition for the semi-nested includes an initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 94°C for 45 seconds, 55°C for 90 seconds and 72°C for 45 seconds and a final additional extension step for 5 minutes in 72°C.

Specific PCRs for detection of *T. luwenshuni* and *T. uilenbergi* (Yin *et al.,* 2008) have been developed, based on the amplification of a 389 and a 388 bp 18S rRNA gene fragment, respectively. Two primer pairs have been designed and PCR amplification was performed in a final volume of 30 µl containing 3 µl of 10× PCR buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm $MgCl_2$, 0.01% gelatin), 200µm of each deoxynucleoside triphosphate, 10 µm of each primer, 100 ng of genomic DNA and 1.5 U of Taq polymerase. The cycling conditions of the PCR were 3 minutes at 94°C for denaturation followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing 1 minute at 57°C for *T. luwenshuni* specific primers or 1 minute at 55°C for *T. uilenbergi* specific primers and extension for 1 minute at 72°C. The final extension step was 7 minutes at 72°C.

A specific PCR was also developed for *Theileria* sp. MK targeting a 757 bp fragment of the *18S ssu rRNA* gene (Altay *et al.,* 2008). The PCR was performed in a total reaction volume of 50 µl containing 5 µl of 10× PCR buffer (750 mM Tris–HCl [pH 8.8], 200 mM $[NH_4]2SO_4$, 0.1% Tween 20), 2 mM $MgCl_2$, 250 µM of each of the four deoxynucleotide triphosphates, 1.25 U Taq DNA polymerase, and 37.5 pmol of each primer and 5 µl of template DNA. The reaction was repeated for 35 cycles under the following conditions: 1 minute at 94°C, 1 minute at 57°C and 1 minute at 72°C. Interestingly, this PCR resulted as sensitive as the RLB as both tests were able to detect one infected cell out of 10^7 sheep erythrocytes.

Target gene	Primer sequences	Amplicon size (bp)	Amplification conditions	Amplification protocol	Reference
Theileria ovis					
ssu rRNA	TSsr 170F: TCG-AGA-CCT-TCG-GGT TSsr 670R: TCC-GGA-CAT-TGT-AAA-ACA-AA	520	50 µl containing: 5 µl of 10× PCR buffer (100 mM Tris–HCl [pH 9], 500 mM KCl, 1% Triton X-100), 250 mM of each dNTP, 2U Taq DNA polymerase , 10 pg of each primer, 5 µl of DNA	• Denaturation at 96°C for 3 minutes • 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes • Final extension of 72°C for 10 minutes	Aktas *et al.,* 2006
SSU rRNA	Primary PCR TSsr 170F: TCG-AGA-CCT-TCG-GGT TSsr 670R: TCC-GGA-CAT-TGT-AAA-ACA-AA Nested PCR TSsr 250FN: CGC-GTC-TTC-GGA-TG TSsr 630RN: AAA-GAC-TCG-TAA-AGG-AGC-AA	520 398	50 µl containing 5 µl of 10× PCR buffer 5100 mM Tris–HCl [pH 9], 500 mM KCl, 1% Triton X-100), 250 µM of each dNTPs, 2 U Taq DNA polymerase, and 10 pg of each primer and: • For primary PCR: 5 µl of the DNA • For nested PCR: 5 µl of a 1/20 dilution of the primary product	• Denaturation at 96°C for 3 minutes • 30 cycles at 94°C for 30 seconds, 54°C for 30 seconds and 72 °C for 2 minutes • Final extension of 72 °C for 10 minutes	Altay *et al.,* 2005
Theileria lestoquardi					
30 kDa mero-zoite surface gene (Tlms)	TlestF: GTG-CCG-CAA-GTG-AGT-CA TlestR: GGA-CTG-ATG-AGA-AGA-CGA-TGA-G	785	50 µl containing: 5 µl of 10× PCR buffer (2.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.55, 16 mM $[NH4]_2SO_4$, 150 µg/ml bovine serum albumin), dNTP mixture 0.2 mM each, primers 0.5 µM each, 0.025 U/µl Taq polymerase and 5 µl DNA	• Denaturation at 94°C for 3 minutes • 40 cycles at 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 2 minutes • Final extension of 72°C for 5 minutes	Kirvar *et al.,* 1998

Target gene	Primer sequences	Amplicon size (bp)	Amplification conditions	Amplification protocol	Reference
SSU-rRNA	• Primary PCR Theil-F: CAC-AGG-GAG-GTA-GTG-ACA-AG Bab-R AAG-AAT-TTC-ACC-TCT-GAC-AG • Emi-nested: ATT-GCT-TGT-GTC-CCT-CCG	426-430 235	Emi-nested : 50 µl containing 1× PCR buffer, 2 mM $MgCl_2$, 250 µM of each dNTPs, 1.25 U Taq DNA polymerase, 50 pmol of each primer, and 10 ng of amplified PCR DNA	Emi-nested: • Denaturation at 95°C for 5 minutes • 35 cycles at 94°C for 45 sec, 55°C for 90 seconds and 72°C for 45 seconds. • Final extension for 5 minutes at 72°C.	Mohammadi *et al.,* 2017
Theileria luwenshuni					
18S rRNA gene	Tluw310s: GGT-AGG-GTA-TTG-GCC-TAC-TGA 374as: TCA-TCC-GGA-TAA-TAC-AAG-T	389	30 µl containing: 3 µl of 10× PCR buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm $MgCl_2$, 0.01% gelatin), 200 µM of dNTPs, 10 µM of each primer, 100 ng of genomic DNA and 1.5 U of Taq polymerase	• Denaturation at 94°C for 3 minutes • 35 cycles at 94°C for 30 seconds, 57°C for 1 minute and 72°C for 1 minute • Final extension of 72°C for 7 minutes	Yin *et al.,* 2008
Theileria uilenbergi					
18S rRNA gene	Tuil310s: GGT-AGG-GTA-TTG-GCC-TAC-CGG 689as: ACA-CTC-GGA-AAA-TGC-AAG-CA	388	30 µl containing: 3 µl of 10× PCR buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm $MgCl_2$, 0.01% gelatin), 200 µM of dNTPs, 10 µM of each primer, 100 ng of genomic DNA and 1.5 U of Taq polymerase	• Denaturation at 94°C for 3 minutes • 35 cycles at 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute • Final extension of 72°C for 7 minutes	Yin *et al.,* 2008
Theileria sp. MK					
18S ssu rRNA gene	Tmk-F: CAT-TGT-TTC-TTC-TCA-TGT-C 990: TTG-CCT-TAA-ACT-TCC-TTG	757	50 µl containing: 1× PCR buffer, 2 mM $MgCl_2$, 250 µM of each dNTPs, 1.25 U Taq DNA polymerase, 37.5 pmol of each primer and 5 µl of DNA	• Denaturation at 94°C for 3 minutes • 35 cycles at 94°C for 1 minute, at 57°C at 1 minute and at 72°C 1 minute • Final extension of 72°C for 10 minutes	Altay *et al.,* 2008

dNTPs: deoxynucleotide triphosphate

2. Serological tests

Detecting antibodies against *Theileria* using serological tests is a useful technique in epidemiological surveys. False positive and negative results due to cross-reactions or weak specific-immune responses are some of the main disadvantages of these tests.

Antibodies first appear at 15 days post-infection (d.p.i) with *T. lestoquardi* in sheep, shortly after the detection of schizonts; an increase in antibody titre of at least four times is observed when compared with samples collected before inoculation. Peak antibody levels, ranging from 1/640 to 1/1280, are reached between 30 and 40 d.p.i., indicating a 32- to 64-fold increase in antibody titres. Serum antibodies are still detectable at 90 d.p.i.

2.1. The indirect fluorescent antibody test

The indirect fluorescent antibody test (IFAT) based on *T. lestoquardi* schizont antigen has been described, but the test has several disadvantages as the subjective operator-dependent interpretation of results and the cross-reactivity of the test against other *Theileria* species. IFAT assay for *T. lestoquardi* showed significant cross-reactivity with *T. annulata* and *T. parva* anti-sera and vice versa. As schizont antigens are mainly used, such cross-reactivity is attributed to shared antigens between *T. lestoquardi* and other tick-borne pathogens.

The protocols for the preparation of schizont antigen and piroplasm antigen as well as the IFAT procedure are provided in Chapter 3.4.15 *Theileriosis* Section B.2.1. As samples from small ruminants are used, in step h) of the procedure it is necessary to use anti-sheep or anti-goat immunoglobulin preparations conjugated to fluorescein isothiocyanate. Schizont antigen are intracytoplasmic schizonts derived from *T. lestoquardi*-infected lymphocyte (mostly B cells) cell lines; piroplasm antigen of transforming *Theileria* are prepared from the blood of infected animals or, in the case of *T. uilenbergi* (non-transforming *Theileria*), from long-term culture of erythrocytic stages (Miranda *et al.,* 2006b).

2.1.1. Preparation of *Theileria uilenbergi* long-term *in-vitro* cultures

Theileria uilenbergi long-term *in vitro* cultures are based on the microaerophilous stationary phase method.

i) A blood sample heparinised from *T. uilenbergi*-infected sheep, with a percentage of parasitised erythrocytes (PPE) of 6% and a haemocrit of 30%, and a blood sample from uninfected sheep (to obtain uninfected RBCs) are washed three times with mVYM (modified Vega and Martinez solution) by centrifugation at 850 ***g*** for 10 minutes at 4°C, the supernatant is discarded and the packed RBCs are used to initiate cultures.

ii) Infected and uninfected RBCs are then prepared in basic culture medium consisting of commercial HL-1 medium supplemented with 20% inactivated lamb serum. Instead of serum, 1% lipid-rich bovine serum albumin supplemented with either 1% NEAA (non-essential amino acids solution) or chemically defined lipids (CDL) can be used. The basic medium is supplemented with 25 mM HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), 2 mM L-glutamine, 24 mM sodium bicarbonate, gentamicin (50 μg/ml), amphotericin B (2.5 μg/ml), 1 mM L-cysteine, 0.02 mM bathocuproinedisulfonic acid disodium salt and 200 μM hypoxanthine.

iii) The parasited and normal RBC suspensions are mixed for a PPE of 3% and a haemocrit of 10%, 0.2 ml of final volume of suspensions are dispensed in duplicate into 96-well culture plates and incubated under O_2 reduced atmosphere (gas mixture of 2% O_2, 5% CO_2 and 93% N_2) in a humidified atmosphere at 37°C.

iv) Change medium daily removing 0.14 ml of medium from the culture well and replacing with an equal volume of fresh medium.

v) For subcultures, remove 0.15 ml of medium from the culture well every 4–5 days and replace with 0.15 ml of a suspension of uninfected RBCs in fresh medium at a final concentration of 1/30. Gently resuspend cultures in 1:8 ratio by trasfering 0.025 ml to new wells and bring to a final volume of 0.2 ml by addition of a suspension of 10% RBCs in fresh colture medium.

2.2. Enzyme-linked immunosorbent assay

The ELISA is easy to perform, reproducible and it allows for high throughput assays. Using clones of the cDNA library followed by bioinformatics analysis, several recombinant proteins were studied to develop indirect ELISA.

Several indirect ELISAs for the detection of *T. lestoquardi* antibodies have been developed that are suitable for epidemiological studies and large-scale studies because of the objectivity of the results and the ability to process a large number of samples every day; no commercial kits are available. The ELISAs are based on crude merozoite antigen (Gao *et al.,* 2002), or partially expressed *T. lestoquardi* recombinant heat shock protein 70 (rTIHSP 70) (Miranda *et al.,* 2006a)., or *T. lestoquardi* recombinant clone-5 schizont surface antigen (Bakheit *et al.,* 2006).

The crude antigen (merozoite lysate)-based ELISA is highly sensitive for detection of antibodies against both *T. uilenbergi* and *T. luwenshuni* (Gao *et al.,* 2002). However, it showed a cross-reaction with antibodies against *Babesia ovis* infection and has the inherent problem of obtaining large amounts of antigen based on time-intensive and expensive and sophisticated procedures. In contrast, large batches can be prepared using recombinant protein-based ELISAs making them amenable for standardisation and increasing specificity.

Recombinant protein-based ELISAs have been established for detection of small ruminant theileriosis in China. The antigens used were heat shock protein 70 from the merozoite of *Theileria* sp. China (TcHSP70) (Miranda *et al.,* 2006a), *T. uilenbergi* immunodominant protein (TuIP) (Liu *et al.,* 2010), *T. luwenshuni* recombinant surface protein (rTlSP) and clone-9 from a merozoite cDNA library of *T. uilenbergi* (Abdo et al., 2010).

The indirect ELISA based on recombinant *T. uilenbergi* immunodominant protein (TuIP) has been validated; it showed a high sensitivity and was able to detect early and persistent infection with *T. uilenbergi*. It was also the most extensively characterised regarding the absence of cross-reaction with related pathogens, and was only found to show cross-reactivity with antibodies against the closely related pathogen *T. luwenshuni* (Liu *et al.,* 2010). *Theileria uilenbergi* and *T. luwenshuni* antibodies can be detected using an ELISA based on crude antigen (merozoite lysate).

An indirect ELISA using the recombinant immunogenic pyroplasmic proteins partially expressed by the clone-9 gene for the detection of circulating antibodies in the sera of sheep infected with *T. uilenbergi*; for this test, no cross-reactivity was observed in the serum of animals infected with *T. lestoquardi.*

Theileria luwenshuni recombinant surface protein (rTlSP)-based ELISA demonstrates reactivity with *T. luwenshuni*, *T. uilenbergi*, *T. ovis*, *T. annulata*, *T. orientalis*, and *T. sinensis* positive sera, but no cross-reactivity was found in sera from *T. lestoquardi*-infected animals. This test could be potentially used for the detection of antibodies against *Theileria* in bovine and ovine sera, but it is currently validated only for cattle.

The recombinant *T. uilenbergi* immunodominant protein (rTuIP) was also used as antigen for a colloidal gold-based immunochromatographic strip (ICS) for the detection of *T. uilenbergi* or *T. luwenshuni* infections (Lu *et al.,* 2015).

C. REQUIREMENTS FOR VACCINES

No commecial vaccines are available for this disease.

For *T. lestoquardi,* an attenuated live vaccine based on inoculation of schizont-infected leukocytes has been used for the control of malignant theileriosis and successfully applied in some countries.

Lambs immunised with 3×10^6 attenuated schizonts obtained at the 63rd passage showed a mild febrile reaction and parasitological reactions. In another trial, sheep immunised with different doses of attenuated *T. lestoquardi*-infected cells at the 105th passage showed a mild reaction with fever lasting for 1–5 days and parasitaemia of <0.2% (Ahmed *et al*., 2013).

Several studies were also conducted to identify antigens for inclusion in a subunit vaccine against *T. lestoquardi* infection in sheep and goats. Sporozoite lestoquardi antigen 1(SLAG-1), p67:SPAG-1 homologue of *T. lestoquardi* and a 73-kDa protein could be identified as possible candidates showing a potential as sporozoite-neutralisation.

Some approaches have been implemented for reducing the risk of tick-borne diseases (TBDs). Vaccines using tick-derived antigens were not intended to prevent tick infestations, but rather to reduce tick populations and the prevalence of tick-borne pathogens (TBPs) by influencing the feeding, fertility, and growth of ticks feeding on immunised animals and ingesting antigen-specific antibodies that interact with and affect protein function. These vaccines should be developed with tick vector species, TBPs, and TBD-affected hosts in mind, and they can be used alone or in conjunction with other control measures. Tick-derived antigens with various functions can be used alone or in combination with pathogen-derived antigens to provide vaccine efficacy in reducing tick infestations, TBP infection and multiplication in the tick, TBP transmission, tick attachment and feeding time, or host TBP infection (de la Fuente *et al.,* 2017).

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NB: There are WOAH Reference Laboratories for theileriosis and ovine theileriosis (please consult the WOAH Web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact WOAH Reference Laboratory for any further information on diagnostic tests, reagents and vaccines for theileriosis

NB: FIRST ADOPTED IN 2022.

SECTION 3.9.

SUIDAE

CHAPTER 3.9.1.

AFRICAN SWINE FEVER (INFECTION WITH AFRICAN SWINE FEVER VIRUS)

SUMMARY

African swine fever (ASF) is an infectious disease of domestic and wild pigs of all breeds and ages, caused by ASF virus (ASFV). The clinical syndromes vary from peracute, acute, subacute to chronic, depending on the virulence of the virus. Acute disease is characterised by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. Soft ticks of the Ornithodoros *genus, especially* O. moubata *and* O. erraticus*, have been shown to be both reservoirs and transmission vectors of ASFV. The virus is present in tick salivary glands and passed to new hosts (domestic or wild suids) when feeding. It can be transmitted sexually between ticks, transovarially to the eggs, or transtadially throughout the tick's life.*

ASFV is the only member of the Asfarviridae *family, genus* Asfivirus*.*

Laboratory diagnostic procedures for ASF fall into two groups: detection of the virus and serology. The selection of the tests to be carried out depends on the disease situation and laboratory diagnostic capacity in the area or country.

Identification of the agent: *Laboratory diagnosis must be directed towards isolation of the virus by inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test and/or the detection of genomic DNA by the polymerase chain reaction (PCR) or real-time PCR. The PCRs are excellent, highly sensitive, specific and rapid techniques for ASFV detection and are very useful under a wide range of circumstances. They are especially useful if the tissues are unsuitable for virus isolation and antigen detection. In doubtful cases, the material is passaged in leukocyte cell cultures and the procedures described above are repeated.*

Serological tests: *Pigs that survive natural infection usually develop antibodies against ASFV from 7–10 days post-infection and these antibodies persist for long periods of time. Where the disease is endemic, or where a primary outbreak is caused by a strain of low or moderate virulence, the investigation of new outbreaks should include the detection of specific antibodies in serum or extracts of the tissues submitted. A variety of methods such as the enzyme-linked immunosorbent assay (ELISA), the indirect fluorescent antibody test (IFAT), the indirect immunoperoxidase test (IPT), and the immunoblotting test (IBT) is available for antibody detection.*

Requirements for vaccines: *At present, there is no vaccine for ASF.*

A. INTRODUCTION

The current distribution of African swine fever (ASF) extends across more than 50 countries in three continents (Africa, Asia and Europe). Several incursions of ASF out of Africa were reported between the 1960s and 1970s. In 2007, ASF was introduced into Georgia, from where it spread to neighbouring countries including the Russian Federation. From there ASF spread to eastern European countries extending westwards and reaching the European

Union in 2014. Further westward and southern spread in Europe has occurred since that time. In all these countries, both hosts – domestic pig and wild boar – were affected by the disease. In August 2018, the People's Republic of China reported its first outbreak of ASF and further spread in Asia has occurred.

ASF virus (ASFV) is a complex large enveloped DNA virus with icosahedral morphology. It is currently classified as the only member of the *Asfaviridae* family, genus *Asfivirus* (Dixon *et al.*, 2005). More than 60 structural proteins have been identified in intracellular virus particles (200 nm) (Alejo *et al.,* 2018). More than a hundred infection-associated proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs (Sánchez-Vizcaíno & Arias, 2012). The ASFV double-stranded linear DNA genome comprises between 170 and 193 kilobases (kb) and contains between 150 and 167 open reading frames with a conserved central region of about 125 kb and variable ends. These variable regions encode five multigene families that contribute to the variability of the virus genome. The complete genomes of several ASFV strains have been sequenced (Bishop *et al.,* 2015; Chapman *et al.,* 2011; De Villiers *et al.,* 2010; Portugal *et al.,* 2015). Different strains of ASFV vary in their ability to cause disease, but at present there is only one recognised serotype of the virus detectable by antibody tests.

The molecular epidemiology of the disease is investigated by sequencing of the 3' terminal end of the B646L open reading frame encoding the p72 protein major capsid protein, which differentiates up to 24 distinct genotypes (Achenbach *et al*., 2017; Boshoff *et al.,* 2007; Quembo *et al.* 2018). To distinguish subgroups among closely related ASFV, sequence analysis of the tandem repeat sequences (TRS), located in the central variable region (CVR) within the B602L gene (Gallardo *et al.,* 2009; Lubisi *et al.,* 2005; Nix *et al.,* 2006) and in the intergenic region between the I73R and I329L genes, at the right end of the genome (Gallardo *et al.,* 2014), is undertaken. Several other gene regions such as the E183L encoding p54 protein, the CP204L encoding p30 protein, and the protein encoded by the EP402R gene (CD2v), have been proved as useful tools to analyse ASFVs from different locations and hence track virus spread.

ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and subclinical infections. Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boar and feral pigs are also susceptible to the disease, exhibiting clinical signs and mortality rates similar to those observed in domestic pigs. In contrast African wild pigs such as warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hylochoerus meinertzhageni*) are resistant to the disease and show few or no clinical signs. These species of wild pig act as reservoir hosts of ASFV in Africa (Costard *et al.,* 2013; Sánchez-Vizcaíno *et al.,* 2015).

The incubation period is usually 4–19 days. The more virulent strains produce peracute or acute haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 4–10 days, sometimes even before the first clinical signs are observed. Case fatality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF. Moderately virulent strains are recognised that induce variable disease forms, ranging from acute to subacute. Low virulence, non-haemadsorbing strains can produce subclinical non-haemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to trauma. Animals that have recovered from either acute, subacute or chronic infections may potentially become persistently infected, acting as virus carriers. The biological basis for the persistence of ASFV is still not well understood, nor it is clear what role persistence plays in the epidemiology of the disease.

ASF cannot be differentiated from classical swine fever (CSF) by either clinical or post-mortem examination, and both diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and CSF. Laboratory tests are essential to distinguish between these diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by the inoculation of pig leukocyte or bone marrow cultures, the detection of genomic DNA by polymerase chain reaction (PCR) or the detection of antigen in smears or cryostat sections of tissues by direct fluorescent antibody test (FAT). Currently the PCR is the most sensitive technique and can detect ASFV DNA from a very early stage of infection in tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. The PCR is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. Pigs that have recovered from acute, subacute or chronic infections usually exhibit a viraemia for several weeks making the PCR test a very useful tool for the detection of ASFV DNA in pigs infected with low or moderately virulent strains. Virus isolation by the inoculation of pig leukocyte or bone marrow cultures

and identification by haemadsorption tests (HAD) are recommended as a confirmatory test when ASF is positive by other methods, particularly in the event of a primary outbreak or a case of ASF.

As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as antibodies are produced from the first week of infection and persist for long periods, they are a good marker for the diagnosis of the disease, particularly in subacute and chronic forms.

ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and Europe. ASF occurs through transmission cycles involving domestic pigs, wild boar, wild African suids, and soft ticks (Sánchez-Vizcaíno *et al.,* 2015). In regions where *Ornithodoros* soft-bodied ticks are present, the detection of ASFV in these reservoirs of infection contributes to a better understanding of the epidemiology of the disease. This is of major importance in establishing effective control and eradication programmes (Costard *et al*., 2013).

ASF is not a zoonotic disease and does not affect public health (Sánchez-Vizcaíno *et al*., 2009).

ASFV should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of bovine anaplasmosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
Virus isolation/ HAD test[(a)]	–	–	++	+++	++	–
FAT	–	–	++	++	+	–
ELISA for antigen detection	+	++	+	+	+	–
Conventional PCR	++	++	++	++	++	–
Real-time PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
ELISA	+++	+++	+++	+	+++	–
IPT[(b)]	+++	+++	+++	+	+++	–
IFAT[(b)]	+++	+++	+++	+	+++	–
IBT[(b)]	++	++	++	+	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
HAD = haemadsorption; FAT= fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; IPT= indirect immunoperoxidase test; IFAT = indirect fluorescent antibody test; IBT = immunoblotting test.
[(a)]As some current ASF virus isolates are non-haemadsorbing, negative HAD results should be confirmed using other tests such as PCR. [(b)]Recommended method as confirmatory serological test.

1. Identification of the agent

Where ASF is suspected, the following samples should be sent to the laboratory: blood in anticoagulant (EDTA for PCR, heparin or EDTA for virus isolation), serum and tissues, mainly spleen, lymph nodes, bone marrow, lung, tonsil and kidney. These should be kept as cold as possible, without freezing, during transit. After the samples arrive at the laboratory, they should be stored at –70°C if processing is going to be delayed. As maintaining a cold chain is not always possible, samples can be submitted in glycerosaline; this may slightly decrease the likelihood of virus isolation, but it may facilitate the submission of samples to the laboratory so that an outbreak can be confirmed.

1.1. Virus isolation

1.1.1. Sample preparation

i) Prepare suspensions of tissues by grinding 0.5–1.0 g pieces with a pestle and mortar containing sterile sand, and then add 5–10 ml of a buffered salt solution or tissue culture medium containing antibiotics. Alternatively, specimens can be prepared by placing tissues in tubes containing buffer or medium and sterile beads or shards then homogenising using a homogeniser.

ii) Clarify the suspensions by centrifugation at 1000 ***g*** for 5 minutes. Use the supernatant for virus isolation.

1.1.2. Haemadsorption test

The haemadsorption (HAD) test (de León *et al.,* 2013) is based on the fact that pig erythrocytes will adhere to the surface of pig monocyte or macrophage cells infected with ASFV and that most virus isolates have a HAD phenotype. A positive result in the HAD test is definitive for ASF diagnosis. A small number of 'non-haemadsorbing' viruses have been isolated, most of which are attenuated or avirulent, but some do produce typical acute ASF. The test is carried out by inoculating blood or tissue suspensions from suspect pigs into primary leukocyte cultures from the blood of naive pigs or into alveolar macrophages cell cultures. It is essential to carry out all procedures in such a way as to prevent contamination of the cultures.

1.1.2.1 Test procedure in primary leukocyte cultures

i) Collect the required volume of fresh defibrinated pig blood.

ii) Centrifuge at 700 ***g*** for 30 minutes and collect the buffy coat cells. Add three volumes of 0.83% ammonium chloride to the leukocytes obtained. Mix and incubate at room temperature for 15 minutes. Centrifuge at 650 ***g*** for 15 minutes and carefully remove the supernatant. Wash pellet in medium or phosphate buffered saline (PBS).

iii) Resuspend the cells at a concentration of 10^6–10^7 cells/ml in tissue culture medium containing 10–30% pig serum and antibiotics. To prevent nonspecific haemadsorption, the medium should contain serum or plasma from the same pig from which the leukocytes were obtained. If a large volume of samples is to be tested, the homologous serum can be replaced by serum that has been identified by pre-screening as capable of preventing the nonspecific auto-rosette formation.

iv) Dispense the cell suspension in 96-well plates with 200 µl per well (300,000 cells/well) and incubate at 37°C in a humidified 5%CO_2 incubator. This procedure can also be performed in aliquots of 1.5 ml in 160 ×16 mm tubes and incubate in a sloping position (5–10° from the horizontal) at 37°C.

Note: For routine diagnosis, only 2–4-day-old cultures are sufficiently sensitive.

v) After 3 days, inoculate three tubes or well plates by adding 0.2 ml/tube or 0.02 ml (1/10 final dilution)/well of prepared samples. It is advisable to inoculate ten-fold and hundred-fold dilutions into cultures, and this is especially important when the field material submitted is in poor condition.

vi) Inoculate positive control cultures with haemadsorbing virus. Uninoculated Non-inoculated negative controls are essential to monitor the possibility of nonspecific haemadsorption.

vii) Add 0.2 ml of a fresh preparation of 1% pig erythrocytes in buffered saline to each tube. In the case of the 96-well plates, add 0.02 ml of 1% pig erythrocytes per well.

viii) Examine the cultures daily for 7–10 days under a microscope for cytopathic effect (CPE) and haemadsorption.

ix) Reading the results

Haemadsorption consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells. A CPE consisting of a reduction in the number of adherent cells in the absence of haemadsorption may be due to the cytotoxicity of the inoculum, Aujeszky's disease virus or non-haemadsorbing ASFV, which can be detected by the FAT on the cell sediment or by use of PCR (see below). If no change is observed, or if the results of the immunofluorescence and PCR tests are negative, subculture the supernatant up to three times into fresh leukocyte cultures. All isolations should be confirmed by PCR and sequencing.

1.1.3. Virus isolation in porcine bone marrow cells

In circumstances where a reliable or consistent supply of defibrinated pig blood cannot be achieved for the HAD test, cultures of primary bone marrow cells may be used as an alternative culture system for the isolation of ASFV. Infected cells are detected by indirect FAT. The advantage of this detection method is that it is not affected by HAD phenotype. This method can also be employed using porcine primary leukocyte or alveolar macrophages cultures, using the procedures described above, substituting HAD detection with pig erythrocytes with FAT.

1.1.3.1 Test procedure in primary bone marrow cell cultures

i) Seed a 25 cm^2 tissue culture flask with 5×10^7 cells/ml in culture medium containing 12.5% fetal calf serum (FCS) and antibiotics and incubate at 37°C for 3 days.

ii) Carefully remove the media. As the monolayer can be easily disturbed, it may be necessary to centrifuge the media (1000 ***g***, 3 minutes) to recover dislodged cells if significant disruption of the monolayer occurs. Inoculate the flask with 1 ml of test specimen and incubate for 1 hour at 37°C.

iii) Remove the inoculum and gently wash the monolayer with PBS. Perform an additional centrifugation step of the inoculum and PBS if significant numbers of cells were removed during this step.

iv) Add fresh media to the flask and incubate for 5–7 days at 37°C in a humidified incubator with 5% CO_2.

v) Harvest the cell culture supernatant by transferring into a centrifuge tube, centrifuge to remove cellular debris and freeze at –70°C for storage or subsequent subculture.

vi) Fluorescence antibody staining of ASFV antigens can be performed on infected cells from the flask that have been dried onto a positively charged microscope slide. First, scrape cells from the surface of the flask using a sterile cell scraper. Aliquot into a centrifuge tube, then pellet the cells at 1000 ***g***, 3 minutes.

vii) Resuspend the cell pellet in 1 ml cell culture media or PBS and deposit 300 µl onto a slide, air dry and fix with acetone for 10 minutes at room temperature.

viii) Wash by immersing slides 3–4 times in fresh PBS, then stain with fluorescein isothiocyanate (FITC)-conjugated anti-ASFV immunoglobulin at the recommended or pre-titrated dilution for 1 hour at 37°C in a humid chamber.

ix) Fix and stain positive and negative control slides similarly.

x) Wash by immersing slides 3–4 times in fresh PBS, mount stained cells in PBS/glycerol, and examine under an upright ultraviolet light microscope with suitable barrier and excitation filters.

xi) *Reading the results*

Test specimens are positive if specific cytoplasmic fluorescence is observed in inoculated porcine marrow cells.

1.2. Antigen detection by fluorescent antibody test

The FAT can be used as an additional method to detect antigen in tissues of suspect pigs in the field or those inoculated at the laboratory. Positive FAT plus clinical signs and appropriate lesions can provide a presumptive diagnosis of ASF. It can also be used to detect ASFV antigen in leukocyte cultures in which no HAD is observed and can thus identify non-haemadsorbing strains of virus. It also distinguishes between the CPE produced by ASFV and that produced by other viruses, such as Aujeszky's disease virus or a cytotoxic inoculum. However, it is important to note that in subacute and chronic disease, FAT has a significantly decreased sensitivity. This reduction in sensitivity may be related to the formation of antigen-antibody complexes in the tissues of infected pigs which block the interaction between the ASFV antigen and detecting antibody conjugate (Sánchez-Vizcaíno & Arias, 2012).

1.2.1. Test procedure

i) Prepare cryostat sections or impression smears of test tissues, or spreads of cell sediment from inoculated leukocyte cultures on slides, air dry and fix with acetone for 10 minutes at room temperature.

ii) Stain with fluorescein isothiocyanate (FITC)-conjugated anti-ASFV immunoglobulin at the recommended or pretitrated dilution for 1 hour at 37°C in a humid chamber.

iii) Fix and stain positive and negative control preparations similarly.

iv) Wash by immersing four times in fresh clean PBS, mount stained tissues in PBS/glycerol, and examine under an ultraviolet light microscope with suitable barrier and exciter filters.

v) *Reading the results*

Tissues are positive if specific granular cytoplasmic fluorescence is observed in paracortical tissue of lymphoid organs or in fixed macrophages in other organs, or in inoculated leukocyte cultures.

1.3. Detection of virus genome by the polymerase chain reaction

PCR techniques have been developed, using primers from a highly conserved region of the genome, to detect and identify a wide range of isolates belonging to all the known virus genotypes, including both non-haemadsorbing viruses and isolates of low virulence. The PCR techniques in use have been shown to be particularly useful for identifying viral DNA in pig tissues that are unsuitable for virus isolation or antigen detection because they have undergone putrefaction, or when there is good reason to believe that virus may have been inactivated before samples are received in the laboratory. Due to its high sensitivity and specificity, together with the possibility for a high throughput application, the PCR is a recommended method for screening and confirmation of suspected cases.

A number of conventional and real-time PCR methods have been described (Agüero *et al.,* 2003; Basto *et al.,* 2006; Fernández-Pinero *et al.,* 2013; King *et al.,* 2003; Tignon *et al.,* 2011) and several commercial PCR kits are available for ASFV detection, including one formally registered with WOAH[1]. Duplex RT-PCR techniques have also been described for simultaneous and differential detection of ASFV and CSFV (Agüero *et al.,* 2004; Haines *et al.,* 2013). Any PCR protocol used must have been validated as fit for the chosen purpose, in accordance with Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* and Chapter 2.2.3 *Development and optimisation of nucleic acid detection assays,* for further details on PCR techniques.

Three validated PCR procedures are described below (Agüero *et al.,* 2003; Fernández-Pinero *et al.,* 2013; King *et al.,* 2003), consisting of a sample preparation followed by the test procedure. These procedures serve as a general guideline and a starting point for the PCR protocol. Optimal reaction conditions (incubation times and temperatures, models and suppliers of equipment, concentrations of assay reagents such as the primers and dNTPs) may vary so the described conditions should be evaluated first.

1 https://www.woah.org/en/scientific-expertise/registration-of-diagnostic-kits/background-information/

1.3.1. Sample preparation procedure

A number of DNA extraction kits are commercially available for the preparation of template suitable for PCR depending on the sample submitted for analysis and may be appropriate for use. Details are given in cited publications (Agüero *et al.,* 2003; Fernández-Pinero *et al.,* 2013; King *et al.,* 2003) and in current methods available from Reference Laboratories[2]. The detection of ASFV can be performed in parallel to that for CSF virus (see Chapter 3.9.3 *Classical swine fever* for CSFV molecular detection methods).

Different samples can be processed for PCR analysis such as cell culture supernatants, EDTA-blood, serum and tissue homogenates. Blood swabs may be useful for sampling wild boar.

For organ and tissue samples, first prepare a 1/10 homogenate of the material in sterile PBS 1×, then centrifuge to clarify at 12,000 ***g*** for 5 minutes. Extract nucleic acids from the resultant supernatant fluid. Sometimes it is recommended to process a 1/10 dilution of the supernatant in parallel (e.g. if organ homogenate looks turbid and/or is suspected to have excessive genomic DNA content.

Control samples for the DNA extraction step: at least one positive and one negative control should be included in each nucleic acid extraction run. The positive control sample should be ASFV-positive serum, EDTA-blood, or 1/10 tissue homogenates (of the same tissue type as the test samples). It is highly recommended that the positive control is prepared to be close to the detection limit of the technique to track the yield of the DNA extraction procedure (e.g. to give a cycle threshold [Ct] value of 32±2 in real-time PCR). The negative control could be water or ASFV-negative EDTA-blood, serum or tissue homogenate. Controls should be processed alongside the test samples. The quality of nucleic acid preparation may affect the efficiency of PCR amplification.

For DNA extraction, follow the kit manufacturer's instructions. Finally undertake the nucleic acid elution, preferably using molecular grade water (this will ensure that both DNA and RNA are recovered, in case an analysis of ASFV and CSFV is required). Use the eluted DNA immediately or store at –20°C for future use.

1.3.2. PCR amplification by conventional PCR (Agüero *et al.,* 2003)

The ASFV primer set described in this procedure can be combined with a specific primer set for CSFV in a multiplex RT-PCR method that allows the simultaneous and differential detection of both virus genomes in a single reaction (Agüero *et al.,* 2004).

Control samples for the DNA amplification step: at least one positive and one negative control should be included in each PCR run. The positive control sample should be 2 µl of ASFV-positive DNA. It is highly recommended that the positive control should be close to the detection limit of the PCR technique to check the yield of the DNA amplification procedure (e.g. to give a Ct value of 32±2 in real-time PCR). The negative reaction control should be 2 µl of nuclease-free sterile water, or DNA extracted from ASFV-negative EDTA-blood, serum or tissue homogenate.

1.3.2.1 Stock solutions

i) Nuclease-free sterile water.

ii) Hot Start DNA polymerase, PCR Buffer, and magnesium chloride are commercially available.

iii) PCR nucleotide mix containing 10 mM of each dNTP is commercially available.

iv) Primers are prepared at a concentration of 20 pmol/µl: Primer PPA-1 sequence 5'-AGT-TAT-GGG-AAA-CCC-GAC-CC-3' (forward primer); primer PPA-2 sequence 5'-CCC-TGA-ATC-GGA-GCA-TCC-T-3' (reverse primer).

v) 10× Loading buffer: 0.2% xylene cyanol, 0.2% bromophenol blue, 30% glycerol.

2 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

vi) TAE buffer (50×) for agarose gel: Tris base (242 g); glacial acetic acid (57.1 ml); 0.5 M EDTA, pH 8.0 (100 ml); distilled water (to 1 litre).

vii) Agarose 2% solution: agarose to prepare a 2% solution in TAE buffer 1× is commercially available.

viii) Molecular weight marker DNA: 100 base-pair ladder is commercially available.

ix) Double-stranded DNA intercalating dye is commercially available.

1.3.2.2. Conventional PCR protocol

i) In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction master mix for the number of samples to be assayed allowing for at least one extra sample.

ii) Prepare the PCR reaction mix to a final volume of 25 µl per sample, including the following reagents at the final concentrations indicated: 1 × PCR buffer, 2 mM magnesium chloride (this may be included in the PCR buffer), 0.2 mM dNTP mix, primer PPA-1, 20 pmol/µl (0.25 µl, final concentration 0.2 µM), primer PPA-2, 20 pmol/µl (0.25 µl, final concentration 0.2 µM), 0.625 U hot-start DNA polymerase and nuclease-free or sterile distilled water.

iii) Add 23 µl of the PCR reaction mix to the required number of 0.2 ml PCR tubes.

iv) Add 2 µl of extracted DNA template to each PCR tube. Include a positive reaction control (2 µl of ASFV DNA) and a negative reaction control (2 µl of distilled water) for each PCR run.

v) Place all the tubes in an automated thermal cycler and run the following programme:

One cycle at 95°C for 10 minutes.

40 cycles at 95°C for 15 seconds, 62°C for 30 seconds and 72°C for 30 seconds.

One cycle at 72°C for 7 minutes.

Hold at 4°C.

Note: the thermocycling programme may vary depending on the DNA polymerase used, but the conditions indicated above serve as a reference and is the programme established in the original procedure (Agüero *et al.,* 2003).

vi) At the end of the programme, remove PCR tubes and add 2.5 µl of 10× loading buffer to each tube.

vii) Load all the samples in a 2% agarose gel in TAE buffer 1× containing DNA intercalating dye (suitable concentration depends on the dye used, follow manufacturer's instructions).

viii) Add marker DNA to one well on each of the gel.

ix) Run the gel at a constant voltage of 150–200 volts for about 30 minutes.

x) Gels are examined over a UV or blue light source. In a positive sample, a discrete band will be present that should co-migrate with the PCR product of the positive control. Calculate the size of the PCR products in the test samples and the positive control by reference to the standard DNA marker. The PCR product of the positive control has a size of 257 base pairs. No bands should be seen in the negative control.

1.3.3. Real-time PCR procedure 1 (King *et al.,* 2003)

1.3.3.1. Stock solutions

i) Nuclease-free or another appropriate sterile water and PCR reaction master mix (2×).

ii) Primers are prepared at a concentration of 50 pmol/µl: Primer 1 sequence 5'-CTG-CTC-ATG-GTA-TCA-ATC-TTA-TCG-A-3' (positive strand); Primer 2 sequence 5'-GAT-ACC-ACA-AGA-TC(AG)-GCC-GT-3' (negative strand).

iii) Fluorescent-labelled hydrolysis probe is included at a concentration of 5 pmol/µl: (5'-[6-carboxy-fluorescein (FAM)]-CCA-CGG-GAG-GAA-TAC-CAA-CCC-AGT-G-3'-[6-carboxy-tetramethyl-rhodamine (TAMRA)]).

1.3.3.2. PCR amplification

i) In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixture described below for each sample. Prepare the master mix for the number of samples to be assayed but allowing for one extra sample.

Nuclease-free or sterile water (7.5 µl); (2× conc.) PCR reaction master mix (12.5 µl); primer 1, 50 pmol (1.0 µl); primer 2, 50 pmol (1.0 µl); fluorescent-labelled probe, 5 pmol (1 µl).

ii) Add 22 µl PCR reaction mix to one well of an optical reaction plate for each sample to be assayed.

iii) Add 3 µl of extracted DNA template or blank extraction control and securely cover each well with a cap.

iv) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.

v) Place the plate in a sequence detection system for PCR amplification (real-time PCR machine equipped with FAM fluorescence channel) and run the following programme:

One cycle at 50°C for 2 minutes,

One cycle at 95°C for 10 minutes,

Forty cycles at 95°C for 15 seconds, 58°C for 1 minute.

Note: If a purpose-built thermal cycler is not available, an ordinary thermal cycler can be used and the PCR products analysed by end-point fluorescence readers or alternatively by electrophoresis on a 1.5% agarose gel. A product of 250 bp is expected.

vi) *Reading the results*

Assign a cycle threshold (Ct) value to each PCR reaction from a scan of all amplification plots (a plot of the fluorescence signal versus cycle number). Negative test samples, uninfected negative or extraction blank controls should have a Ct value >40.0. Positive test samples and controls should have a Ct value < 40.0 (strongly positive samples have a Ct value <30.0).

Modifications of this protocol using a range of commercial amplification kits can provide even higher PCR yields, however these amplification kits should be fully validated prior to use. One modification of this using a fast amplification protocol has been validated for diagnostic purposes by Fernández-Pinero *et al.,* 2010 (detailed protocol available in Fernández-Pinero *et al.,* 2013).

1.3.4. Real-time PCR procedure 2 (Fernández-Pinero *et al.,* 2013)

This real-time PCR method has been shown to exhibit the highest sensitivity for detection of ASFV DNA both at very early stages of infection and in long-term chronically infected animals where the viraemia level is usually quite low (Fernández-Pinero *et al.,* 2013; Gallardo *et al.,* 2015a).

1.3.4.1. Stock solutions

i) Nuclease-free sterile water.

ii) A range of real-time PCR kits is commercially available. Selected kits should be validated before use for diagnostic purposes.

iii) Primers are prepared at a concentration of 20 pmol/µl: primer ASF-VP72-F sequence 5'-CCC-AGG-RGA-TAA-AAT-GAC-TG-3' (forward primer); primer ASF-VP72-R sequence 5'-CAC-TRG-TTC-CCT-CCA-CCG-ATA-3' (reverse primer).

Note: the nucleotide code, R = A+G mixed base position.

iv) Labelled hydrolysis probe (10 pmol/µl, labelled with FAM reporter dye) is commercially available ready to use (see Fernández-Pinero *et al.,* 2013 for details).

Note: If access to the specific probe is not possible, it could be substituted by the following standard probe using identical concentration and reaction conditions: (5'-[6-carboxy-fluorescein (FAM)]-TCC-TGG-CCR-ACC-AAG-TGC-TT-3'-[black hole quencher (BHQ)])

1.3.4.2. PCR amplification

Control samples for the DNA amplification step: at least one positive and one negative control should be included in each PCR run. The positive control sample should be 2 µl of ASFV-positive DNA. It is highly recommended that the positive control should be close to the detection limit of the PCR technique to check the yield of the DNA amplification procedure (e.g. to give a Ct value of 32±2 in real-time PCR). The negative control should be 2 µl of nuclease-free sterile water.

i) In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed allowing for at least one extra sample.

ii) The PCR reaction mixture includes: nuclease-free or sterile distilled water (7 µl), master mix 2× (10 µl), primer ASF-VP72-F, 20 pmol/µl (0.4 µl), primer ASF-VP72-R, 20 pmol/µl (0.4 µl), fluorescent-labelled hydrolysis probe 10 pmol/µl (0.2 µl).

iii) Add 18 µl of the PCR reaction mix to the required number of 0.2 ml optical PCR tubes.

iv) Add 2 µl of extracted DNA template to each PCR tube. Include a positive reaction control (2 µl of ASFV DNA) and a negative reaction control (2 µl of distilled water) for each PCR run.

v) Place all the tubes in a real-time PCR thermal cycler (equipped with FAM fluorescence channel) and run the following programme:

One cycle at 95°C for 5 minutes.

45 cycles at 95°C for 10 seconds, 60°C for 30 seconds. Programme the fluorescence collection in FAM channel at the end of each cycle.

Note: the incubation programme may vary depending on the DNA polymerase used, but the indicated one serves as a general one and it is the programme established in the original procedure publication (Fernández-Pinero *et al.,* 2013).

vi) Reading the results

The point where the fluorescence measurement is above the background signal and reaches the detectable level is called the cycle threshold (Ct), and this is determined automatically by the PCR equipment software. It will be the starting fluorescence point for considering a sample as positive.

In a positive sample, a sigmoid-shaped amplification curve will be obtained where the Ct value will be <40. Samples giving a Ct value ≥38 should be considered as doubtful if a sigmoidal plot is observed and the analysis should be repeated for confirmation. A negative sample will maintain the fluorescence profile under background fluorescence level and the equipment will not report any Ct value.

Samples reporting a Ct value >40 can be considered as negative unless the results of serological techniques or the epidemiological information suggest the possibility of ASFV infection. In this case, the analysis should be repeated to confirm the negative PCR result.

1.4. Antigen detection enzyme-linked immunosorbent assay

Antigen detection ELISA may be performed as an alternative method but, as its sensitivity is much lower than PCR or HAD, it should not be used as the only method for virus detection and results should be confirmed by PCR or HAD. A commercial kit is available for use on specimens of porcine blood, spleen or lymph nodes, in accordance with recommendations of the kit manufacturer. The kit uses a double antibody sandwich ELISA. An MAb specific for the virus capsid protein is coated on the plate and binds to ASFV in positive samples. After washing a second MAb specific to a different epitope on the capsid protein and conjugated to peroxidase is added. Binding of this second antibody to the captured antigen is detected by adding an appropriate substrate.

2. Serological tests

Serological assays are the most commonly used diagnostic tests due to their simplicity, relatively low cost and the fact that they require little specialised equipment or facilities. For ASF diagnosis, this is particularly relevant given that no commercial vaccine is available against ASFV, which means that the presence of anti-ASFV antibodies

always indicates infection. Furthermore, anti-ASFV antibodies appear soon after infection (7–10 days) and persist for several months or even years. Domestic pigs and wild boar infected with virulent strains usually die before a specific antibody immune response is raised. In areas with a well established ASFV infection, where attenuated and low virulent virus isolates are also circulating, serological detection is crucial for identifying recovered and asymptomatically infected animals. There are no fully neutralising antibodies.

The most commonly used is the ELISA (Gallardo *et al*., 2015b; Sánchez-Vizcaíno, 1987), which is suitable for examining serum or plasma. Confirmatory testing of ELISA-positive samples should be carried out using an alternative test, such as the IFAT, IPT or immunoblotting (Gallardo *et al.,* 2015b; Pastor *et al.,* 1989). Antibody is usually not detected in pigs infected with virulent ASFV as they die before it is produced. Antibodies are produced in pigs infected with low or moderately virulent ASF viruses, A penside test (lateral flow assay) for antibody detection has been validated and is commercially available.

In recent years, extensive studies were carried out to assess the specificity and sensitivity of ASF serological tests in the different epidemiological scenarios of Africa and Europe. These studies included currently circulating ASFV genotype II isolates in Eastern Europe and the eastern African isolates exhibiting more variability. The results showed the WOAH tests recommended to certify animals prior to movement (see Table 1) are able to detect the presence of antibodies to ASFV in all the epidemiological situations evaluated with accuracy and suitable sensitivity (Gallardo *et al.,* 2013; 2015b).

Where ASF is endemic, confirmation of suspected cases of disease can be done using a standard serological test (ELISA), combined with an alternative serological test (IFAT, IPT, IBT) and an antigen-detection test. In some countries, over 95% of positive cases have been identified using a combination of IFATs and FAT.

It should be noted that when pigs have been infected with avirulent isolates or those of low virulence, serological tests may be the only way of detecting infected animals.

2.1. Enzyme-linked immunosorbent assay

The ELISA is a direct test that can detect antibodies to ASFV in pigs that have been infected by viruses of low or moderate virulence. Currently, several commercial ELISA kits, based on a competition or indirect format, are available for the detection of antibodies to ASFV, validated for use under different epidemiological situations. A cheaper alternative is to prepare a soluble antigen for use in an indirect ELISA, and procedure using this soluble antigen is described below.

Carrying out a second confirmatory test such as the IBT, IFAT or IPT described below is recommended in the case of a doubtful result or a positive result when sera are suspected to be poorly preserved.

2.1.1. Antigen preparation for ELISA

The ELISA antigen is prepared from infected cells grown in the presence of pig serum (Escribano *et al.,* 1989).

i) Infect MS (monkey stable) cells at multiplicity of infection of 10 with adapted virus, and incubate in medium containing 2% pig serum.

ii) Harvest the cells at 36–48 hours post-infection, when the CPE is extensive. Wash in PBS, sediment at 650 ***g*** for 5 minutes, wash the cell pellet in 0.34 M sucrose in 5 mM Tris/HCl, pH 8.0, and centrifuge to pellet cells.

Carry out steps (iii) to (v) on ice:

iii) Resuspend the cell pellet in 67 mM sucrose in 5 mM Tris/HCl, pH 8.0 (1.8 ml per 175 cm^2 flask), and leave for 10 minutes with agitation after 5 minutes.

iv) Add nonionic detergent Nonidet P-40 to a final concentration of 1% (w/v), and leave for 10 minutes (with agitation after 5 minutes) to lyse the cells.

v) Add sucrose to a final concentration of 64% (w/w) in 0.4 M Tris/HCl, pH 8.0, and centrifuge at 1000 ***g*** for 10 minutes to pellet nuclei.

vi) Collect the supernatant and add EDTA (2 mM final concentration), beta-mercaptoethanol (50 mM final concentration) and NaCl (0.5 M final concentration) in 0.25 mM Tris/HCl, pH 8.0, and incubate for 15 minutes at 25°C.

vii) Centrifuge at 100,000 ***g*** for 1 hour at 4°C over a layer of 20% (w/w) sucrose in 50 mMTris/HCl, pH 8.0.

Remove the band immediately above the sucrose layer and use as the ELISA antigen. Store at –20°C.

2.1.2. Indirect ELISA procedure (Pastor *et al.,* 1990)

i) Coat 96-well ELISA micro plate(s) with antigen by adding 100 µl of the recommended or pretitrated dilution of antigen in 0.05 M carbonate/bicarbonate buffer, pH 9.6, to each well.

ii) Incubate at 4°C for 16 hours (overnight) and then wash five times with 0.05% Tween 20 in PBS, pH 7.2.

iii) Dilute the test sera and positive and negative control sera 1/30 in 0.05% Tween 20 in PBS, pH 7.2, and add 100 µl of each diluted serum to duplicate wells of the antigen-coated plate(s).

iv) *Incubate* plates at 37°C for 1 hour (optionally on a plate shaker), and then wash five times with 0.05% Tween 20 in PBS.

v) To each well add 100 µl of protein-A/horseradish-peroxidase conjugate (Pierce) at the recommended or pretitrated dilution in 0.05% Tween 20 in PBS.

vi) Incubate the plates at 37°C for 1 hour, and then wash five times with 0.05% Tween 20 in PBS.

vii) Substrate: Add 200 µl of substrate DMAB/MBTH to each well prepared as follows:

The volume required per plate is 10 ml of DMAB 80.6 mM Solution + 10 ml of MBTH 1.56 mM solution + 5 µl H_2O_2 30%.

a) DMAB/MBTH substrate preparation

DMAB – 3-dimethylaminobenzoic acid; MBTH – 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohidrate.

b) DAMB 80.6 mM solution

Dissolve 13.315 g of DAMB acid in 1000 ml of 0.1 M phosphate buffer, pH 7 (5.3 g KH_2PO_4, 8.65 g Na_2HPO_4 made up to 1000 ml in distilled water) by continuous agitation for 1 hour at room temperature, adjusting the pH to 7 with NaOH (5 M). Filter through a funnel.

c) MBTH 1.56 mM solution

Dissolve 0.3646 g of MBTH in 1000 ml of 0.1 M phosphate buffer, pH 7 (5.3 g KH_2PO_4, 8.65 g Na_2HPO_4 made up to 1000 ml in distilled water) by continuous agitation for 1 hour, adjusting the pH to 6.25 with concentrated hydrochloric acid. Filter through a funnel.

Substrate can be prepared as stock solutions, aliquoted and kept at –20°C. Mix the DAMB and the MBTH solutions (1:1) just before use and add the required quantity of 30% H_2O_2.

Alternatively 0.04% orthophenylene-diamine (OPD) can be used instead of DMAB/MBTH. It is prepared in phosphate/citrate buffer, pH 5.0 at the rate of 10 µl/25 ml. Add 100 µl of substrate to each well. **Note:** when OPD substrate is used, the number of false positive reactions is increased.

viii) Incubate at room temperature for approximately 6–10 minutes (before the negative control begins to be coloured). The time necessary for the colour to develop will depend on both the temperature of the substrate when added to the wells, and the room temperature.

ix) Stop the reaction by adding 100 µl of 3 N sulphuric acid to each well.

x) Reading the results: Positive sera have a clear colour (yellow in case of OPD substrate, blue in case of DMAB/MBTH substrate) and can be read by eye, but to ensure that all positive sera are identified, it is necessary to read the absorbance in each well spectrophotometrically, at 600–620 nm (in case of DMAB/MBTH) or 492 nm (OPD substrate) in an ELISA reader. Using DMAB/MBTH substrate, the test is validated when the mean absorbance value of the positive control is more than four times the mean of absorbance of the negative control. Using OPD substrate, any serum is considered to be positive if it has an absorbance value of more than twice the mean absorbance value of the control negative sera on that plate.

To correctly interpret the results it is necessary to calculate a cut-off point which enables the differentiation of negative, inconclusive and positive results. The cut-off point is established by the following equation:

The cut-off point = optical density negative serum × 1 + optical density positive serum × 0.2.

a) Sera with an optical density below the cut-off point – 0.1 can be considered negative.

b) Sera with an optical density higher than cut-off point + 0.1 can be considered positive.

c) Sera with an optical density between cut-off point ± 0.1 can be considered inconclusive and the result needs to be confirmed by the IPT, IFAT or IBT.

This indirect ELISA procedure has been improved and validated and has higher sensitivity than that obtained with the previous procedure for serum samples collected at earlier stages of infection, by adjusting the incubation time, incubation temperatures, buffers, concentrations of the antigen and the samples, as well as the type and concentration of the conjugate and substrate (Fernández-Pacheco *et al*., 2016). Briefly, test and control sera are diluted 1/10 in blocking buffer (PBS containing 0.05% Tween 20, 2% skim milk and 2% normal porcine serum), and one well of the ELISA plate is left blank as a control well (100 µl of blocking buffer). Sera are incubated for 2 hours at 37±2°C on a plate shaker. Pre-titrated conjugate at a working dilution (suggested range 1:5000–1:20000 of protein A, 1 mg/ml) is maintained for 45 minutes at 37±2°C on a plate shaker. Finally, a new substrate is used, adding 100 µl/per well of substrate solution (ABTS [2,2'-azino-di(3-ethyl-benzothiazoline)-6-sulfonic acid]-diammonium salt) and incubating for 30 minutes at room temperature in the dark. The reaction is stopped by the addition of 100 µl/well stopping solution (1% sodium dodecyl sulphate) and results are obtained at 405 nm wavelength. For interpretation of the results, the test is validated when the mean of the optical density obtained in the blank (ODblank) is <0.250. A value cut-off (VCO) is calculated as mean OD NC (optical density negative control) × 2. The index cut-off (ICO) will define the result and is calculated as ICO = (mean OD sample)/VCO. Serum samples with ICO ≤1 are considered as negatives, ICO >1 ≤1.25 as doubtful, and serum samples with ICO >1.25 are considered as positives.

2.2. Indirect Immunoperoxidase test (IPT) (Gallardo *et al.,* 2015b)

The IPT is an immune-cytochemistry technique on fixed cells to determine the antibody–antigen complex formation through the action of the peroxidase enzyme. In this procedure, African green monkey kidney (Vero) or MS cells are infected with ASFV isolates adapted to these cell cultures. The infected cells are fixed and are used as antigens to determine the presence of the specific antibodies against ASF.

IPT should be used as a confirmatory test for sera from areas that are free from ASF and are positive in the ELISA, and for sera from endemic areas that give an inconclusive result in the ELISA. Given its superior sensitivity and its performance, this is the best test to test blood, fluids or exudate tissue samples (Gallardo *et al.,* 2015b).

2.2.1. Preparation of 96-well plates coated with fixed ASFV adapted viruses

i) Subculture MS or Vero cells at a 1:2 split ratio and distribute the diluted cell suspension into the required number of 96-microwell plates (growth area/well 0.32 cm^2).

ii) Incubate for 24 (Vero) or 48 (MS) hours at 37±3°C in a 5% CO_2 humidified incubator to get an 80–90% confluent plate. After 24 or 48 hours' incubation, carefully decant the medium of the cell cultures grown in the 96-microwell plates.

iii) In a separate bottle prepare the appropriate dilution (in culture medium without FCS) of the adapted ASFV to inoculate with an m.o.i. (multiplicity of infection) between 0.025 and 0.05.

The dilution factor is calculated using the following formula:

$$\text{Dilution factor} = \frac{0.7 \times \text{virus titre} \times \text{volume}}{\text{Number of cells} \times \text{m.o.i}}$$

iv) Inoculate the plates with 100 µl/well of the inoculum and incubate at 37±3°C in 5% CO_2 humidified incubator for:

a) 18±1 hours for Vero infected cell plates.

b) 24±1 hours for MS infected cell plates: In this case, after 2 hours at 37±3°C, complete the volume to 200 µl with medium +4% FCS (final concentration of 2% SFBi).

v) Fix the cells: the inocula are removed by vacuum suction and the ASFV-infected cell sheets are fixed with a cold solution containing 70% methanol and 30% acetone for 8±2 minutes at room temperature. Finally, the plates are washed with PBS 3–5 times for 5 minutes each, on a plate shaker.

vi) The fixed and dry IPT plates can be used directly or stored at <–10°C.

2.2.2. IPT procedure

i) Keep the ASFV IPT plates at room temperature (18–25°C) for 30 minutes after defrosting.

ii) Blocking step: Block the plates by adding 100 µl per well of blocking solution (PBS/0.05%Tween 20, pH 7.2 [±0.2]/milk 5%). Incubate for 1 hour at 37±2°C on a plate shaker.

iii) Sample pre-incubation: In a separate 96-well microtitre plate dilute at 1/40 the samples (sera, blood, fluid or exudate tissues) and controls (positive, limit and negative) in blocking solution (PBS/0.05% Tween 20, pH 7.2 [±0.2]/milk 5%) containing 2% SFB. Add 100 µl per well and incubate for 1 hour at 37±2°C on a plate shaker.

iv) Sample incubation: After 1 hour discard the blocking solution from the ASFV-IPT-plates and add 100 µl per well of the pre-incubated samples and controls. Incubate for 45 minutes at 37±2°C on a plate shaker.

v) Washing step: Wash three times with 100 µl/per well of PBS 1× for 5 minutes at 37±2°C on a plate shaker.

vi) Add 100 µl of protein A peroxidase conjugate per well diluted at 1/5000 in blocking solution. Incubate for 45 minutes at 37±2°C on a plate shaker.

vii) Washing step: Wash three times with 100 µl/per well of PBS 1× for 5 minutes at 37±2°C on a plate shaker.

viii) Add 50 µl/well of substrate solution and incubate 5–10 minutes at room temperature (18–25°C).

Substrate solution: The substrate solution must be prepared when it is going to be used. Mix 300 µl of stock solution in 5 ml of acetate buffer + 5 µl of H_2O_2 (this volume is recommended for one 96 well plate).

a) Stock solution (20 mg/one tablet AEC (3-amino-9-etilcarbazol) in 2.5 ml dimethylformamide (keep at 4 ±3°C in the dark).

b) Acetate buffer 74 ml solution A + 176 ml solution B. Store at room temperature. Expiry date 6 months.

1. Solution A: 0.2 N acid acetic glacial (1.155 ml acetic in 100 ml water). Store at room temperature.

2. Solution B: 0.2 M sodium acetate (2.72 g [±0.05] AcNa tri-hydrated in 100 ml water). Store at room temperature.

ix) Add PBS 1 × 100 µl/per well to stop the reaction.

x) Reading the results: In the wells with positive samples against ASF, an intense red cytoplasmic coloration will be observed in the ASFV-infected cells. The red cytoplasmic coloration is interpreted as a positive result against ASF and the absence as a negative result.

In some specific situations related to samples collected from vaccinated animals against other diseases, some slight background can be observed with a nonspecific red coloration in the wells. In these cases the samples must be analysed against non-infected cells in parallel with the infected cells.

2.3. Indirect fluorescent antibody test

This test (Sánchez-Vizcaíno, 1987) should be used as a confirmatory test for sera from areas that are presumed free from ASF and are positive in the ELISA, and for sera from endemic areas that give an inconclusive result in the ELISA.

2.3.1. Test procedure

i) Prepare a suspension of ASFV-infected pig kidney or monkey cells at a concentration of 5×10^5 cells/ml, spread small drops on glass slides, air dry and fix with acetone at room temperature for 10 minutes. Note that slides can be stored at –20°C until ready for use.

ii) Heat inactivate test sera at 56°C for 30 minutes.

iii) Add appropriate dilutions of test sera and positive and negative control sera in buffered saline to slides of both infected and uninfected control cells, and incubate for 1 hour at 37°C in a humid chamber.

iv) Wash the slides by immersing four times in fresh clean PBS and then distilled water.

v) Add predetermined or recommended dilutions of anti-pig immunoglobulin/FITC or protein-A/FITC conjugate to all slides, and incubate for 1 hour at 37°C in a humid chamber.

vi) Wash the slides by immersing four times in fresh clean PBS and then distilled water, mount in PBS/glycerol, and examine under an ultraviolet light microscope with suitable barrier and exciter filters.

vii) *Reading the results:* The control positive serum on infected cells must be positive and all other controls must be negative before the test can be read. Sera are positive if infected cultures show specific fluorescence.

2.4. Immunoblotting test (Pastor *et al.*, 1989)

This test should be used as an alternative to the IFAT and IPT to confirm equivocal results with individual sera. It gives suitable results for weak-positive samples for ASF antibody detection from the second week post-infection. Viral proteins that induce specific antibodies in pigs have been determined. These polypeptides have been placed on antigen strips and have been shown in the immunoblotting test to react with specific antibodies from 9 days post-infection.

2.4.1. Preparation of antigen strips

i) Prepare cytoplasmic soluble virus proteins as described for the preparation of ELISA antigen in Section B.2.1.

ii) Electrophorese through 17% acryl-amide/N,N'-diallyltartardiamide (DATD) gels with appropriate molecular weight standards.

iii) Transfer the proteins on to a 14×14 cm^2 nitrocellulose membrane by electrophoresis at a constant current of 5 mA/cm in transfer buffer (20% methanol in 196 mM glycine, 25 mMTris/HCl, pH 8.3).

iv) Dry the membrane and label the side on to which the proteins were electrophoresed.

v) Cut one strip from the edge of the filter and carry out the immunoblotting procedure described below. Identify the region containing proteins of 23–35 kDa by comparison with

the molecular weight standards run in parallel, and cut this region into 0.5 cm wide strips. Label each strip on the side on to which the proteins were electrophoresed.

These strips (approximately 4 cm long) constitute the antigen strips used for immunoblotting and contain proteins with which antibodies in both acute and convalescent pig sera will react. These antibodies persist for life in some pigs.

2.4.2. Preparation of chloranaphthol substrate solution

This solution must be prepared immediately before use.

i) Dissolve 6 mg of 4-chloro-1-naphthol in 2 ml of methanol and add this solution slowly to 10 ml of PBS while it is being stirred.

ii) Remove the white precipitate that is formed by filtration through Whatman No.1 filter paper (optional).

iii) Add 4 µl of 30% H_2O_2 (hydrogen peroxide).

2.4.3. Test procedure

The antigen strips must be kept with the labelled side uppermost during the immunoreaction procedure.

i) Incubate the antigen strips in blocking buffer (2% non-fat dried milk in PBS) at 37°C for 30 minutes with continuous agitation.

ii) Prepare 1/40 dilutions of test sera and positive and negative control sera in blocking buffer.

iii) Incubate the antigen strips in the appropriate serum at 37°C for 45 minutes with continuous agitation. Incubate one antigen strip in positive control serum and one in negative control serum. These two strips are controls. Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.

iv) Add protein-A/horseradish-peroxidase conjugate at the recommended or pretitrated dilution (usually at 1/1000 dilution) in blocking buffer to all antigen strips. Incubate at 37°C for 45 minutes with continuous agitation. Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.

v) Prepare the substrate solution, add to the antigen strips, and incubate at room temperature for 5–15 minutes with continuous agitation.

vi) Stop the reaction with distilled water when the protein bands are suitably dark.

vii) *Reading the results:* Positive sera react with more than one virus protein in the antigen strip; they must give a similar protein pattern and have the same intensity of colour as the antigen strips stained with positive control serum.

C. REQUIREMENTS FOR VACCINES

At present there is no commercially available vaccine for ASF.

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NB: There are WOAH Reference Laboratories for African swine fever
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for African swine fever

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.9.2.

ATROPHIC RHINITIS OF SWINE

SUMMARY

Description and importance of disease: *Atrophic rhinitis is an infectious disease of swine characterised by serous to mucopurulent nasal discharge, shortening or twisting of the snout, atrophy of the turbinate (conchal) bones and reduced productivity. It may occur enzootically or more sporadically, depending on a variety of factors including herd immunity. The most severe progressive form is caused by infection with toxigenic strains of* Pasteurella multocida *alone or in combination with* Bordetella bronchiseptica*. Infections with* B. bronchiseptica *alone can cause a mild to moderate form with nonprogressive turbinate bone atrophy. Turbinate atrophy may only be obvious at slaughter or may be detected in the live animal by use of radiography or tomography. Environmental and management factors also contribute to the severity and incidence of this disease. A large proportion of apparently normal pig herds may be infected with* B. bronchiseptica *or nontoxigenic* P. multocida *and show a mild degree or low prevalence of turbinate atrophy.*

Identification of the agents: *The diagnosis of atrophic rhinitis depends on clinical and post-mortem observations in affected swine assisted by the recovery and characterisation of* P. multocida *and* B. bronchiseptica*. The isolation of both organisms is often complicated by the more profuse growth of other bacteria. Isolation rates are improved by preservation of the nasal or tonsillar swab at 4–8°C in a non-nutritive transport medium and by using a selective culture medium.*

Pasteurella multocida *and* B. bronchiseptica *can be identified by traditional biochemical tests.* Pasteurella multocida *isolates may be further characterised by their capsular and somatic antigens. Capsular type D is most prevalent in many areas of the world, but in some regions type A predominates. Capsular antigens may be distinguished serologically by indirect haemagglutination or immunofluorescence, chemically by flocculation in acriflavine, or by susceptibility to hyaluronidase. Somatic antigen types can be distinguished by a gel diffusion precipitation test, with type 3 found most frequently in swine. Toxigenicity of* P. multocida *isolates can be demonstrated by testing for cytotoxicity in cultured cells or with a commercially available toxin-specific enzyme-linked immunosorbent assay (ELISA). The ELISA is also suitable for detection of toxin production by bacteria from primary culture plates without the need for prior isolation and identification of individual colonies.*

Assays based on the use of conventional or real-time polymerase chain reaction (PCR) provide rapid, sensitive and highly specific detection of B. bronchiseptica *and both toxigenic and nontoxigenic* P. multocida *for those laboratories with the capability to perform them. A multiplex PCR useful for capsular typing of* P. multocida *has also been described.*

Serological tests: *Detection of antibodies to* P. multocida *and* B. bronchiseptica *is of little value as nontoxigenic strains of* P. multocida *share cross-reactive antigens with toxigenic strains and* B. bronchiseptica *can be isolated from many swine herds. ELISAs for detection of antibodies to the* P. multocida *toxin have been described but their usefulness is limited as not all infected swine develop such antibodies. Widespread vaccination with* P. multocida *toxoid induces antibodies of vaccinal origin, complicating interpretation of results.*

Requirements for vaccines: *Several vaccines are available commercially that contain bacterins of* B. bronchiseptica *and a mixture of toxigenic and/or nontoxigenic strains of* P. multocida*, or a toxoid derived from* P. multocida *or from a recombinant* Escherichia coli.

A. INTRODUCTION

1. Description and impact of the disease

Initial clinical signs include sneezing, snuffling and eye discharge with resultant dark tear-staining and subsequent nasal discharge, which can vary from serous to mucopurulent; in some cases pigs may show epistaxis. Atrophy of the nasal turbinate and septal deviation may lead to shortening or twisting of the snout and, in severe cases, difficulty in eating. Increased severity is associated with overstocking and poor management, housing and environmental conditions. Reduced productivity is generally associated with moderate to severe atrophic rhinitis, although the precise relationship between infection with the causative bacteria and reduced weight gains has not been thoroughly elucidated.

Bordetella bronchiseptica or toxigenic *P. multocida* may be present in a herd without clinical evidence of disease, especially when other respiratory pathogens are absent and environmental and management conditions are optimal. Such carrier herds pose a risk of transmitting these agents to other herds in which progression to severe disease may occur. *Bordetella bronchiseptica* and toxigenic *P. multocida* are commonly found in many domesticated and wild animal species that could potentially transmit the bacteria to swine herds.

2. Nature and classification of the pathogens

Atrophic rhinitis is an infectious disease of swine characterised by stunted development or deformation of the nasal turbinate (conchal bone) and septum.

Two forms of atrophic rhinitis have been recognised, depending on the causal agent(s) (Brockmeier *et al*., 2012):

i) A severe progressive form caused by toxigenic isolates of *Pasteurella multocida,* most commonly capsular types D or A, alone or in combination with *Bordetella bronchiseptica.*

ii) A less severe, nonprogressive form with mild to moderate turbinate atrophy, often without significant snout changes, caused by *B. bronchiseptica*.

3. Zoonotic potential and biosafety and biosecurity requirements

Infection of humans by *B. bronchiseptica* is rare and occurs most often in immunocompromised persons exposed to infected or vaccinated pets; transmission from swine to humans has not been reported. *Pasteurella multocida* can be a serious human pathogen but most zoonotic infections are associated with exposure to pets or wild animals. *Pasteurella multocida* has also been frequently isolated from healthy human carriers working in swine production sites or living nearby and has sometimes been associated with chronic or acute disease in such individuals (Donnio *et al.,* 1999; López *et al.,* 2013; Marois *et al.,* 2009). Transmission occurs primarily through bites or scratches and wound contamination from infected material, but may also result from inhalation of aerosols. Appropriate precautions should be observed by persons having contact with swine infected with *P. multocida*, particularly those who may be immunocompromised. Clinical specimens and cultures of *B bronchiseptica* and *P. multocida* should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

4. Differential diagnosis

Porcine cytomegalovirus also causes rhinitis in young pigs but does not progress to turbinate atrophy or nasal distortion. Asymmetric bone development resulting from habitual biting or chewing of stalls or drinkers may lead to a noticeable misalignment of the jaw in some pigs. Careful inspection can distinguish this condition from shortness or deviation of the snout characteristic of atrophic rhinitis.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of atrophic rhinitis of swine and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Culture and biochemical testing	+++	+++	+++	+++	+++	–
Real-time PCR	++	++	++	++	++	–
Conventional PCR	++	++	++	++	++	–
ELISA for *P. multocida toxin*	++	++	++	++	++	–
CPE for *P. multocida* toxin	+	+	+	+	+	–
Detection of immune response						
ELISA for *P. multocida* toxin	–	–	–	–	–	++
ELISA or agglutinaton testing for *B. bronchiseptica*	++	++	n/a	++	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; CPE = cytopathic effect in tissue culture.
[(a)] A combination of agent identification methods applied on the same clinical sample is recommended.

The diagnosis of atrophic rhinitis depends on clinical, pathological and microbiological investigations, with the latter being particularly important for herds infected subclinically. It is generally accepted that a herd in which toxigenic *P. multocida* is present be defined as affected with progressive atrophic rhinitis, whether or not clinical signs of the disease are evident (Pedersen *et al.,* 1988). Control in many countries has, therefore, centred on detection of infection, even in subclinically infected animals considered to be potential carriers.

1. Pathological diagnostic criteria

Turbinate atrophy may only be seen at slaughter when snout sections at the level of the first/second premolar tooth are examined. Subjective assessment of turbinate atrophy is convenient and often useful for monitoring herds (Brockmeier *et al*., 2012), but objective scales of measurement (Gatlin *et al.,* 1996) are best suited for studies requiring data analysis. Radiography (Done, 1976) and tomography (Magyar *et al.,* 2013) can provide objective observations from live animals; tomography reveals not only severe lesions but more subtle changes that may not be apparent by radiography. However, these techniques are of limited use due to the equipment and expertise required. Diagnosis is assisted by detection of characteristic histopathological features including fibrous

replacement of the bony plates of the ventral conchae with varying degrees of inflammatory and reparative changes.

2. Identification of the agents

2.1. *In vitro* culture

As *P. multocida* preferentially colonises the tonsil, tonsillar swabs or biopsies will provide the highest isolation rates (Ackermann *et al.,* 1994). Nasal swabs are preferred for isolation of *B. bronchiseptica*. When sampling the tonsil is not practical, nasal swabs suffice for isolation of both organisms. Swabs with flexible shafts should be used; sample collection in young pigs is facilitated by the use of mini-tipped swabs. A single swab is used to sample both sides of the nasal cavity and should then be placed in a non-nutritive transport medium (e.g. phosphate buffered saline) and kept at 4–8°C during transit to avoid overgrowth by other faster-growing bacteria. Transit time should not exceed 24 hours.

Although both *P. multocida* and *B. bronchiseptica* grow readily on blood agar, a selective medium is preferred as overgrowth of other bacteria that are present in higher numbers often interferes with their detection. An additional difficulty related to *B. bronchiseptica* is that this organism grows more slowly than most other bacteria present in clinical samples. Various formulations of media containing antibiotics have been used for isolation of *P. multocida,* but comparison of studies in the literature indicates that the highest isolation rates are obtained with modified Knight medium (bovine blood agar containing 5 μg/ml clindamycin, 0.75 μg/ml gentamicin) (Lariviere *et al*., 1993) or KPMD (bovine blood agar containing 3.75 U/ml bacitracin, 5 μg/ml clindamycin, 0.75 μg/ml gentamicin, and 2.25 μg/ml amphotericin B) (Ackermann *et al*., 1994). MacConkey agar with 1% glucose and 20 μg/ml furaltadone is used by many laboratories for selective growth of *B. bronchiseptica* from nasal swabs, but a modified Smith-Baskerville medium (a peptone agar formulation containing 20 μg/ml penicillin, 20 μg/ml furaltadone, and 0.5 μg/ml gentamicin) appears superior, especially when the number of *B. bronchiseptica* present is low (Lariviere *et al*., 1993; Smith & Baskerville, 1979). A further improvement in isolation rate was reported using blood agar containing 40 μg/ml cephalexin (Lariviere *et al*., 1993). A selective blood agar medium for simultaneous isolation of both *P. multocida* and *B. bronchiseptica*, containing 5 mg/litre clindamycin-HCl, 0.75 mg/litre gentamicin sulphate, 2.5 mg/litre K-tellurite, 5 mg/litre amphotericin-B and 15 mg/litre bacitracin, has also been described (De Jong & Borst, 1985). However, it should be noted that K-tellurite has sometimes been found to be inhibitory to the growth of type D *P. multocida* (Lariviere *et al*., 1993).

2.2. Biochemical characteristics

Pasteurella multocida is a Gram-negative, bipolar, pleomorphic rod and forms nonhaemolytic, greyish colonies on blood agar with a characteristic, 'sweetish' odour. It fails to grow on MacConkey agar but yields positive oxidase and catalase reactions and produces indole.

Bordetella bronchiseptica is also a Gram-negative rod, forming convex colonies 1–2 mm in size, usually haemolytic, on blood agar or Bordet-Gengou medium after 48 hours of growth. It is nonfermentative, but positive for oxidase, catalase, citrate and urea and grows in 6.5% NaCl.

Agglutination tests using specific antisera have been described for confirming the identity of presumptive *B. bronchiseptica* isolates but appropriate sera are not widely available for use.

2.2.1. Capsular typing of *P. multocida*

Capsular typing of *P. multocida* is useful for epidemiological purposes, as *P. multocida* often has a mucoid capsule. Serotyping by indirect haemagglutination has traditionally been used (Carter, 1955) but only a few laboratories throughout the world make and maintain the antisera required. However, simpler chemical methods can usually distinguish most swine isolates. Those producing a type D capsule form a heavy flocculate in 1/1000 aqueous acriflavine (Carter & Subronto, 1973), while capsular type A strains can be identified by inhibition of growth in the presence of hyaluronidase (Carter & Rundell, 1975). A small proportion of swine isolates are noncapsulated.

2.2.2. Acriflavine test procedure for capsular type D *P. multocida*

i) Inoculate a tube containing 3 ml of brain–heart infusion broth, using a freshly grown bovine blood agar culture, for each *P. multocida* isolate to be tested. Include a known type D strain and a known type A strain as positive and negative controls.

ii) Incubate inoculated tubes at 37°C for 18–24 hours.

iii) Pellet bacteria by centrifugation and remove 2.5 ml of the supernatant.

iv) Add 0.5 ml of a 1/1000 aqueous solution of acriflavine neutral. Acriflavine solution should be freshly prepared each week and stored at 4°C, protected from light.

v) Mix to resuspend the bacterial pellet and incubate the tube at room temperature, without shaking.

vi) Observe at 5 minutes for the presence of a heavy flocculent precipitate.

2.2.3. Hyaluronidase test procedure for capsular type A *P. multocida*

i) Prepare fresh bovine blood agar cultures of the isolates to be tested. Include a known type A strain and a known type D strain as positive and negative controls.

ii) Inoculate each strain to be examined on a separate trypticase soy blood agar plate with 5% sheep blood or 6% bovine blood by streaking several parallel lines of growth, approximately 3–5 mm apart, across the diameter of the plate. For maximum production of hyaluronic acid it is important that the plates be fresh and not dehydrated.

iii) Heavily streak a hyaluronidase-producing strain of *Staphylococcus aureus* at right angles to the lines of *P. multocida* growth.

iv) Incubate the plates at 37°C, in a humidified atmosphere, and observe periodically for up to 24 hours. Type A strains will exhibit a marked inhibition of growth in the region adjacent to the growth lines of *S. aureus*.

2.2.4. Somatic antigen typing of *P. multocida*

Differences in the cell wall lipopolysaccharide among *P. multocida* strains provide the basis for somatic antigen typing. Sixteen types can be distinguished by a gel diffusion precipitation test (Heddleston *et al*., 1972), with type 3 found most frequently in swine. Although the required antisera are not widely available, many reference laboratories and some diagnostic laboratories offer somatic antigen typing.

2.2.5. Detection of the *P. multocida* toxin

Diagnosis of progressive atrophic rhinitis depends upon characterisation of *P. multocida* isolates as toxigenic. The heat-labile toxin of *P. multocida* produces dermonecrosis in guinea-pigs and is lethal in mice following intraperitoneal injection. Toxigenicity can also be demonstrated *in vitro* by testing for cytopathic effects on monolayers of embryonic bovine lung (EBL) cells (Rutter & Luther, 1984), African green monkey kidney (Vero) cells (Pennings & Storm, 1984) or bovine turbinate cells (Eamens *et al*., 1988). The bacteria are grown in brain–heart infusion broth incubated at 37°C for 24 hours and then pelleted by centrifugation. The supernatant is sterilised by filtration and titrated in monolayer cultures prepared in microtitre plates. Following incubation at 37°C for 2–3 days, the monolayers are stained with crystal violet and examined microscopically to detect cytopathic effects. A rapid cell culture test, in which the suspect colonies are grown on an agar overlay of EBL cells (Chanter *et al*., 1986), permits more efficient analysis of large numbers of isolates.

An enzyme-linked immunosorbent assay (ELISA) for detection of the *P. multocida* toxin, commercially available in some countries, can be used to test mixtures of bacteria recovered from primary isolation media. This is an important advantage as swine may be colonised simultaneously with a mixture of toxigenic and nontoxigenic strains (Ackermann *et al*., 1994; Brockmeier *et al*., 2012). Cell culture methods would require every colony of *P. multocida* in the sample to be tested, which is clearly impractical, to achieve the same level of sensitivity as the ELISA. The ELISA is also suitable for use with individual isolates. Though highly specific, a positive result without previous history of disease or suspicious signs should be thoroughly investigated to recover toxigenic isolates from the animals sampled.

2.3. Molecular methods

Colony morphology and biochemical testing remain the basis for identification of toxigenic *P. multocida* and *B. bronchiseptica* in many laboratories. However, assays based on polymerase chain reaction (PCR) for detection of these agents from swine, including conventional PCRs (Kamp *et al*., 1996; Lichtensteiger *et al*., 1996; Nagai *et al*., 1994; Register & DeJong, 2006) and a real-time PCR (Scherrer *et al*., 2016) provide faster, more specific and more sensitive diagnostic tools. Diagnostic laboratories are increasingly using PCR for identification of these agents as the equipment and expertise become more readily available. Proper in-house validation with known controls as well as standardised, ongoing quality control measures are essential (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

Nucleotide sequences of primer pairs used by Register & DeJong (2006) for conventional PCR detection of *B. bronchiseptica* and toxigenic *P. multocida*, respectively, written 5' to 3', are:

Fla4: TGG-CGC-CTG-CCC-TAT-C/Fla2: AGG-CTC-CCA-AGA-GAG-AAA-GGC-TT

toxA-7: ACT-ACA-GAT-TCC-TAA-CAA-AGG-TTC-TGG/toxA-6: TGC-TCA-AAT-CCT-AAA-TCA-CCT-TGT

Nucleotide sequences of the primers and probe reported for real-time PCR detection of toxigenic *P. multocida* (Scherrer *et al*., 2016), written 5' to 3', are:

toxA-F: GAA-ATG-GCT-GGA-AAA-ACC-AGT-G/toxA-R: GAA-AAG-GCG-CTG-AAA-TTA-CTG-TAT-C

toxA-probe: CGG-CTG-ATT-TAA-TAC-GCT-TTG-CCT-TGC

A multiplex PCR assay for capsular typing of *P. multocida* (Townsend *et al*., 2001) appears to provide more reliable results than phenotypic methods and is frequently used in suitably equipped diagnostic laboratories.

Various DNA fingerprinting or sequence-based typing techniques, including restriction endonuclease analysis (REA), ribotyping, pulsed-field gel electrophoresis, multilocus sequence typing, and PCR-based methods have been evaluated by numerous groups for the purpose of differentiating *P. multocida* isolates. Few direct comparisons between methods have been carried out using strains from pigs with atrophic rhinitis, but REA currently appears to be the method of choice for epidemiologic investigations as it provides a high level of discrimination without the need for specialised equipment or reagents (Djordjevic *et al*., 1998; Gardner *et al*., 1994; Harel *et al*., 1990).

3. Serological tests

At present there are no satisfactory serological tests that can be relied on to detect those animals infected with toxigenic *P. multocida* and capable of developing or spreading the disease. Detection of antibodies to *P. multocida* is not helpful, as nontoxigenic strains share many cross-reacting antigens with toxigenic strains. ELISAs for detection of antibodies to the *P. multocida* toxin have been described (Foged, 1992; Takada-Iwao *et al*., 2007). However, many animals infected with toxigenic *P. multocida* fail to produce antibodies to the toxin, and widespread use of toxoid-containing vaccines limits the diagnostic value of these ELISAs to herds with no history of vaccination (for which results are definitive only when positive) or to detection of vaccine response in vaccinated herds.

Infection with *B. bronchiseptica* can be detected serologically by agglutination testing with formalin-treated bacteria or with a more sensitive ELISA (Venier *et al*., 1984). Unless monitoring the status of a negative herd, *B. bronchiseptica* serology may be of little value as the organism is present in many apparently healthy pig herds.

C. REQUIREMENTS FOR VACCINES

1. Background

There are several commercially available vaccines that contain whole-cell bacterins of *B. bronchiseptica* combined with a toxigenic *P. multocida* bacterin and/or a *P. multocida* toxoid. The toxigenic *P. multocida* bacterin component is most often capsular type D but some vaccines additionally include a type A strain, which may be toxigenic or

nontoxigenic. Live, attenuated *B. bronchiseptica* vaccines are also available. Vaccines containing only *B. bronchiseptica* are not suitable for control of progressive atrophic rhinitis, but may be of benefit in herds with the nonprogressive form. *Pasteurella multocida* and *B. bronchiseptica* vaccines appear to reduce the level of colonisation by these bacteria, but do not eliminate them or prevent infection. Most commercially available vaccines contain either an oil adjuvant or aluminium hydroxide gel.

The *P. multocida* toxin is the single most important protective antigen with respect to progressive atrophic rhinitis. Vaccines based on a *P. multocida* toxoid offer specific protection against the action of the toxin, which, by itself, can be used to reproduce all of the major signs of this disease (see Foged, 1992 for review). The level of toxin produced by *P. multocida* is relatively low and the toxin-specific antibody response induced by bacterin-only vaccines may not be optimal. Purified toxoid (inactivated by formaldehyde) is more immunogenic than crude toxoid and the immunogenicity of the inactivated form is not affected by mixture with a *B. bronchiseptica* bacterin. However, the difficulty and expense of large-scale purification from cultures of *P. multocida* prevent routine incorporation of native, chemically inactivated toxoid into vaccines. Recombinant vaccines containing subunit toxin proteins or genetically detoxified derivatives of the full-length toxin are highly efficacious and less costly to produce (Foged *et al.,* 1992; Hsuan *et al.,* 2009). A DNA vaccine encoding a full-length but enzymatically inactive toxoid was shown to be highly immunogenic in pigs but has so far not been evaluated for efficacy against challenge (Register *et al*., 2007).

Bordetella bronchiseptica produces a variety of toxins and adhesins that are potential virulence factors in swine. Only one, the outer membrane protein pertactin, has been shown to protect against disease in pigs (Kobisch & Novotny, 1990). Despite this fact, a dermonecrotic toxin produced by *B. bronchiseptica*, unique from the toxin produced by *P. multocida*, has traditionally been regarded as the primary virulence factor and protective immunogen in swine (Brockmeier *et al*., 2012). Several studies strongly implicate the toxin as a virulence factor and it undoubtedly plays a role in pathogenesis and, perhaps, in protection. However, the role of pertactin and several additional virulence factors in protective immunity is most likely equal to, or perhaps exceeds, that of the toxin.

Bordetella bronchiseptica is subject to phenotypic variation under certain growth conditions (e.g. temperatures below 37°C or the presence of chemical modulators such as $MgSO_4$ or nicotinic acid), in which production of most virulence factors is reversibly turned off. Spontaneously occurring mutants, permanently unable to produce most virulence factors, also arise with a low frequency during culture. Careful attention to colony morphology, on an area of the plate with well-separated colonies, is essential to retain cultures in the phase I (also known as Bvg^+), or virulent, mode. Phase I colonies are small (1–2 mm in diameter), domed, and haemolytic on blood agar. Loss of haemolysis and the appearance of larger, flat colonies indicate conversion to the avirulent form. Whenever possible, cultures should be propagated using single haemolytic colonies to minimise the slow accumulation of avirulent clones within the culture.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given below and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

The seed-lot system should be employed for the bacterial strains used to prepare whole-cell bacterins, as well as for the strains from which purified antigens are derived.

In the case of whole-cell bacterins, the origin and history of both the *P. multocida* and *B. bronchiseptica* strains should be described and the full characterisation of the master seeds should be laid down in a master seed batch protocol. *Bordetella bronchiseptica* used for vaccine production should be a phase I virulent culture and the *P. multocida* isolates used should be toxigenic.

Working seeds used for vaccine production should be derived from the master seed and checked for all relevant properties as described in the master seed batch protocol.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Both the master seed and the working seed must be pure cultures, free from bacterial, mycotic, mycoplasmal and viral contamination. Related guidelines are provided in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

Identity of the bacterial species and the production of relevant antigens should be confirmed.

2.2. Method of manufacture

2.2.1. Procedure

Precise details of standards for the production of effective commercial vaccines are not available, but they are known to contain 10^{10} cells of formalin-killed *B. bronchiseptica* and 10 μg of *P. multocida* toxoid per dose. *Bordetella bronchiseptica* should be confirmed to be a phase I culture and, for *P. multocida*, it should be confirmed that the culture contains sufficient levels of toxin. A defined number of passages should be used to give the production culture. *Bordetella bronchiseptica* cells, and either *P. multocida* cells and/or toxin, are inactivated, detoxified and formulated with an adjuvant. As the toxin of *P. multocida* has an intracellular location and is released on cell lysis during the stationary phase, the culture supernatant should be harvested approximately 48 hours after the end of the exponential phase of growth.

2.2.2. Requirements for ingredients

All bacterial strains should be propagated in media that support efficient growth and allow optimal expression of the antigens that are relevant for the induction of protective antibodies.

2.2.3. In-process controls

Seed and production cultures are inoculated on blood agar plates and incubated. No nonspecific colonies should grow on these plates.

Cultures are inactivated with formaldehyde. Tests are performed to check the effectiveness of the inactivation process and to test for residual formaldehyde.

Quantification of antigens is carried out by performing a total cell count using a bacterial counting chamber for enumerating whole cells or an antigenic mass determination for defined antigens, e.g. *P. multocida* toxin, by quantitative enzyme immunoassay.

2.2.4. Final product batch tests

i) Sterility

Every batch of vaccine should be tested for sterility according to standard methods (see chapter 1.1.9) described in the European Pharmacopoeia or the United States Code of Federal Regulations.

ii) Identity

Identity testing should be conducted on every batch of vaccine using standard methods, such as those described in the European Pharmacopoeia or the United States Code of Federal Regulations.

iii) Safety

Every batch of vaccine should be tested for safety in the target animal, by giving a double dose by the recommended route of vaccination, and a second, single dose 2 weeks later. No abnormal local or systemic reactions should occur. When a preservative is used, the concentration should be measured for each batch. It must not exceed the maximum permitted level. Batch-to-batch safety tests are required unless safety of the product is demonstrated and approved in the registration dossier and production is consistent with that described in chapter 1.1.8.

iv) Batch potency

Every batch of vaccine should be tested for potency using a validated serological test that correlates with the protection obtained in the efficacy experiment, as described under Section C.2.3.2. The potency test is not necessarily carried out in the target animal – mice or rabbits can be used. In these latter cases, correlation has to be shown with protective antibody levels in the target animal.

2.3. Requirements for relevant regulatory approval

2.3.1. Manufacturing process

For regulatory authorisation of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and Section C.2.2 *Method of manufacture*) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Reversion-to-virulence for attenutated/live vaccines and environmental considerations

Attenuating genetic changes in modified live *B. bronchiseptica* vaccines are generally not well-characterised, but reversion to virulence has not been reported. Regulatory approval may require backpassage studies, in which the vaccine is serially passaged in the target animal for a specified number of times, with no evidence of an increase in virulence.

ii) Precautions (hazards)

Although inactivation of the bacterial cultures by a validated method is a standard procedure, both *B. bronchiseptica* and *P. multocida* produce dermonecrotic toxins; detoxification of these toxins should be confirmed when toxoids are used as vaccine components.

When an oil emulsion is used as the adjuvant, accidental injection of the operator can cause a severe local reaction. Medical attention should be sought immediately, treating the wound as a grease-gun injury.

Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

Normally the vaccine is applied during the late stage of pregnancy, so that progeny will be protected by the uptake of colostral antibodies. The efficacy of a trial vaccine should be measured by vaccinating groups of pregnant sows. Their progeny should be challenged by virulent cultures of *B. bronchiseptica* and toxin-producing *P. multocida*. Significant protection should be obtained against the clinical signs of the progressive form of atrophic rhinitis, i.e. turbinate atrophy. The clinical signs induced in the controls and vaccinates may be compared according to the scoring system of Done (1976).

2.3.4. Duration of immunity

As part of the relevant regulatory approval procedure, the manufacturer should be required to demonstrate the duration of immunity of a given vaccine by either challenge or alternative test at the end of the claimed period of protection.

When the vaccine may be applied irrespective of the stage of pregnancy, duration of immunity should be at least 6 months, so that booster vaccinations twice a year should maintain effective antibody levels.

2.3.5. Stability

As part of the relevant regulatory approval procedure, the manufacturer should be required to demonstrate the stability of all the vaccine's properties at the end of the claimed shelf-life period. Storage temperature shall be indicated and warnings should be given if product is damaged by freezing or ambient temperature.

Every batch of vaccine should be subjected to an accelerated shelf-life test, which has been correlated with real-time shelf-life testing.

3. Vaccines based on biotechnology

A number of vaccines incorporating enzymatically inert *P. multocida* toxin subunits or whole toxin rendered inactive through mutation have been described and evaluated under experimental conditions. The recombinant proteins are generally expressed in *Escherichia coli* and purified prior to use. At present, only a few vaccines that include recombinant toxin or toxin subunits are commercially available; they may or may not include a *P. multocida* bacterin. Evidence so far suggests such vaccines elicit levels of toxin-specific antibody equal to or greater than those obtained with vaccines containing native, chemically inactivated toxoid.

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NB: At the time of publication (2018) there were no WOAH Reference Laboratories for atrophic rhinitis of swine (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.9.3.

CLASSICAL SWINE FEVER (INFECTION WITH CLASSICAL SWINE FEVER VIRUS)

SUMMARY

Classical swine fever (CSF) is a contagious viral disease of domestic and wild pigs. The causative virus is a member of the genus Pestivirus *of the family* Flaviviridae*, and is closely related to the viruses of bovine viral diarrhoea and border disease. There is only one serotype of CSF virus (CSFV).*

The disease may run an acute, subacute, chronic, late-onset, inapparent course or persistent infection, depending on a variety of viral and host factors of which the age of the animals, the virulence and load of the virus and the time of infection (pre- or post-natal) are of greatest importance. Adult pigs usually display less severe signs of disease than young animals and stand a better chance of survival. In pregnant sows, the virus may cross the placental barrier and reach the fetuses. In-utero *infection with strains of the virus of moderate or low virulence can result in what is referred to as the 'carrier sow' syndrome followed by prenatal or early post-natal death, the birth of diseased piglets or an apparently healthy but persistently infected litter. The virus has also the ability to induce persistent infection following post-natal infection and may be transmitted to the sows through boar semen. An outbreak of CSF in domestic pigs has serious consequences for trade in pigs and pig products.*

The highly variable clinical picture of CSF precludes a diagnosis on clinical and pathological grounds alone. Laboratory methods are therefore essential for an unambiguous diagnosis. Detection of virus or viral nucleic acid in anticoagulated whole blood, tonsil and of antibodies in serum are the methods of choice for diagnosing CSF in live pigs, whereas detection of the virus, viral nucleic acid or antigen in organ samples is most suitable when the pig is dead.

Detection of the agent: *The isolation of CSFV should be attempted in pig kidney (PK-15, SK-6) cell lines, or other CSFV permissive cell lines. The cultures, which are generated from stocks that are* Pestivirus*-free (and preferably free of other contaminants, e.g. mycoplasmas, porcine circovirus), are examined for virus growth by immunofluorescence or immunoperoxidase staining; positive isolates are further characterised by partial genetic sequencing or, if that method is not available, by the use of monoclonal antibodies (MAbs). Reverse-transcriptase polymerase chain reaction protocols for the identification of CSFV nucleic acid are internationally accepted and are used in many laboratories, both for detection of the agent and differentiation from other pestiviruses. The direct fluorescent antibody test (FAT) on cryostat sections of organs from affected pigs can be used for the detection of CSF antigen. A panel of MAbs is used to determine whether the fluorescence is caused by CSF or non-CSF* Pestivirus *antigens. Antigen-capture enzyme-linked immunosorbent assays (ELISAs) are also useful for herd screening, but must not be used on a single animal basis.*

Serological tests: *Detection of virus-specific antibodies is particularly useful in herds suspected of having been infected at least 21 days previously with CSFV. Serological methods are also valuable for monitoring and for prevalence studies, and are essential if a country wishes to be internationally recognised as being free from the disease in the absence of vaccination.*

As CSFV cross-reactive antibodies against other pestiviruses are occasionally observed in pigs, screening tests have to be followed by confirmatory tests that are CSFV-specific. Certain ELISAs are relatively CSFV-specific, but the definitive method of choice for differentiation is the comparative neutralisation test, which compares the neutralising titre of antibodies to different Pestivirus *isolates.*

Requirements for vaccines: *Modified live vaccines (MLVs) against CSF are based on the live virus that has been attenuated by passage through cell cultures or through a suitable host species that is not of the family* Suidae*. The production of these MLVs is based on a seed-lot system that has been validated with respect to virus identity, sterility, purity, safety, non-transmissibility, stability and immunogenicity.*

Effective inactivated, conventional whole virus vaccines are not available. In contrast to conventional MLV vaccines, a new generation MLV 'marker vaccine' capable of inducing antibodies that can be distinguished from antibodies induced by field virus based on an appropriate companion discriminatory diagnostic test, is available in some regions and more are in development. Subunit 'marker vaccines', based on the major envelope glycoprotein (E2-subunit) of CSFV are produced in insect or mammalian cells (depending on the expression system used).

A. INTRODUCTION

The viruses that cause classical swine fever (CSF), bovine viral diarrhoea (BVD) and border disease (BD) are members of the family *Flaviviridae*, genus *Pestivirus*, and are closely related to each other, both antigenically and structurally. Clinical signs and lesions seen at post-mortem examination in pigs affected with CSF are highly variable due to both viral and host factors. Furthermore, congenital infections with ruminant pestiviruses in pigs occasionally give rise to a clinical disease that is indistinguishable from CSF. Recent reviews of CSF disease are provided by Blome *et al.* (2017c), Ganges *et al.* (2020), and Postel *et al.* (2018).

CSF affects the immune system, the main characteristic being generalised leukopenia, which can often be detected before the onset of fever. Immunosuppression may lead to concurrent infections that can mask the clinical picture.

The clinical signs can vary considerably and depend on many factors, including the age of the affected pigs and the virulence of the circulating viral strain. Pyrexia, huddling, inappetence, dullness, weakness, conjunctivitis and constipation followed by diarrhoea are the prevailing signs of disease in all age groups. In addition, animals may display a staggering gait, ataxia or convulsions. Several days after the onset of clinical signs, the ears, abdomen and inner thighs may especially show petechial haemorrhages or a purple discoloration. Animals with acute disease die within 1–4 weeks. Sudden death in the absence of clinical illness is not a sign of CSF.

Under certain circumstances determined by the animals' age, breed, health condition and the virus strain involved, infected animals may develop subacute or chronic clinical illness including subclinical forms. These forms of infection can be protracted for several weeks or months, or even longer. Chronic illness leads to a stunting of growth, anorexia, intermittent pyrexia and diarrhoea. Animals with subclinical forms can develop persistent infections, showing variable viral load, from high to low in serum in the absence of antibody induction.

Congenital persistent infections may go undetected for months and may be confined to only a few piglets in the herd or may affect larger numbers. Persistently infected piglets do not produce antibodies against CSF virus (CSFV), show life-long viraemia and viral shedding in most cases. The clinical signs are nonspecific: wasting in the absence of pyrexia. Chronic and persistent infections always lead to the death of the animal. Herd mortality rates may be slightly above the expected level.

In acute cases, gross pathological lesions might be inconspicuous or absent. In typical cases, the lymph nodes are swollen and marbled red, and haemorrhages occur on serosal and mucosal membranes of the intestinal organs. Splenic infarctions may occur. In subacute and chronic cases, necrotic or 'button' ulcers may be observed in the mucosa of the gastrointestinal tract, epiglottis, tonsils and larynx, in addition to the above lesions.

Histopathological findings are not pathognomonic. Lesions may include parenchymatous degeneration of lymphatic tissue, cellular proliferation of vascular interstitial tissue, and a nonsuppurative meningo-encephalomyelitis, with or without vascular cuffing.

The variability of the clinical signs and post-mortem lesions does not provide firm evidence for unequivocal diagnosis. Other viral diseases, such as African swine fever, porcine dermatitis and nephropathy syndrome (PDNS), and PCV2-systemic disease (PCV2-SD), thrombocytopenic purpura and various septicaemic conditions including, amongst others, salmonellosis (especially caused by *Salmonella choleraesuis*), erysipelas, pasteurellosis, actinobacillosis (caused by *Actinobacillus suis*) and *Haemophilus parasuis* infections may be confused with acute CSF. In fact, these bacteria often cause concurrent infections, and isolating these pathogens may obscure the CSFV

infection as the actual cause of the disease. Similarly, concurrent PDNS can lead to oversight of an underlying CSF infection.

A tentative diagnosis based on clinical signs and post-mortem lesions must therefore be confirmed by laboratory investigations. With regard to diagnosis and vaccination of CSF, recent reviews have been published and research gaps have been defined (Blome *et al.,* 2017b; Ganges *et al.,* 2020; Postel *et al.,* 2018).

There is no known risk of human infection with CSFV. The virus has a high risk of spread from the laboratory, and biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Countries lacking access to an appropriately equipped laboratory should send specimens to a WOAH Reference Laboratory.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of classical swine fever and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Virus isolation	–	+	–	+++	–	–
RT-PCR	+	+++	++	+++	++	–
ELISA (antigen)	++	+	+	+	–	–
FAT	–	–	+	+	–	–
Detection of immune response						
ELISA (antibody)	+++	+++	+++	–	+++	+++
VN (FAVN or NPLA)	+	+++	++	++	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcriptase polymerase chain reaction;
ELISA = enzyme-linked immunosorbent assay; FAT = fluorescent antibody test;
VN = virus neutralisation; FAVN = fluorescent antibody virus neutralisation; NPLA = neutralising peroxidase-linked assay.
[(a)]A combination of agent detection methods applied on the same clinical sample is recommended.

Pyrexia is one of the first signs of CSF and is accompanied by viraemia. Detection of virus or viral nucleic acid in whole blood, collected in heparin (may interfere with polymerase chain reaction [PCR]) or ethylene diamine tetra-acetic acid (EDTA), or in tissues (especially in tonsil) collected from febrile animals, is the method of choice for detecting infected herds at an early stage. This is all the more necessary in view of the serious consequences of an outbreak of CSF for trade in pigs and pig products.

Laboratory methods for diagnosis of CSF are aimed at detection of the virus, viral nucleic acid or viral antigens, or detection of specific antibodies. Targeted and risk-based sampling should be performed, random sampling only being applied in cases where no clinical signs of disease are present. To increase the sensitivity of detection of virus, viral antigen or nucleic acid, clinically diseased animals and febrile animals should primarily be sampled. For the

detection of antibodies, animals that have recovered from disease or animals that have been in contact with infected or diseased animals should be primarily targeted.

For a correct interpretation of the test results, the inspecting veterinarian should pay particular attention to the simultaneous and clustered occurrence of two or more of the prevailing signs of disease listed above. In suspected primary cases an initial positive test result needs to be confirmed using a second test method.

Antibodies develop in the third week of illness and may persist in the surviving animal for years or even life (except for chronic cases). Antibodies develop 10–14 days post-vaccination and may persist in the vaccinated animals for years or even longer following extensive vaccination programmes. Samples for antibody detection are collected in ordinary (non-anticoagulant) tubes from convalescent pigs, from contact herds and populations post-vaccination. All methods and protocols need to be validated in the respective laboratory and the laboratory has to prove that it is capable of performing the tests it uses for diagnostic purposes with satisfactory results. Laboratories should maintain appropriate quality management in respect of the testing carried out and validation should be done in accordance with WOAH validation standard (see Chapter 1.1.5 *Quality Management in Veterinary Laboratories* and Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases for terrestrial animals*).

1. Detection of the agent

1.1. Isolation of virus

Isolation of virus in cell cultures is a more sensitive but slower method for diagnosis of CSF than immunofluorescence on frozen sections. Organ preparations, leukocyte preparations, whole blood samples, sera, plasma, swab samples or semen can be used. Isolation is best performed in rapidly dividing PK-15 cells used as a pre-formed monolayer or seeded as cell suspension for simultaneous infection with a 2% suspension of the tonsil or another appropriate sample in growth medium. Other pig cell lines may also be used, but should be demonstrably at least as sensitive as PK-15 cells for isolation of CSFV and must be free of pestiviruses and *Pestivirus* antibodies. It is generally advantageous to use more than one porcine cell line for inoculation, to enhance the chances of a positive result. As growth of the virus does not cause a cytopathic effect, its presence must be demonstrated by an immunostaining method, which may be carried out after one (when used for screening purpose) or two virus passages. This can be done by examining the cultures for fluorescent foci by FAT after 24–72 hours or by immunoperoxidase staining after preferably 72 hours (minimum; 2 days; maximum: 5 days) incubation. NB: Positive and negative controls always need to be included.

The tonsil is the most suitable organ for virus isolation from pigs that died or were killed for diagnostic purposes. Alternatively, or in addition, spleen, kidney, ileum or lymph nodes can also be used.

Fetal bovine serum (FBS) used in any diagnostic assay always needs to be free of pestiviruses and *Pestivirus* antibodies. It might not be sufficient to rely on manufacturers' declarations and for this reason it is recommended that each lot of FBS be tested for the presence of pestiviruses and *Pestivirus* antibodies prior to its use in diagnostic assays. The percentage of FBS (5–10%) in the growth medium depends on the conditions used for adaptation and propagation of the cells in the individual laboratory.

1.1.1. Virus isolation – Test procedure 1

i) Prepare a 100-fold strength glutamine–antibiotic stock solution: dissolve glutamine (2.92 g) in 50 ml distilled water (solution A) and sterilise by filtration. Dissolve each of the following antibiotics in 5–10 ml sterile distilled water: penicillin (10^6 International Units [IU]); streptomycin (1 g); mycostatin (5×10^5 U); polymixin B (15×10^4 U); and kanamycin (1 g). Pool these solutions (solution B). Mix aseptically solutions A and B, make up to 100 ml with sterile distilled water, and store in 5 ml aliquots at –20°C. Exact antibiotic constitution is not critical, provided sterility is achieved and cells are not affected.

ii) Cut 1–2 g of tissue (organ sample of approx. 1 cm^3) into small pieces and, using a mortar and pestle or other device, grind in a small amount of cell culture medium with sterile sand into a homogeneous paste. Alternatively, use an appropriate crushing machine or automatic homogeniser at 4°C; in this case, organ samples are cut into pieces with a scalpel and an amount, of 10–30 mg, (or an amount suitable for the specific homogeniser) is put into a tube. (Attention: high speeds can heat the sample and reduce virus viability!)

iii) Make a 10% (w/v) suspension by adding Hanks' balanced salts solution (BSS) or Hanks' minimal essential medium (MEM); 1 ml of the glutamine–antibiotic stock is added for each 10 ml of suspension. This mixture is held at room temperature for 1 hour.

iv) Centrifuge at 1000–2500 ***g*** for 15 minutes. The supernatant is used for inoculation of cell cultures. A 1/100 dilution can be processed in parallel in case of cytotoxic effects. Sterile filtration can be performed, if considered necessary using syringe filters (0.45 μm followed by 0.22 μm).

v) A PK-15 monolayer is trypsinised, the cell suspension is centrifuged at 160 ***g*** for 10 minutes. The supernatant is discarded and the pellet is resuspended to contain approximately 2×10^6 cells/ml in growth medium (Eagle's MEM with Earle's salts; 5–10% FBS free of ruminant pestiviruses and *Pestivirus* antibodies; and 0.2 ml of the glutamine–antibiotic stock solution per 10 ml cell suspension). As a guide, one 75 cm^2 flask will give approximately 50 ml of cell suspension at the appropriate concentration. It usually contains about 8.5×10^6 cells.

Alternatively, a protocol without centrifugation can be performed:

Growth medium is removed from a PK-15 monolayer and cells are washed once or twice with 5 ml of adjusted trypsin/versene (ATV) solution (5 ml ATV for a 250 ml flask). ATV is removed and replaced with fresh ATV (2 ml ATV for a 250 ml flask). The flask is incubated at 37°C for 5–15 minutes or until cells are detached. It is then filled with cell culture medium containing 5–10% FBS (8 ml medium for a 250 ml flask) and the cells are resuspended.

vi) Either:

Suspension inoculation: mix nine parts of cell suspension (from step v) and one part of sample supernatant fluid (from step iv) and inoculate 1.0–1.5 ml into 6–8 Leighton tubes with cover-slips or other appropriate cell culture flasks or plates. Three tubes are inoculated with 1.0–1.5 ml of cell suspension alone as controls. After completion of the sample inoculations, three tubes are inoculated with CSFV as positive controls. Careful precautions must be taken to avoid cross-contamination with this known positive virus suspension. Negative cultures should also be prepared. Incubate at 37°C in a 5% CO_2 incubator.

Or:

Pre-formed monolayer inoculation: for each tissue, inoculate 1.0–1.5 ml of cell suspension (prepared as in step v) into 6–8 Leighton tubes with cover-slips or other appropriate cell culture flasks or plates. Incubate at 37°C for a minimum of 4 hours and a maximum of 36 hours (until 50–80% confluency is reached). Then drain the medium and inoculate 0.2 ml of supernatant fluid (from step iv), incubate for 1–2 hours at 37°C, with gently shaking once or twice to avoid cells from drying out and dying, rinse once with PBSM (phosphate-buffered saline [PBS] without Ca/Mg), overlay with 1 ml of growth medium and incubate at 37°C in a 5% CO_2 incubator.

vii) At 1, 2 and 3 days after inoculation, two cultures, together with a positive and negative control culture are washed twice for 5 minutes each in Hanks' BSS, Hanks' MEM or PBS and fixed. Different methods for cell fixation can be applied and are described in Section B.2.1. Cell fixation can be performed by 100% acetone (analytical grade) for 5 minutes for cell cultures grown on glass surfaces. Alternative fixation protocols (e.g. heat fixation at 80°C for at least 4 hours or overnight) can be used.

viii) After fixation, staining with a direct or indirect anti-CSFV conjugate at its appropriate working dilution is performed as described in Section B.1.2. After washing in PBS three times for 5 minutes each, the cover-slip cultures are mounted in 90% carbonate/ bicarbonate buffered glycerol, pH>8.0, and examined for fluorescent foci.

Instead of Leighton tubes, 6-well plates with cover-slips can be used. Alternatively, cultures growing on flat-bottomed microtitre plates or M24-plates can also be used for virus isolation. In such case, plates are fixed and stained as described later for the neutralising peroxidase-linked assay (NPLA; Section B.2.1).

ix) If the 2% tonsil suspension proves to be toxic for the cells, then the test should be repeated using a higher dilution or another organ. Use of the method employing pre-formed monolayers (above) will help to avoid this problem.

1.1.2. Virus isolation – Test procedure 2

Whole blood (heparin or EDTA-treated) from clinically diseased pigs is a suitable sample for early CSF diagnosis. The leukocyte fraction or other components may be used, but for reasons of simplicity the use of whole blood is more practical and therefore preferred. The procedure is as follows:

i) Freeze a sample of whole blood at –20°C and thaw in a water bath at 37°C to lyse the cells.

ii) Inoculate 100–300 µl haemolysed blood on to a PK-15 monolayer grown to approximately 50–80% confluence* in an M24-plate or Leighton tubes with cover slips, and allow adsorption for 1–2 hours at 37°C, with gently shaking once or twice to avoid the cells from drying out and dying. Duplicate cultures of each sample should always be prepared.

iii) Remove inoculum, wash the monolayer once with Hanks' BSS or Hanks' MEM or PBSM, and add fresh growth medium.

iv) After a further incubation period of 3–4 days at 37°C in a 5% CO_2 incubator, the plates are washed, fixed and stained, as described later for the NPLA, using in each step a volume of 300 µl to compensate for the larger cell surface.

Note: This method is less sensitive than conventional virus isolation (test procedure 1) for the detection of acute CSF.

1.1.3. Virus isolation – Test procedure 3

To improve the sensitivity, virus isolation can be performed over two passages:

i) Inoculate a cell culture tube with 200–300 µl of organ preparation or blood lysate (see above). Duplicate cultures should always be prepared.

ii) Incubate the cell cultures for 37°C for 1–2 hours, gently shake once or twice to avoid the cells from drying out and dying, and wash twice with PBSM.

iii) Incubate the cultures for 72 hours at 37°C in a 5%CO_2 incubator. Eagle's MEM with 5–10% FBS is the ideal medium for virus growth. Simultaneous inoculation is possible if the sample is fresh and a cytotoxic effect is unlikely.

iv) Freeze the cell culture tubes or plates at –80°C for at least 1 hour and then thaw at room temperature.

v) When using cell culture tubes, the tubes are centrifuged for 10 minutes at 778 ***g***.

vi) Incubate 200–300 µl of the supernatant for 1–2 hours on cell cultures in a well of a multi-dish plate or Leighton tube as described above.

vii) Wash the cell culture tubes or plates with PBSM, refill with cell culture medium and incubate for 72–96 hours in a 5%CO_2 incubator.

viii) Cells are fixed and stained as described in Section B.2.1.

If a slow-growing isolate is suspected, a second passage in a culture tube can be done, leading to a third passage in a culture dish.

Positive and negative controls must always be included and processed in the same way.

1.1.4. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Many methods for RT-PCR have been described or are being developed (Ganges *et al.,* 2020; Postel *et al.,* 2018). By using RT-PCR techniques, infected animals can be detected early during the incubation period, later persistent infection period and for a longer period of time in cases where the pigs recover. RT-PCR detects viral nucleic acid only and positive results may be obtained in cases where virus isolation or other techniques yield negative results. RT-PCR is

* Simultaneous inoculation, though slightly more sensitive, is less suitable as the anticoagulant may interfere with the adhesion of cells on to the surface.

therefore more sensitive than other techniques (such as virus isolation, antigen-capture ELISA, and FAT).

Owing to its speed and sensitivity, RT-PCR is a suitable approach for screening and confirmation of suspected cases of disease, and is accepted by many countries, including the European Union (EU). It is however important to bear in mind that false positive results due to laboratory contamination can occur, as well as false negative results due to PCR inhibitors contained in the sample. Therefore, any positive results from primary outbreaks must always be confirmed by other tests, for example virus isolation or a second RT-PCR targeting a different genome region or real-time RT-PCR. It is mandatory to include an adequate number of positive and negative controls in each run; it is also strongly recommended that internal controls be included. See Chapters 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases* and 2.2.3 *Development and optimisation of nucleic acid detection assays* for further details on PCR techniques.

The test can be applied to EDTA blood and serum samples as well as solid organs and cell culture supernatants and has been used successfully in case of outbreaks.

Isolation of RNA is a critical step in RT-PCR analysis. RNA integrity is at the highest risk prior to and after extraction. Thus, treatment of samples prior to RNA extraction and storage of isolated RNA have to be carefully considered as they will influence the quality of the yielded RNA and the test result. Different methods for RNA isolation have been described and a wide variety of extraction kits is commercially available (including robotic extraction systems). RNA isolation must also be validated in the laboratory.

Several conventional RT-PCR and real-time RT-PCR protocols have been described (Hoffmann *et al*., 2005; McGoldrick *et al*., 1998; Paton *et al*., 2000a; Risatti *et al*., 2005) and a suitable protocol may be obtained from the literature or from the WOAH Reference Laboratories for CSF [1]. Evaluation of RT-PCR results can either be performed by agarose gel electrophoresis (standard RT-PCR) or by real-time techniques, with the latter providing the significant benefit of reduced risk of laboratory contamination with PCR products. Other RT-PCR tests have been developed to differentiate between field and C-strain vaccine viruses. Some tests focus on the detection of the vaccine virus and others on the detection of a specific CSFV field strain (Liu *et al.,* 2011; Zhao *et al.,* 2008). Any RT-PCR protocol to be used must be thoroughly validated in each individual laboratory to show that the method is fit for purpose, before it can be used for diagnosis in that laboratory. Any RT-PCR protocol used must prove to be at least as sensitive as virus isolation. The real-time RT-PCR protocol described by Hoffmann *et al*. (2005) is widely used with the following primer sequences: forward primer CSF 100-F: 5'-ATG-CCC-AYA-GTA-GGA-CTA-GCA-3'; slightly modified reverse primer CSF 192-R: 5'-CTA-YTG-ACG-ACT-RTC-CTG-TAC-3'. The sequence of the CSF probe is: 5'-FAM-TGG-CGA-GCT-CCC-TGG-GTG-GTC-TAA-GT-TAMRA-3' (or BHQ1-3'). The method yielded consistent results in international interlaboratory comparison testing.

In principle, pooling of samples is possible, but sensitivity must be shown to be at least as high as the sensitivity of virus isolation performed on single samples. Pooling must be properly validated prior to its use in each individual laboratory.

Quality control is an essential issue in PCR diagnosis and prevention of laboratory contamination is crucial.

1.1.5. Molecular epidemiology and genetic typing

The molecular epidemiology of CSF is based on the comparison of genetic differences between virus isolates. RT-PCR amplification of CSFV RNA followed by nucleotide sequencing is the simplest way of obtaining the sequence data to make these comparisons, as well as to study virus evolution, epidemiological tracing of the origin of viral strains and to monitor the efficacy of vaccination in conferring virological protection. A number of different regions of the CSFV genome may be targeted for molecular epidemiological studies (Paton *et al*., 2000b). Two regions have been extensively studied and provide large sets of sequence data with which new isolates

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

can be compared. One of these regions lies within the 5'-nontranslated region (5'NTR) of the genome (150 nucleotides) and the other lies within the E2 major glycoprotein gene (190 nucleotides). In brief, the method used involves extracting virus RNA from clinical samples or cell cultures infected with low passage CSFV, performing RT-PCR to amplify one or both targets within the 5'NTR or the E2 gene, and then determining the nucleotide sequence of the products and comparing with stored sequence information held in the databases. A database of these sequences is available from the WOAH Reference Laboratory for CSF in Germany (Postel *et al.,* 2016). Analysis of multiple regions to type accurately CSFV and other *Pestivirus* strains by this method was described previously (Becher *et al.,* 2003). To discriminate between closely related CSFV isolates, analysis of the complete E2 encoding region (1119 nucleotides) was demonstrated to be a useful strategy (Postel *et al.,* 2012). CSFV isolates from primary outbreaks should be sent to a WOAH Reference Laboratory for investigation of molecular epidemiology. The receiving laboratory should be contacted first and an import permit should be obtained prior to dispatch. It is highly recommended that detailed characterisation of isolates from primary outbreaks be performed, including determination of complete genome sequences e.g. by high throughput sequencing.

1.2. Immunological methods

1.2.1. Fluorescent antibody test

The fluorescent antibody test (FAT) is a rapid test that can be used to detect CSFV antigen in cryostat sections of tonsils, spleen, kidney, lymph nodes or distal portions of the ileum. Tissues should be collected from several (febrile and/or diseased) animals (Bouma *et al*., 2001) and transported without preservatives under cool conditions, but not frozen. Cryostat sections are stained directly with anti-CSF immunoglobulin conjugated to a fluorescence marker such as fluorescein isothiocyanate (FITC) or indirectly using a secondary fluorescent conjugate and examined by fluorescence microscopy. During the first stage of the infection, tonsillar tissue is the most suitable, as this is the first to become affected by the virus irrespective of the route of infection. In subacute and chronic cases, the ileum is frequently positive and occasionally may be the only tissue to display fluorescence, tonsil is also a suitable sample in living animals.

A negative FAT result does not completely rule out CSF infection. When suspicion of CSF continues, further samples should be obtained or attempts made at RT-PCR or virus isolation in cell culture. In some cases, during the terminal stage of disease, neutralising antibodies can mask a positive reaction.

There is a relatively high risk of false (positive and negative) results when FAT is used by laboratories not thoroughly acquainted with the method. Thus FAT should only be used by laboratories that have experience of using the technique, perform the technique on a routine basis and have had training in interpreting the fluorescence.

1.2.1.1. Test procedure

Include positive and negative control sections in each series of organ samples to be examined. In indirect labelling, an infected control section should also be included, which is treated without incubation of the first antibody. The control sections can be prepared in advance and stored after acetone fixation for 2–3 years at –70°C until use.

i) Cut out a piece of tonsil, spleen, kidney and ileum of approximately 1 × 1 × 0.5 cm, and mount it with a cryo-embedding compound or distilled water on a cryostat table.

ii) Freeze the piece of organ on to the cryostat table. The freezing temperature should be –15°C to –20°C. Shock-freezing of the tissue in n-Heptane cooled with liquid N_2 is ideal.

iii) Cut sections not more than 4–8 µm thick and mount these on to grease-free cover-slips. It is helpful to mark these cover-slips by one cut-off corner and to mount them with this corner in the same position (e.g. top right).

iv) Prepare several cover slips for each tissue sample.

v) Dry for 20 minutes at room temperature.

vi) After drying, fix the mounted sections for 10 minutes in acetone (analytical grade) at –20°C or air-dry for 20 minutes at 37°C.

vii) Immerse the sections briefly in PBS, remove excess fluid with tissue paper and place them (cut off corner top right) on a frame in a humid incubation chamber.

viii) Dispense the anti-CSF immunoglobulin at working dilution (dilution in PBS) on to the entire section and incubate in a dark, closed chamber for 30 minutes at 37°C. Check afterwards that the conjugate solutions have not evaporated and that the tissues have not dried out.

If a secondary FITC conjugate is required, wash the section five times for 2 minutes each in PBS at room temperature, then add the FITC conjugate at working dilution and incubate as previously described.

ix) Wash the sections five times for 2 minutes (or three times for 5 minutes) each in PBS at room temperature.

x) Immerse the section briefly in double-distilled water (solvent).

xi) If necessary, counterstain in Evans Blue for 30 seconds.

xii) Remove the remaining fluid by touching the cover-slip against tissue paper and mount the cover-slip (with the section between cover-slip and slide) with mounting buffer on to a microscope slide.

xiii) Remove excess mounting fluid with tissue paper and examine the sections for fluorescence using a UV microscope.

A CSF-positive section shows brilliant fluorescence in the cytoplasm of infected cells. In the tonsils, fluorescence in the epithelial lining of the crypts is particularly evident. In kidney sections, fluorescence is most abundant in the proximal and distal tubules of the renal cortex and the collecting ducts in the medulla. In the ileum, fluorescence is most prominent in the epithelial cells of the Lieberkühn glands, whereas in the spleen reactivity is more diffuse, with concentrations of lymphoid cells in the periarterial lymphoid sheath (PALS).

It is recommended to use anti-CSFV gamma-globulins prepared from polyclonal antibodies against CSFV raised in specific pathogen free pigs. This ensures that no minor variant viruses will be missed, but has the disadvantage that the test will not distinguish between the antigens of different pestiviruses. Thus, pigs infected with other pestiviruses can yield a positive result. To differentiate CSFV from other pestiviruses, especially in CSFV-free areas, duplicate samples from FAT-positive samples should be examined using monoclonal antibodies (MAbs) that can distinguish between CSFV and other pestiviruses (especially BVDV and BDV) (Section B.1.2.2). Alternatively, confirmatory diagnosis should await results of RT-PCR (followed by genetic typing) or virus isolation in cell culture with subsequent typing by MAbs.

Strains of modified live virus (MLV) vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Pigs vaccinated with MLV strains may yield a positive FAT for 2 weeks after vaccination (Ogawa *et al*., 1973). RT-PCR followed by nucleic acid sequencing of the RT-PCR amplicon allows differentiation between field isolates and vaccine strains of CSFV.

The working dilution of the conjugates (at least 1/30) should combine a maximum brilliance with a minimum of background. The test should only be performed on samples from freshly dead animals, as autolysis and bacterial contamination can often result in high background staining.

1.2.2. Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies

The use of a panel of three MAbs that are conjugated to either horseradish peroxidase (HRPO) or a fluorescence marker, or used in conjunction with an anti-mouse conjugate and capable of specifically detecting all field strains of CSFV, vaccine strains of CSFV and BVDV, respectively, would allow an unambiguous differentiation between field and vaccine strains of CSFV on the one hand, and between CSFV and BVDV on the other (Edwards *et al*., 1991). A prerequisite is that the MAb against CSFV recognises all field strains and that the anti-vaccine MAb recognises all vaccine strains used in the country. *No single MAb selectively reacts with all other pestiviruses* (Edwards *et al*., 1991). The use of a MAb to differentiate a CSF vaccine strain can be omitted in non-vaccinated areas. A polyclonal anti-CSF immunoglobulin conjugated to HRPO serves as a positive control. Caution should be exercised when using evidence of a single MAb as sole confirmation of an isolate as CSF. Advice on suitable MAbs and their suppliers should be sought

from the WOAH Reference Laboratories for CSF. The occurrence of various BDV genotypes that may infect pigs under natural conditions and the detection of a growing number of additional ruminant pestiviruses can hamper the differentiation of CSFV from other pestiviruses by MAbs. For reliable differentiation of CSFV from other pestiviruses it is therefore recommended to perform RT-PCR and followed by nucleotide sequencing.

Positive and negative control sections need to be included in each series of organ samples to be examined. In the case of indirect labelling, an infected control section, which is treated without incubation of the first antibody, should also be included. The control sections can be prepared in advance and stored after acetone fixation for 2–3 years at –70°C until use.

1.2.2.1. Test procedure

i) Cut eight or more cryostat sections (4–8 μm) of the FAT-positive tonsil, or another positive organ if the tonsil is not available (as described above for the FAT method).

ii) Place the sections on to cover-slips, allow to dry for 20 minutes at room temperature and fix for 10 minutes in acetone (analytical grade) at –20°C and allow to air dry.

iii) Prepare working dilutions of the respective MAb-peroxidase conjugates in PBS + 0.01% Tween + 5% horse serum, pH 7.6. (FITC–MAb can be used as well as unconjugated MAb provided that a secondary conjugate is used.)

iv) Immerse the sections briefly in PBS, remove excess fluid with tissue paper and place them (cut off corner top right) on a frame in a humid incubation chamber.

v) Overlay two sections each with the working dilution of the respective monoclonal conjugates, and two sections with the working dilution of the polyclonal conjugate (controls).

vi) Incubate in a dark, closed chamber for 30 minutes at 37°C. Check afterwards that the solutions have not evaporated and that the tissues have not dried out.

vii) Wash the sections six times for 10 seconds each in PBS at room temperature.

viii) Stain the sections with freshly prepared chromogen–substrate solution* for 5–15 minutes at room temperature.

ix) Rinse the sections in 0.05 M sodium acetate, pH 5.0, in distilled water and mount them on microscope slides.

x) Examine sections with a light microscope. Dark red staining of the cytoplasm indicates recognition of the virus isolate by the respective conjugate, and is considered to be positive.

xi) Interpretation of the test:

Polyclonal antibody	Monoclonal antibody specific for			Interpretation
	CSF strain	CSF vaccine strain	BVDV strain	
+	+	–	–	CSF field strain
+	+/-	+	–	CSF vaccine strain
+	–	–	+	BVD/BD strain
+	–	–	–	Other non-CSF *Pestivirus*[†]

*The existence of novel strains of CSF should always be considered and any isolate from cases where CSF is still suspected should be sent to a WOAH Reference Laboratory.

* **Chromogen–substrate solution**

A. Stock solution of chromogen: 0.4% 3-amino-9-ethyl carbazole; N,N-dimethyl-formamide (1 ml).
Caution **TOXIC compound**. Both chemicals are carcinogens and irritants to eyes, skin and respiratory tract.

B. 0.05 M sodium acetate, pH 5.0; 19 ml (sterile filtered through a membrane).

C. Stock solution of substrate (30% hydrogen peroxide).

Keep stock solutions A and C at 4°C in the dark and solution B at room temperature. Stock solution A can be kept at 4°C for at least 6 months and solution C for 1 year. Immediately before use, dilute 1 ml of solution A in 19 ml of solution B. Then add 10 μl of stock solution C. Mix well and stain the sections.

1.2.3. Antigen-capture assay

For rapid diagnosis of CSF in live pigs, antigen-capture enzyme-linked immunosorbent assays (ELISAs) have been developed for screening herds suspected of having been recently infected. The ELISAs are of the double-antibody sandwich type, using monoclonal and/or polyclonal antibodies against a variety of viral proteins in either serum, the blood leukocyte fraction or anticoagulated whole blood; in addition, some test kits can be used to test clarified tissue homogenates or serum. The technique is relatively simple to perform, does not require tissue culture facilities, is suitable for automation and can provide results within half a day. The disadvantage of being less sensitive than virus isolation, especially in adult pigs and mild or subclinical cases may be compensated by testing all pigs of the suspect herd showing pyrexia or clinical signs of disease. However, considering the lowered specificity of these tests other agent detection tests (i.e. virus isolation, RT-PCR or FAT) are recommended.

The test is not suitable for the diagnosis of CSF in a single animal, but should only be used at the herd level.

In any primary case, positive results must be confirmed using another test (i.e. virus isolation, RT-PCR or FAT).

2. Serological tests

Due to the immunosuppressive effect of CSFV, antibodies cannot be detected with certainty until at least 21 days post-infection. Serological investigations aimed at detecting residual foci of infection, especially in breeding herds, may be useful in a terminal phase of CSF eradication. Antibody titres provide valuable epidemiological information and may be of help in determining the entry route of the virus. Antibody detection has become the most important method for evaluating the immune response after CSF vaccination, and can be a useful component of a vaccination programme.

As the incidence of infection with ruminant pestiviruses may be high, particularly in breeding stock, only tests that will discriminate between CSF and BVD/BD antibodies are useful. Virus neutralisation (VN) and the ELISA using MAbs satisfy the requirements for sensitivity, but positive results should be confirmed by comparative VN testing.

Neutralisation tests are performed in cell cultures using a constant-virus/varying-serum method. As CSFV is noncytopathic, any non-neutralised virus must be detected, after multiplication, by an indicator system. The NPLA and the fluorescent antibody virus neutralisation (FAVN) test are the most commonly used techniques. Both tests can be carried out in microtitre plates. The NPLA system is now favoured, being easier to read and having the advantage that the results can be determined by use of an inverted light microscope.

2.1. Neutralising peroxidase-linked assay

The NPLA is carried out in flat-bottomed microtitre plates. For complement inactivation and to lower the risk of bacterial contamination, it is recommended to incubate the sera at 56°C for 30 minutes prior to use in the VN test. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution). For surveillance schemes within a country, a screening dilution of 1/10 (1/20 final dilution) may suffice. Appropriate controls to ensure specificity and sensitivity of reactions are incorporated into each test.

2.1.1. Test procedure

i) Dispense dilutions of serum in growth medium (Eagle's MEM, 5–10% FBS and antibiotics) in 50 µl volumes into duplicate wells of a microtitre plate. The FBS must be free from both ruminant pestiviruses and antibodies to BVDV and other pestiviruses. A third well should be included for each sample. This well contains serum and not virus and is used as a serum control (for cytotoxicity and/or nonspecific staining).

ii) Add 50 µl of virus suspension to the wells, diluted in growth medium to contain approximately 100 $TCID_{50}$ (50% tissue culture infective dose)/50 µl, and mix the contents on a microplate shaker for 20 seconds. A commonly used virus is CSF Alfort 187 (genotype 1.1). Although there is only one CSFV serotype, it is strongly recommended that recent genotypes or field virus isolates circulating in the country or relevant other countries should

also be used as antibody titres can vary depending on the virus genotype used in the assay. Vaccine strains can also be used as recommended viruses, given the safety concerns regarding laboratory operations.

iii) Incubate the plates in a CO_2 incubator in a moist chamber for 1 hour at 37°C.

iv) Add to all wells 50 µl of growth medium containing 2×10^5 PK-15 cells/ml.

v) Back titrate the virus and incubate together with the neutralisation plate.

vi) Allow the cells to grow at 37°C in 5% CO_2 to become confluent, usually within 3–4 days.

vii) Discard the growth medium and rinse the plates once in 0.15 M NaCl or PBS.

viii) Drain the plates by blotting on a towel.

ix) The cell monolayers may be fixed, and the virus inactivated, in one of several ways:

a) The plates are incubated for 45 minutes at 37°C, and then for at least a further 45 minutes at –20°C. The plates are removed from the freezer, the wells are filled with 100 µl 4% paraformaldehyde in PBS and re-incubated for 5–10 minutes at room temperature. The paraformaldehyde is discarded and the plates are rinsed with 0.15 M NaCl;

or

b) The plates are incubated at 70–80°C for 2–3 hours;

or

c) The plates are fixed with 80% acetone and incubating at 70–80°C for 1 hour;

or

d) The plates are fixed in 20% acetone in PBS for 10 minutes followed by thorough drying at 25–30°C for 4 hours. (This can be done quickly with the aid of a hair-dryer: after 3–5 minutes complete dryness is obtained as observed by the whitish colour of the cell monolayer.)

or

e) The plates are washed with ice-cold 99.9% ethanol and fixed with 99.9% ethanol for 45 minutes at 4°C. (Staining should be done immediately.)

x) Add to each well (of a 96-well plate) 50 µl of a hyperimmune porcine CSF antiserum or MAb, diluted in 0.5 M NaCl containing 1% Tween + 0.1% sodium azide, pH 7.6; Tween 20 or Tween 80 can be used. Incubate at 37°C for at least 15–30 minutes. The working dilution of the antiserum should be determined by prior titration: i.e. a serum with an NPLA titre of 1/30,000 could be used at 1/100.

xi) Wash the plates three to five times with 0.15 M NaCl containing 1% Tween, pH 7.6 or PBS containing Tween and once in distilled water.

xii) Add to each well 50 µl of an anti-porcine or anti-murine (as appropriate) IgG-HRPO conjugate, diluted to its working concentration in 0.5 M NaCl with 1% Tween, pH 7.6, and then incubate in the dark for at least 15–30 minutes at 37°C.

xiii) Wash the plates three to five times in 0.15 M NaCl containing 1% Tween, pH 7.6.

xiv) Add 50 µl of chromogen–substrate solution to each well and stain for 15–30 minutes at room temperature. This solution is described in Section B.1.2.2 Immunoperoxidase procedure for differentiation of pestiviruses by MAbs.

xv) Discard the supernatant and wash once with 1/3 PBS/distilled water.

xvi) The test is read visually. Infected cell sheets are completely or partially stained reddish brown in the cytoplasm. The monolayer should be examined by low-power microscopy to determine the end-point of the titration. The cytoplasm of infected cells is stained dark red. Neutralisation titres are expressed as the reciprocal of the highest serum dilution that prevents virus growth in 50% of two replicate wells. The titre can be calculated according to the equation of Karber (1931)

xvii) The following controls are included in the test: cell control, positive serum and back titration of test virus. The virus dilution added to the neutralisation plate undergoes a back-titration, which should cover a range of 4 log dilutions. The back-titration, which acts as an internal quality control, should confirm that virus has been used at a concentration of between 30 and 300 $TCID_{50}$/50 µl. A CSF antibody positive reference serum with known titre needs to be included. If the reference serum does not give the expected result and the back-titration is out of the limit, the test has to be repeated. Reference sera should be monitored over time using internal laboratory tracking charts.

xviii) The back-titration titre is calculated using the method described by Karber (1931).

Note: The incubation times given above are for guidance only. Longer incubation times, with reagent dilutions optimised to such times, may be used, to conserve reagents.

2.2. Fluorescent antibody virus neutralisation test

2.2.1. Leighton tube or other appropriate cell culture flasks or plates method

i) Seed a suspension of PK-15 cells at a concentration of 2×10^5 cells/ml into Leighton tubes with a cover-slip, or other appropriate cell culture flasks or plates.

ii) Incubate the cultures for 1–2 days at 37°C until they reach 70–80% confluency.

iii) Inactivate the complement in the sera by incubation for 30 minutes at 56°C. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution).

iv) Incubate the diluted serum with an equal volume of a virus suspension that contains 200 $TCID_{50}$ of CSFV for 1–2 hours at 37°C; in this way, a constant amount of CSFV of 100 $TCID_{50}$ is used for each reaction well.

v) Discard the medium from the cell cultures, overlay the cell sheet with the serum/virus mixture (from step iv) and incubate for 1 hour at 37°C in a humid atmosphere.

vi) Put an appropriate volume of maintenance medium into the cell cultures for 2–3 more days.

vii) Discard the medium from the cell culture, wash the monolayers twice for 5 minutes each in PBS, pH 7.2, fix in pure acetone or paraformaldehyde for 30 minutes and stain with the working dilution of the conjugate for 30 minutes at 37°C before washing.

viii) If Leighton tubes are used, mount the cover-slips on grease-free microscope slides with 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examine for fluorescence. If a cell culture flask or plate is used, put an appropriate volume of PBS into it to prevent the cells from over-drying before fluorescence examination.

When the FAVN test is performed in microtitre plates, the procedure for the NPLA (see Section B.2.1.1) can be followed up to step ix. The plates are then stained with the working dilution of the conjugate for 30 minutes at 37°C and examined for fluorescence. **Note**: When detecting fluorescence, microplates are best examined from above, using a long focal-length objective and an inverted microscope.

2.3. Comparative neutralisation test for discrimination between infection with CSFV and infection with other pestiviruses

Sera from pigs infected with BVDV, BDV or other pestiviruses may show cross-neutralising antibody titres that react in the FAVN or NPLA and produce false positive results indicating the pigs were infected with CSFV. The extent of cross-reactivity depends on the *Pestivirus* involved and the interval between infection and time of sampling (Wensvoort *et al*., 1989). Comparative tests using one or more strains of CSFV, a strain of BVDV and at least one strain of BDV, that are representative for the country or region, should be used to confirm positive results from serological tests that do not discriminate between infection with CSFV or other pestiviruses. Comparative neutralisation tests are end-point titrations in which the same series of twofold dilutions of the suspected serum sample is tested in duplicate against 100 $TCID_{50}$ of each selected virus strain. The comparative tests are performed according to the protocols described for the FAVN or NPLA; the cell lines used must be suitable for BVDV and BDV. Neutralisation titres are expressed as the reciprocal of the highest serum dilution that prevents virus growth in 50% of two replicate wells. A three-fold difference or more between end-points of two titrations (using two-fold dilution series) should be considered decisive for an infection by the virus species yielding the highest

titre. It may be necessary to use different strains of the same genotype, or to test several pigs from an affected herd to obtain a definitive result.

2.4. Enzyme-linked immunosorbent assay

Several ELISA techniques have been developed that are mainly based on two formats: competitive or blocking ELISA and non-competitive ELISA (e.g. indirect, double-antigen ELISA). The commercial assays primarily detect antibodies against the E2 glycoprotein. Serum or plasma samples that are derived from individual pigs are analysed by the ELISA. A large number of samples can be investigated in a short time, making such assays suitable for surveillance at the herd level. Likewise, ELISAs allow post-vaccination seropositivity to be monitored, although only in countries where the virus is not circulating and vaccination is still being applied. In general, the ELISA should be sensitive enough to score any positive serum sample from convalescent animals (i.e. at least 21 days post-inoculation) that are positive in the neutralisation test and should demonstrate minimal cross-reactivity with BVDV, BDV and other pestiviruses. The test system must ensure the identification of all CSFV infections regardless of the CSFV genotype. If the ELISA procedure is not CSF-specific, then positive samples should be further examined by differential tests to distinguish between CSFV and other pestiviruses.

2.4.1. Antigen

The antigen should be derived from or correspond to viral proteins of one of the recommended CSFV strains. Cells used to prepare antigen must be free from any other *Pestivirus* infection.

2.4.2. Antisera

Polyclonal antisera for competitive or blocking assays should be raised in pigs or rabbits by infection with one of the recommended CSFV strains or with the lapinised C strain. MAbs should be directed against or correspond to an immunodominant viral protein of CSFV. MAbs should be used preferentially. Indirect assays should use an anti-porcine immunoglobulin reagent that detects both IgG and IgM.

The use of marker vaccines depends on a discriminatory test able to distinguish between vaccinated and naturally infected animals. In combination with the E2 subunit vaccine or other marker vaccines that induce CSFV E2-specific antibodies in the absence of a CSFV E^{rns}-specific immune response, an ELISA detecting CSFV E^{rns}-specific antibodies can be used as discriminatory tests. However, commercially available E^{rns}-specific ELISAs are less sensitive and specific than conventional CSF E2 antibody ELISAs. It is recommended to use such discriminatory tests on a herd basis and not for diagnostic analysis on samples of single animals (European Commission, 2003; Meyer *et al.,* 2017; Pannhorst *et al.,* 2015).

More information on commercial kits for diagnosis can be obtained from the WOAH Reference Laboratories. Even though commercial test kits may have been thoroughly validated before licensing, each lab must perform batch control with selected (positive and negative) reference sera prior to use. This must also be applied with regard to the use of in-house ELISAs.

C. REQUIREMENTS FOR VACCINES

1. Background

CSF has severe clinical and socio-economic consequences for pig production worldwide. The control of the disease is usually a national responsibility, and in many countries vaccination is carried out as part of a national control programme under the auspices of the veterinary authority.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements. Varying additional requirements relating to quality, safety and efficacy will apply in particular countries or regions for manufacturers to obtain an authorisation or licence for a veterinary vaccine.

Wherever live CSFV is handled, the appropriate biosecurity procedures and practices should be used. The CSF vaccine production facility should meet the requirements for containment outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

The optimal CSF vaccine should have the following general characteristics: short- and long-term safety for target and non-target species (especially for oral vaccines), stability, rapid induction of a stable, preferably life-long immunity, efficacy against all strains and genotypes of field viruses, full clinical protection and protection against carrier states, prevention of horizontal and vertical transmission. Furthermore, marker vaccines will have to be accompanied by reliable discriminatory tests. Manufacture should provide for consistency of production and validation.

Modified live vaccines (MLVs) based on several attenuated virus strains (e.g. C-strain, Thiverval, PAV-250, GPE-, K-strain) are most widely used, and many of them have proven to be both safe and efficacious. In addition, E2 subunit vaccines produced in baculovirus or other systems are available. Inactivated whole virus vaccines are presently not available.

Information regarding these vaccines can be found in review publications (Blome *et al.,* 2017b; Ganges *et al.,* 2020; Postel *et al.,* 2018).

Early CSF marker vaccines were based on recombinant CSFV glycoprotein E2 expressed in insect cells. Pigs vaccinated with these subunit vaccines do not produce antibodies against other CSFV proteins (e.g. E^{rns}), allowing reliable differentiation between vaccinated and infected animals (DIVA). However, compared with animals vaccinated with live vaccines, two major disadvantages of these subunit vaccines were a significantly delayed onset of immunity and two inoculations were required. While a single administration of the E2 subunit vaccine was able to prevent clinical signs and mortality, along with reduced transmission following challenge infection, it did not prevent transplacental transmission.

New generations of marker vaccines are also being developed and a chimeric pestivirus encompassing the E2 from CSFV in a ruminant pestivirus backbone has been granted regulatory approval by the European Medicines Agency (EMA) and has also been authorised for use in a USA vaccine bank. This vaccine, CP7_E2alf, possesses many characteristics of an optimal vaccine including: genetic stability; innocuousness for target and relevant non-target species; absence of vaccine virus transmission to in-contact animals or shedding through urine, faeces or semen; rapid onset of protection following a single intramuscular injection; duration of immunity for at least 6 months; and protection against different CSFV genotypes (Blome *et al.,* 2017a). The CP7_E2alf vaccine capacity to prevent transplacental transmission after a single vaccine dose has been proven in pregnant sows against a moderately virulent CSFV strain (Henke *et al.,* 2018). The protection from transplacental transmission against CSFV highly virulent strains remains to be evaluated.

Different DIVA strategies are available by using serological methods (e.g. ELISA) or genome detection methods (e.g. RT-PCR). A opinion published by the European Food Safety Authority (EFSA, 2008) demonstrated that the combination of a vaccine that uses the C-strain with RT-PCR to detect viral genome in slaughtered animals can be successfully used in a vaccination-to-live strategy (Zhao *et al*, 2008). See also Section B.1.1.4 of this chapter.

For the approved chimeric vaccine CP7_E2alf, the use of a ruminant pestivirus backbone provides serological differentiation by use of CSFV E^{rns}-ELISAs. However, evaluation of two such assays for DIVA capability revealed that test specificity may be compromised by infection with ruminant pestiviruses, resulting in induction of cross-reactive antibodies (Meyer *et al.,* 2017; 2018; Pannhorst *et al.,* 2015).

There is therefore still room for improvement with respect to marker vaccines and their companion diagnostic tests. Information regarding the current state-of-the-art with respect to CSFV vaccine candidates was reviewed by Blome *et al*., 2017b and Ganges *et al.,* 2020.

CSF vaccines are used in different epidemiological settings and situations. Most countries free of the disease have adopted a control strategy without prophylactic vaccination but established legal provisions for emergency vaccination scenarios. In endemic situations, vaccination is mainly used to lower the impact of the disease or as a first step in an eradication programme. During epidemic incidents in previously free areas, emergency vaccination can be an additional tool to control and eradicate the disease and DIVA vaccines promise a valuable additional tool in this context.

Moreover, oral vaccination of affected wild boar populations may be considered. These different scenarios and the different systems of pig production may require different vaccine characteristics or may influence the focus of requirements.

2. Outline of production and minimum requirements for live vaccines

2.1. Characteristics of the seed

CSF vaccines prepared in live animals do not follow WOAH animal welfare principles. Their production and use should be discontinued.

2.1.1. Biological characteristics of the master seed

MLVs are produced from CSFV strains that have been attenuated by passage either in cell cultures or in a suitable host species not belonging to the family *Suidae*. Production is carried out in cell cultures, based on a seed-lot system.

Master seed viruses (MSVs) for MLVs should be selected and produced, based on their ease of growth in cell culture, virus yield and stability.

The exact source of the underlying CSFV isolate, its sequence, and the passage history must be recorded.

2.1.2. Quality criteria

Only MSVs that have been established as sterile, pure (free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and those listed by the appropriate licensing authorities) and immunogenic, should be used for vaccine virus (working seed viruses and vaccine batches) production. Live vaccines must be shown not to cause disease or other adverse effects in target animals injected in accordance with chapter 1.1.8 (section on *Safety tests* [for live attenuated MSVs]).

Identity of the MSV has to be confirmed using appropriate methods (e.g. through the use of specific MAbs or vaccine strain-specific genome detection methods).

2.1.3. Validation as vaccine strain

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy.

Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy (TSE) agents, consideration should also be given to minimising the risk of transmission by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

The vaccine virus in the final product should generally not differ by more than five passages from the master seed lot. The commercial vaccine should be produced in batches in lyophilised form as a homogeneous product.

2.2. Method of manufacture

2.2.1. Procedure

The virus is used to infect an established cell line. Such cell culture should be proven to be free from contaminating microorganisms and shall comply with the requirements in chapter 1.1.8.

Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze–thaw cycles). The harvest can be further processed by filtration and other methods. A stabiliser may be added as appropriate. The vaccine is homogenised before lyophilisation to ensure a uniform batch/serial.

2.2.2. Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

2.2.3. In-process controls

In-process controls will depend on the protocol of production: they include virus titration of bulk antigen and sterility tests.

2.2.4. Final product batch/serial test

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Appropriate methods (specific antibodies or specific genome detection methods) should be used for confirmation of the identity of the vaccine virus.

iii) Residual moisture

The level of moisture contained in desiccated products should be measured as described in chapter 1.1.8.

iv) Safety

Batch safety testing is to be performed unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions.

For batch/serial safety testing, use two healthy piglets, 6–10 weeks old, that do not have antibodies against pestiviruses. Administer to each piglet by a recommended route a tenfold dose of the vaccine. Observe the piglets daily for at least 14 days. The vaccine complies with the test if no piglet shows notable signs of disease or dies from causes attributable to the vaccine.

v) Batch/ serial potency

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in pigs and titre of the modified live vaccine *in vitro*.

In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (see Section C.2.3.3).

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For regulatory approval of a vaccine, all relevant details concerning preparation of MSV, manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches originating from the same MSV, with a volume not less than 1/3 of the typical industrial batch volume.

The in-process controls are part of the manufacturing process.

2.3.2. Safety requirements

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily.

Vaccines should be tested for any pathogenic effects on healthy pigs, and in sows to evaluate the safety in pregnant animals and their offspring.

i) Safety in young animals

Carry out the test for each recommended route of application using in each case piglets not older than the minimum age recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Use no fewer than eight piglets of 6–8 weeks of age that do not have antibodies against pestiviruses. Administer to each piglet a quantity of the vaccine virus equivalent to not less than ten times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the piglets daily for at least 14 days. The body temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the vaccine, at the time of administration, 4 hours after and then daily for at least 14 days. The vaccine complies with the test if the average body temperature increase for all piglets does not exceed 1.50°C, no piglet shows a temperature rise greater than 1.50°C for a period exceeding 3 consecutive days, and no piglet shows notable signs of disease or dies from causes attributable to the vaccine.

Blood samples are taken at 7 days after vaccination and tested for leukopenia. The average white blood cell (WBC) count should exceed 7×10^6 cells/ml.

In addition, the vaccines in their commercial presentation should be tested for safety in the field (see chapter 1.1.8, section on *Field tests* [*safety and efficacy*]).

ii) Safety test in pregnant sows and test for transplacental transmission

Carry out the test with vaccination by a recommended route using no fewer than eight healthy sows or gilts of the same age and origin, between the 55th and 70th days of gestation, that do not have antibodies against pestiviruses. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each sow a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Clinical observation of animals is carried out daily until farrowing. Blood samples should be taken from newborn piglets before ingestion of colostrum.

The test is invalid if the vaccinated sows do not seroconvert before farrowing. The vaccine virus complies with the test if no abnormalities in the gestation or in the piglets are noted. No sow or gilt shows notable signs of disease or dies from causes attributable to the vaccine.

Vaccine virus or antibodies against CSFV must not be present in blood samples from newborn piglets.

iii) Non-transmissibility

Keep together for the test no fewer than 12 healthy piglets, 6–10 weeks old and of the same origin, that do not have antibodies against pestiviruses. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Administer by a recommended route to no fewer than six piglets a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine.

Maintain no fewer than six piglets as contact controls. The mixing of vaccinated piglets and contact piglets is done 24 hours after vaccination.

After 45 days, kill all piglets humanely. Carry out appropriate tests on the piglets to detect antibodies against CSFV and on the control piglets to detect CSFV in the tonsils. The vaccine complies with the test if antibodies are found in all vaccinated piglets and if no antibodies and no virus are found in the control piglets.

iv) Reversion-to-virulence

The test for increase in virulence consists of the administration of the vaccine virus from the master seed lot or one or two passages above to piglets that do not have antibodies against pestiviruses.

This protocol is repeated five times. Administer to each of two healthy piglets free of antibodies to pestiviruses, 6–10 weeks old, by a recommended route, a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Collect an appropriate quantity of blood from each piglet daily between day 2 and day 7 after administration of the vaccine virus, and pool the samples taken on the same day. Then kill the piglets and take the tonsils of both of them, pool the tonsils and prepare a 10% suspension in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage. At the same time, the presence of CSF antigens is confirmed at each passage. Blood and pooled tonsillar tissue are used to inoculate two further pigs of the same age and origin by the same route as before.

Administer 2 ml of the pooled material (blood and tonsillar tissue) with the highest virus titre by a recommended route to each of two other piglets of the same age and origin. If no virus is found, repeat the administration once again with the same material and another two piglets. If no virus is found at this point, end the process here. If, however, virus is found, carry out a second series of passages by administering 2 ml of positive material by a recommended route to each of two other piglets of the same age and origin.

Carry out this passage operation no fewer than four times (in total five groups from the start of the test should be vaccinated), verifying the presence of the virus at each passage in blood and tonsils. Care must be taken to avoid contamination by virus from previous passages.

The vaccine virus complies with the test if no indication of increasing virulence (monitored by clinical observations) of the maximally passaged virus compared with the unpassaged virus is observed.

If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2.3.3. Efficacy requirements

i) Protective dose

Vaccine efficacy is estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge and is expressed by the number of 50% protective doses (PD_{50}) for pigs contained in the vaccine dose.

The test consists of a vaccination/challenge trial in piglets aged 6–10 weeks using different dilutions of the vaccine in question and five piglets per dilution. An additional group of two piglets of the same age and origin are used as controls. All animals have to be free from antibodies against pestiviruses prior to the trial. Each group of piglets, except the control group, is vaccinated with an appropriate dilution of the reconstituted vaccine (e.g. 1/40 and 1/160 using a suitable buffer solution.

Fourteen days after the single injection of vaccine, challenge the piglets by a suitable route with a dose of a virulent strain of CSFV that kills at least 50% of the non-vaccinated piglets in less than 21 days. Observe the piglets for 21 days and record the body temperature 3 days before challenge and daily after challenge for 21 days. The PD_{50} content of the vaccine is calculated from the number of animals protected in each group using the Spearman-Kärber method.

The test is invalid if less than 50% of the control piglets display typical signs of serious infection with CSFV, and die, and if less than 100% of the control piglets show clinical signs of disease within the 21 days following challenge.

The vaccine complies with the test if the minimum dose corresponds to not less than 100 PD_{50}.

In addition, protection from virus replication conferred by the vaccine should be evaluated in the blood and tissues from vaccinated animals using the protocols previously described in Sections B.1.1.3 and B.1.1.4.

ii) Protection against transplacental infection

Use eight sows that do not have antibodies against pestiviruses, randomly allocated to either the vaccine group (n = 6) or the control group (n = 2).

Between the 34th and 49th day of gestation, all sows allocated to the vaccine group are vaccinated once with 1 dose of vaccine containing not more than the minimum titre stated on the label. Three weeks after vaccination, all eight sows are challenged by a suitable route with a dose of virulent strain of CSFV that would be sufficient to kill at least 50% of non-vaccinated piglets in less than 21 days.

Just before farrowing, the sows are killed humanely and their fetuses are examined for CSFV. Serum samples from sows and fetuses are tested for the presence of antibodies against CSFV. Isolation of CSFV is carried out from blood of the sows (collected 7 and 9 days after challenge and at euthanasia), and from homogenised organ material (tonsils, spleen, kidneys, lymph nodes) of the fetuses.

The real-time RT-PCR assay can be performed following the methodology described in Section B.1.1.4., although always in correlation with the virus isolation test. The test is valid if virus is found in at least 50% of the fetuses from the control sows (excluding mummified fetuses).

The vaccine complies with the test if no virus is found in the blood of vaccinated sows and in fetuses from the vaccinated sows, and antibodies against CSFV should not be found in the serum of the fetuses from the vaccinated sows.

In addition, where appropriate, the vaccines should be tested for efficacy in the field (see chapter 1.1.8, section on *Field tests* [*safety and efficacy*]).

2.3.4. Duration of immunity

As part of the authorisation procedure the manufacturer should demonstrate the duration of immunity of a given vaccine by either challenge or the use of a validated alternative test, at the end of the claimed period of protection.

At least ten vaccinated pigs are each inoculated with an amount of virus corresponding to 10^5 PID_{50} (median pig infectious dose) of a virulent strain of CSFV and observed for 3 weeks. The vaccinated animals have to remain healthy, only the controls should die.

The duration of immunity after vaccination against CSF shall not be less than 6 months.

2.3.5. Stability

The stability of all vaccines should be demonstrated as part of the shelf-life determination studies for authorisation.

The period of validity of lyophilised CSF vaccine should be shown to be at least 1 year.

3. Requirements for other vaccines

3.1. Oral vaccine

3.1.1. Background

The most widely applied concept of oral bait vaccination of wild boar against CSF, including bait design and immunisation scheme was developed, evaluated, and optimised by Kaden *et al.* (2010). The respective vaccines are conventional MLVs. Immunisation occurs by uptake of the

oral vaccine through the lymphoid tissues of the oral mucosa and tonsils, where expression of virus stimulates the immune system (Kaden *et al.,* 2000; 2010).

Safety is of paramount consideration for oral vaccine use, not only for the target animals, but for the environment (see chapter 1.1.8) and other species that may come in contact with the vaccine.

3.1.2. Outline of production and minimum requirements for vaccines

In addition to the outline of production described for injectable vaccines above, the following specific requirements must be met:

i) Method of manufacture

a) Final product batch/serial test

After combining all of the ingredients, the final blend contains the definitive formulation that is usually used in liquid form. The last step in production of a batch/serial is filling the final blend into blisters/capsules to be included in baits or filling directly into the bait. This final batch/serial is tested as described for the injectable vaccines, with the following differences:

- Residual moisture test

The residual moisture test does not apply if the oral vaccine is presented in liquid form.

- Safety

Administer orally by syringe to each piglet a volume corresponding to ten oral doses as indicated by the manufacturer.

ii) Requirements for authorisation/registration/licensing

In addition to the requirements described for injectable vaccines, the following specific requirements must be met.

a) The bait

The bait is an integral part of the product and should ideally meet the following criteria:

- Designed for and attractive to the target species and adapted to the mode of distribution.
- Keep its form and shape under a wide range of temperature and weather conditions.
- Ingredients are non-harmful, comply with animal feed standards and should not interfere with vaccine activity.
- Feature a labelling system with a public warning and identification of the product.

b) Safety requirements

For all the tests the liquid vaccine is administered orally with a syringe (not in the final bait formulation) to ensure that each animal receives the full dose.

- Precaution hazards

The release of oral vaccines in the environment shall comply with the requirements in chapter 1.1.8.

c) Efficacy requirements

Efficacy should be proven using the liquid vaccine administered by syringe to ensure that each animal receives the full dose. Proof-of-concept studies for the final formulation (vaccine integrated into bait) should be provided.

3.2. Recombinant E2 glycoprotein-based vaccines

3.2.1. Background

As described in Guideline 3.3 Section E, conventional, live attenuated CSF vaccines have a rapid onset of immunity and are effective at preventing transmission of infection (Ganges *et al.,* 2020; Postel *et al.,* 2018), but have the disadvantage that it is not possible using serological methods (e.g. ELISA) to differentiate infected pigs from those that have merely been vaccinated. Commercial E2 subunit vaccines (Marker vaccine) have a slower onset of immunity and reduce, but may not completely prevent, viral shedding and transplacental infection. However, these vaccines enable a DIVA strategy to be followed thereby facilitating a 'vaccination to live' strategy.

The vaccine only elicits antibodies against the E2 glycoprotein and therefore antibodies against other CSFV antigens, such as the E^{RNS} antigen, can be used as markers of infection. New E2-based formulations, with improved efficacy and/or produced in non-baculovirus based expression systems have been (Suarez *et al.,* 2017), or are being developed (Blome *et al*., 2017b; Gong *et al.,* 2019*;* Ganges *et al.,* 2020).

3.2.2. Outline of production

i) Characteristics of the seed

E2 subunit marker vaccine is prepared by the use of *Baculovirus* expressing the E2 antigen of CSFV. The vaccine therefore does not contain any CSFV while the baculo (vector) virus is chemically inactivated.

a) Biological characteristic of the master seed

Production is carried out in insect cell cultures, based on a seed-lot system.

Selection of MSVs should ideally be based on their ease of growth in cell culture, virus yield and stability.

The exact source of the isolate including its sequence and passage history should be recorded.

b) Quality criteria

Only MSVs that have been established as sterile and pure (free of extraneous agents as described in chapter 1.1.9 and those listed by the appropriate licensing authorities), and immunogenic, shall be used for preparing the vaccine virus production.

Appropriate methods (specific antibodies or specific genome detection methods) should be used for confirmation of the identity of the MSV.

c) Validation as vaccine strain

The vaccine prepared from the MSV is shown to be satisfactory with respect to safety and efficacy for the swine for which it is intended.

In accordance with chapter 1.1.8, consideration should also be given to minimising the risk of transmission of TSE agents by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

The vaccine virus used to produce the final product should not differ by more than five passages from the material used for validating the seed lot. The commercial vaccine is inactivated for residual baculovirus and adjuvanted.

ii) Method of manufacture

a) Procedure

The baculovirus is used to infect an established insect cell line. Such cell culture should be proven to be free from contaminating microorganisms and shall comply with requirements in chapter 1.1.8.

Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus. The harvest can be further processed by filtration and other methods. Inactivation of residual baculovirus is performed, preferably using a first order inactivant. The antigen is homogenised before formulation with adjuvant.

b) Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

c) In-process controls

Infectivity, sterility and antigenic mass are monitored. After inactivation a test for innocuity is carried out on every batch of antigen. The cells used to test for absence for residual live baculovirus are the same cell line used for production or potentially equally or more sensitive cells.

d) Final product batch/serial test

- Sterility

Must comply with chapter 1.1.8.

- Identity

The identity test is performed by a specific MAb-based virus neutralisation against CSFV or an appropriate molecular identification. Sera prepared to be used for identity testing should not be prepared using the homologous vaccine virus or baculovirus expressed subunit antigen but from another source. This test may be combined with the potency test (see below).

- Safety and prove of marker concept

Batch safety testing is to be performed unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions.

For batch/serial safety testing, use two healthy piglets, 6–10 weeks old, that do not have antibodies against pestiviruses. Administer to each piglet by a recommended route a double dose of the formulated vaccine. Observe the piglets daily for at least 14 days for local and systems reactions to vaccination. After 14 days they are each injected with a second single dose of vaccine.

Any adverse reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. The vaccine should elicit antibodies against CSFV E2 but not against CSFV-E^{RNS} antigen.

- Batch/serial potency

Induction of specific anti-E2 antibodies in vaccinated pigs can be used to confirm the potency of each batch once the titre has been correlated with the results of the efficacy test.

iii) Requirements for authorisation /registration/ licensing

a) Manufacturing process

See Section C.2.3.1.

b) Identity

The identity test is performed by virus neutralisation using immune sera against CSFV. Sera prepared to be used for identity testing should not be prepared using the homologous vaccine virus or baculovirus expressed subunit antigen but from another source.

c) Safety requirements

- Safety in young animals

For the purposes of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration in eight piglets of 6–8 weeks of age. Single-dose and repeat-dose tests using vaccines formulated to contain the maximum permitted payload should be conducted. The repeat dose test should correspond to the primary vaccination schedule (e.g. two injections) plus the first revaccination (i.e. a total of three injections). The animals are observed for local and systemic reaction to vaccination for no fewer than 14 days after each injection. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine. It has to be proven that the vaccine does not elicit antibodies against CSFV-E^{RNS} antigen.

- Safety in pregnant sows

For the purpose of gaining regulatory approval a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration corresponding to the primary vaccination schedule (e.g. two injections) in eight pregnant sows. The sows are observed for local and systemic reactions to vaccination. The observation period must last until parturition to examine any harmful effects during gestation or on progeny. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine. It has to be proven that the vaccine does not elicit antibodies against CSFV-E^{RNS} antigen.

d) Efficacy requirements

- Protective dose

Vaccine efficacy is estimated in animals vaccinated according to the manufacturer's recommendation, following the methods described in Section C.2.3.3.

- Protection against transplacental infection

The trial vaccine should comply with the test described in Section C.2.3.3.

e) Duration of immunity

As part of the authorisation procedure the manufacturer should demonstrate the duration of immunity (see Section C.2.3.4).

f) Stability

The stability of all vaccines should be demonstrated as part of the shelf-life determination studies for authorisation. The period of validity of a batch of biotechnology-based CSF vaccine should be shown to be at least 1 year (see Section C.2.3.5).

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NB: There are WOAH Reference Laboratories for classical swine fever
(please consult the WOAH web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for classical swine fever

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.9.4.

NIPAH VIRUS ENCEPHALITIS

See Chapter 3.1.15. Nipah and Hendra virus diseases.

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CHAPTER 3.9.5.

PORCINE CYSTICERCOSIS (INFECTION WITH *TAENIA SOLIUM*)

See Chapter 3.10.3. Cysticercosis (*including infection with* Taenia solium)*.*

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CHAPTER 3.9.6.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (INFECTION WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS)

SUMMARY

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory problems of piglets and growing pigs. The disease is caused by the PRRS virus (PRRSV), a virus currently classified as a member of the order Nidovirales, suborder Arnidovirineae, family Arteriviridae, subfamily Variarterivirinae, genus Betaarterivirus. The primary target cells of the virus are differentiated macrophages of the pig, mainly pulmonary alveolar macrophages and pulmonary intravascular macrophages, but the virus may also replicate in monocyte-derived macrophages in lymphoid tissues and, to a lesser extent, in dendritic cells and monocyte-derived macrophages residing in most organs in perivascular space. There is an increasing body of evidence that highly pathogenic strains of PRRS virus are also able to infect endothelial cells in lung, heart and brain. This attribute is thought to be associated with higher pathogenicity and may also be of value in characterising new strains.

According to the newest classification, previous genotypes are now considered to be two distinct species named Betaarterivirus suid 1 and Betaarterivirus suid 2, classified within two different subgenera, Eurpobartevirus *and* Ampobartevirus, respectively. *In the present chapter, commonly accepted and recognised conventional names (PRRSV-1 and PRRSV-2) will be used to denote two PRRSV species, respectively. Historically, PRRSV-1 (previously described as genotype 1, Type 1 or European – EU) was restricted to Europe and PRRSV-2 (previously genotype 2, Type 2 or North American – NA) to North America; currently they are distributed globally. The virus is primarily transmitted via direct contact but also by contact with faeces, urine, semen and fomites. The possibility of insect vectors (houseflies and mosquitos)* and *aerogenic spread for short distances has also been confirmed through this route is considered to be of minor importance. PRRS occurs in most major pig-producing areas throughout the world. The reproductive failure is characterised by infertility, late fetal mummification, abortions, stillbirths, and the birth of weak piglets that often die soon after birth from respiratory disease and secondary infections. Older pigs may demonstrate mild signs of respiratory disease, usually complicated by secondary infections. In 2006, a highly pathogenic PRRSV strain emerged in China (People's Rep. of) causing high fever (40–42°C) in all age groups, abortions in sows and high mortality in sucking piglets, weaners and growers.*

Detection of the agent: *Virological diagnosis of PRRSV infection is difficult; the virus can be isolated from serum or organ samples such as lungs, tonsils, lymph nodes and spleen of affected pigs. As porcine alveolar macrophages are one of the most susceptible culture systems for virus of both species, these cells are recommended for virus isolation. Recent findings show that porcine monocyte-derived macrophages can also be used for PRRSV isolation and propagation in culture. MARC-145 (MA-104 clone) cells are suitable for isolation of PRRSV-2. There is variability between batches of macrophages in their susceptibility to PRRSV. Thus, it is necessary to identify a batch with high susceptibility, and maintain this stock in liquid nitrogen until required. The virus is identified and characterised by immunostaining with specific antisera or monoclonal antibody. Additional techniques, such as immunohistochemistry and* in-situ *hybridisation on fixed tissues, reverse-transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR, have been developed for laboratory confirmation of PRRSV infection.*

Serological tests: *A wide range of serological tests is currently available for the detection of serum, oral fluid and meat juice antibodies to PRRSV. The immunoperoxidase monolayer assay and immunofluorescence assay using alveolar macrophages or MARC-145 cells can be used for the detection of antibodies specific to PRRSV-1 or PRRSV-2. Commercial or in-house enzyme linked immunosorbent assays (ELISA) are now most often used for PRRSV diagnosis. An indirect ELISA and a blocking ELISA have been described as well as a double blocking ELISA, using antigen from both PRRSV-1 and PRRSV-2, that can distinguish between serological reactions to the two species. Commercial ELISAs specifically designed for detection of PRRSV seroconversion in oral fluid also exist.*

Requirements for vaccines: *Vaccines can be of value as an aid in the prevention or control of reproductive and respiratory forms of PRRS. Vaccination with modified live virus may result in shedding of vaccinal virus in semen and vertical and horizontal transmission between sows and piglets and between vaccinated and non-vaccinated pigs. Subsequent vaccine-virus-induced adverse signs have been reported. Modified live virus vaccines can persist in vaccinated herds. Whole virus inactivated vaccines are also available.*

A. INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory disease in pigs (as reviewed by Zimmerman *et al.,* 2019). PRRS was first recognised in 1987 in the United States of America, in 1989 in Japan and in 1990 in Germany. Within a few years it became a pandemic. The disease is caused by the PRRS virus (PRRSV), discovered in 1991 in The Netherlands and in 1992 in USA (Zimmerman *et al.,* 2019). PRRSV is a single-stranded positive-sense RNA virus and the biology of the virus has been well characterised. Apart from domestic pigs, feral swine and wild boars, no other species are known to be naturally infected with PRRSV. The virus does not pose a zoonotic risk and it is not infectious for humans or for cells of human origin. Soon after the discovery of the virus it became apparent that the European and North American isolates represent two antigenically distinct groups, initially recognised as two genotypes (Zimmerman *et al.,* 2019). According to the newest, revised classification of the International Committee on Taxonomy of Viruses (ICTV, Virus Taxonomy: 2019 Release), previous genotypes are now considered to constitute two distinct species named Betaarterivirus suid 1 and Betaarterivirus suid 2 and classified within two different subgenuses *Eurpobartevirus* and *Ampobartevirus* in the genus *Betaarterivirus* (subfamily Variarterivirinae, Family Arteriviridae, Suborder Arnidovirinae, Order Nidovirales). In the present chapter, commonly accepted and recognised conventional names (PRRSV-1 and PRRSV-2) will be used to denote two PRRSV species. Additional investigations have demonstrated regional differences within each continent. These differences are now becoming blurred as PRRSV-2 has been introduced into Europe and PRRSV-1 virus has been discovered in North America. Most PRRSV isolates from South America and much of Asia are of species 2 and it is assumed these viruses were introduced through the movement of swine or semen. Most highly virulent PRRSV-2 viruses in South-East Asia (highly pathogenic PRRSV) are characterised by amino acid deletions in the NSP2 region of the genome. However, there is good experimental evidence that these deletions do not determine virulence (Shi *et al*., 2010a; Zhou *et al.,* 2009; Zhou & Yang, 2010).

There is an increasing diversity among strains of the two species, which has been attributed to the high error rate inherent in PRRSV replication and recombinations between strains (Murtaugh *et al*., 2010). There have also been descriptions of east European strains of PRRSV-1 with a high degree of polymorphism, providing further insights into the emergence of the relatively new pathogen of pigs. This polymorphism has been proposed to distinguish subtypes 1, 2 and 3 within PRRSV-1. Moreover, mounting evidence indicates that an additional subtype (subtype 4) might exist (Stadejek *et al.,* 2008; 2013). The effects of such diversity on diagnostics and vaccines are largely unknown, but do raise concerns and should be considered. Subtype 3 (Lena, SU1-bel) and subtype 2 Bor strains have been shown to have higher virulence than subtype 1 strains (Karniychuk *et al*., 2010; Morgan *et al.,* 2013; Stadejek *et al*., 2017). Trus *et al.* (2014) showed that subtype 1 modified live vaccine partially protects against challenge with subtype 3 Lena strain. Although nine different genetic lineages have been identified in PRRSV-2, the overall level of diversity within species 2 does not exceed that observed for subtype 1 of PRRSV-1 (Shi *et al*., 2010b; Stadejek *et al*., 2013).

The reproductive syndrome is recognised by late-gestation abortions and early or delayed farrowings that contain dead and mummified fetuses, stillborn pigs, and weak-born pigs. An increase in repeat breeders during the acute phase of the epizootic is commonly reported. Infrequently, there are reports of early- to mid-gestation reproductive failure. Most probably the cause of PRRSV-related reproductive disorders is virus-induced damage to the placenta and endometrium (Karniychuk & Nauwynck, 2013). In boars and unbred replacement gilts and sows, transient fever

and anorexia may be observed. The respiratory syndrome is recognised by dyspnoea ("thumping"), fever, anorexia, and listlessness. Younger pigs are more affected than older animals with boars and sows (unbred) frequently having subclinical infection. An increase in secondary infections is common and mortality can be high. In PRRSV-infected boars and boars that have been vaccinated with live attenuated vaccine, PRRSV can be shed in semen, and changes in sperm morphology and function have been described (Christopher-Hennings *et al.,* 1997). The virus is primarily transmitted directly via contact with infected pigs but also with faeces, urine and semen. It can also be spread by insects (houseflies and mosquitoes) and indirectly, presumably via aerosol routes, leading to chronic re-infections of herds in swine dense areas, and possibly by mechanical vectors. Gross and microscopic lesions consistent with PRRSV infection have been well described (Zimmerman *et al.,* 2019). In general, the lesions are more severe in younger animals than older ones. Differences in virulence between PRRSV isolates within a genotype and between genotypes were proved to exist based on field observations and experimental studies (Karniychuk *et al*., 2010; Stadejek *et al*. 2017; Weesendorp *et al*., 2013). This variability has been reinforced with the emergence in 2006 of a PRRSV lineage in South-East Asia associated with porcine high fever disease, a syndrome causing high mortality in all ages of swine (Tian *et al*., 2007). Although there is now an extensive body of research completed since the discovery of PRRSV, there are still many gaps in the knowledge base about the apparent link between PRRSV and other diseases as well as understanding the PRRSV immune response.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of porcine reproductive and respiratory syndrome and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Virus isolation	–	++	–	+++	–	–
Conventional RT-PCR	+++	+++	+++	+++	++	–
Real-time RT-PCR	+++	+++	+++	+++	++	–
IHC	–	–	–	++	–	–
ISH	–	–	–	++	–	–
Detection of immune response[b]						
ELISA	+++	++	+++	++	+++	++
IPMA	++	++	++	+	++	+++
IFA	++	++	++	+	++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; IHC = immunohistochemistry method;
ISH = *in-situ* hybridisation; ELISA = enzyme-linked immunosorbent assay; IPMA = immunoperoxidase monolayer assay,
IFA = immunofluorescence assay.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]One of the listed serological tests is sufficient.

1. Detection of the agent

Identification of PRRSV can be accomplished by virus isolation, the detection of nucleic acids, and the detection of viral proteins. Following infection, swine develop a viraemia and lung infection that can persist for weeks in young pigs and days in adult animals making serum and bronchoalveolar lung lavage ideal samples to collect for detection of PRRSV.

1.1. Virus isolation

Isolation of PRRSV can be difficult as not all virus isolates (especially PRRSV-1 viruses) can easily infect MARC-145 cells and CL-2621, clones derived from the MA-104 monkey kidney cell line (Provost *et al*., 2012; Zimmerman *et al.,* 2019). Recent findings show that porcine monocyte-derived macrophages can also be used for PRRSV isolation and propagation in cell culture (Garcia-Nicolas *et al*., 2014). These can be differentiated *in vitro* from porcine peripheral blood mononucleated cells (PBMCs) without slaughtering animals, as opposed to collection of the lung for porcine alveolar macrophage (PAM) preparations. Moreover, several genetically modified cell lines supporting PRRSV replication have been developed including immortalised PAM cell line expressing CD163, immortalised porcine monomyeloid cells, PK-15 expressing CD163 and sialoadhesin as well as porcine, feline and baby hamster kidney cells expressing CD163 (Delrue *et al*., 2010; Provost *et al*., 2012). Other, non-recombinant cell lines permissive for PRRSV infection have also been described (Feng *et al*., 2013; Provost *et al*., 2012). PAM will support replication of most, if not all PRRSV isolates. However, the collection of PAM is not an easy task as only pigs of high health status and less than 8 weeks of age should be used as the PAM source (Feng *et al*., 2013). Different batches of PAM are not always equally susceptible to PRRSV; it is thus necessary to test each batch before use. PAM can be stored in liquid nitrogen until needed as described below. Isolation of PRRSV using PAM is a technique that can be performed in most diagnostic laboratories. This technique should be sensitive for isolation of all PRRSV strains and will be explained in detail. Samples for virus isolation should be refrigerated at 4°C immediately after collection and shipped to the laboratory within 24–48 hours. The half-life of the virus in serum at this temperature was estimated as 155 hours. However, infectivity is rapidly lost outside of pH 6.5–7.5 range (Zimmerman *et al*., 2019). For longer storage freezing at –70°C is recommended.

1.1.1. Harvesting of alveolar macrophages from lungs

Lungs should preferably be obtained from specific pathogen free pigs or from a herd of pigs that is proven to be free from PRRSV infection. Best results are obtained with pigs that are under 8 weeks of age. The macrophages should be harvested from the lung on the same day that the pig is slaughtered. The lungs should be washed three or four times with a total volume of approximately 200 ml sterile phosphate-buffered saline (PBS). The harvested wash fluid is then centrifuged for 10 minutes at 300 ***g***. The resulting pellet of macrophages is resuspended in PBS and centrifuged (washed) twice more. The final pellet is resuspended in 50 ml PBS, and the number of macrophages is counted to determine the cell concentration. The macrophages can then be used fresh, or can be stored in liquid nitrogen according to standard procedures at a final concentration of approximately 6×10^7 macrophages/1.5 ml vial. Macrophage batches should not be mixed.

1.1.2 Batch testing of alveolar macrophages

Before a batch of macrophages can be used it should be validated. This should be done by titrating a standard PRRSV with known titre in the new macrophages, and by performing an immunoperoxidase monolayer assay (IPMA) with known positive and negative sera on plates seeded with the new macrophages. The cells are suitable for use only if the standard PRRSV grows to its specified titre, ($TCID_{50}$ or 50% tissue culture infective dose). Alveolar macrophages and fetal bovine serum (FBS) to supplement culture medium must be pestivirus free.

1.1.3 Virus isolation/titration on alveolar macrophages

Alveolar macrophages are seeded in the wells of flat-bottomed tissue-culture grade microtitre plates. After attachment, the macrophages are infected with the sample. Samples can be sera or 10% suspensions of tissues, such as tonsils, lung, lymph nodes, and spleen. In general, the PRRSV gives a cytopathic effect (CPE) in macrophages after 1–2 days of culture, but sometimes viruses are found that give little CPE or give a CPE only after repeat passage. After a period of 1–2 days or

once CPE has been observed, the presence of PRRSV needs to be confirmed by immunostaining with a specific antiserum or monoclonal antibody (MAb).

i) Seeding macrophages in the microtitre plates

Defrost one vial containing 6×10^7 macrophages/1.5 ml. Wash the cells once with 50 ml PBS and centrifuge the cell suspension for 10 minutes at 300 ***g*** (room temperature). Collect the cells in 40 ml RPMI 1640 medium supplemented with 1% glutamine, 10% FBS and 1–2% antibiotic mixture (growth medium). Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of 10^5 cells in each well of the plates).

ii) Preparation of sample (serum, 10% tissue suspension) dilutions in a dummy plate

Dispense 90 µl of growth medium into each well of a microtitre plate. Add 10 µl samples to the wells of rows A and E (duplicate 1/10 dilution). Shake the plates and transfer 10 µl from rows A and E to rows B and F (1/100 dilution). Shake the plates and transfer 10 µl from rows B and F to rows C and G (1/1000 dilution). Shake the plates and transfer 10 µl from rows C and G to rows D and H (1/10,000 dilution). Shake the plates. For virus isolation without titration, dilutions of 1/10 and 1/100 are sufficient.

iii) Incubation of samples

Transfer 50 µl of the sample dilutions from the dilution plates to the corresponding wells of the plate with macrophages (first passage). Incubate for 2–5 days and observe daily for a CPE. At day 2, seed macrophages in new microtitre plates (see above). Transfer 25 µl of the supernatants from the plates of the first passage to the corresponding wells of the freshly seeded plates (second passage). Incubate for 2–5 days and observe daily for a CPE.

iv) Reading and interpreting the results

Wells in which macrophages show CPE in the first passage only are considered to be false positive because of the toxicity of the sample. Wells in which macrophages show CPE in both passages, or in the second passage only, are considered to be suspect positive. All wells with macrophage monolayers that do not show CPE need to be identified as PRRSV negative by immunostaining with a PRRSV-positive antiserum or MAb. CPE-positive samples need to be identified as PRRSV positive by culturing CPE-positive supernatant samples, or the original sample dilutions, for both 24 and 48 hours in macrophages, followed by immunostaining with a PRRSV-positive antiserum or MAb.

v) Immunostaining with a PRRSV-positive antiserum or MAb

Infect macrophages with 50 µl of supernatant or tissue sample as described in Section B.2.1, and grow the infected cells for 24 and 48 hours. Prepare an appropriate dilution of a PRRSV-positive serum in dilution buffer, and immunostain the macrophages as described in Section B.2.1 or B.2.2.

1.2. RNA detection methods

One of the most commonly used diagnostic techniques is detection of PRRSV nucleic acid with reverse-transcription polymerase chain reaction (RT-PCR), nested RT-PCR, and real-time RT-PCR (Kleiboeker *et al.,* 2005; Wernike *et al*., 2012a; 2012b). The advantages of RT-PCR are high specificity and sensitivity as well as rapid evaluation of a current infection status. However, inactivated virus cannot be differentiated from infectious virus using this technique. RT-PCR-based tests are commonly used to detect nucleic acid in tissues and serum. It has been suggested that oral fluids testing also give reliable results for pen-based diagnosis (Kittawornrat *et al*., 2010). Another population-based sample that can be used for monitoring PRRSV infections in breeding herds is processing fluid, obtained as an exudate from tissues collected during piglet castration and tail-docking (Zimmerman *et al*., 2019). The above-mentioned assays are also useful when virus isolation is problematic, such as when testing semen (Christopher-Hennings *et al.,* 1997) and when testing tissues partially degraded by autolysis or by heat during transport of specimens for virus isolation. Most of the in-house protocols and currently available commercial kits provide the possibility of differentiating species of PRRSV-1 and 2 (Kleiboeker *et al.,* 2005; Wernike *et al*., 2012a;

2012b). False-negative results related to high genetic diversity, and primer and probe mismatches are the major concern when using RT-PCR. Currently, no single RT-PCR assay is capable of detecting all PRRSV strains, especially within highly diverse European subtypes 2–4 of PRRSV-1. RT-PCR results should be carefully evaluated, and continual validation based on recently circulating PRRSV strains is strongly recommended (Wernike *et al*., 2012a). Reverse-transcription – loop-mediated isothermal amplification (RT-LAMP) is an alternative technique not requiring advanced equipment unlike the real-time RT-PCR (Zimmerman *et al.,* 2019). All of these nucleic acid tests are more rapid than virus isolation and do not require cell culture infrastructure, with the added advantage of differentiating between species.

The following example RT-PCR protocols allow for differentiation between PRRSV-1 and PRRSV-2 based on the gel electrophoresis of amplified fragments (conventional RT-PCR) or specific fluorescent-labelled probes (real-time RT-PCR). The oligonucleotides presented were shown to recognise a wide range of diverse PRRSV strains (Wernike *et al.,* 2012a). However, when relying on RT-PCR methods it is important to determine the sequence of locally circulating strains, and, if necessary, to adjust the primers and probe sequences accordingly. Cycling conditions should be adapted depending on the exact composition of the reaction mix, enzymes characteristics and thermal cycler applied. Positive (PRRSV-1 and PRRSV-2) and negative controls should be included in every run. Additional controls – positive and negative extraction controls and internal controls for real-time PCR – are recommended. To minimise the possibility of contamination, separation of consecutive protocol stages (RNA extraction, assembling the reaction mix, adding the RNA template, gel electrophoresis) is highly recommended.

1.2.1. Conventional RT-PCR

i) Perform RNA extraction.

ii) Reverse transcription:

Reverse transcription may be performed separately (e.g. 30 minutes at 50°C), or included as a part of a one-tube RT-PCR reaction, as indicated below.

iii) Prepare the reaction mix for a one-step RT-PCR (final volume 25 µl)

RT-PCR buffer	1× concentrated (including 2.5 mM $MgCl_2$)
dNTPs mix	final concentration 0.4 mM
Forward and reverse primers	final concentration of each primer 0.5 µM
Primers amplify partial ORF7/3'UTR region of PRRSV (Wernike *et al.,* 2012a):	
Forward primer	5'-ATG-GCC-AGC-CAG-TCA-ATCA-3'
Reverse primer	5'-TCG-CCC-TAA-TTG-AAT-AGG-TGA-CT-3'
RT-PCR enzyme mix	Recommended No. of Units of each enzyme
RNA	5 µl
RNase-free water	up to 25 µl

iv) Perform a one-step RT-PCR reaction according to the following thermal protocol:

Reverse transcription 50°C for 30 minutes

Reverse transcriptase inactivation and polymerase activation: depending on the enzyme characteristics

35 cycles of

Denaturation	94°C for 30 seconds
Annealing	55°C for 40 seconds
Extension	72°C for 50 seconds

Final extension 72°C for 10 minutes

v) Gel electrophoresis

Analyse obtained amplicons through electrophoresis on a 1.5% agarose, staining with an intercalating dye (ethidium bromide or its equivalent) and visualisation in a UV light.

Differentiation between PRRSV-1 and PRRSV-2 is based on the length of the amplicons, which are 398 bp and 433 bp, respectively

1.2.2. Real-time RT-PCR

i) Perform RNA extraction

ii) Reverse transcription

Reverse transcription stage may be performed separately (e.g. 30 minutes at 50°C) or included as a part of a one-tube RT-PCR reaction.

iii) Prepare the reaction mix using the reagents appropriate for hydrolysis probe-based detection. Most often, the reaction is performed in a volume of 25 µl, including 2-5 µl of extracted RNA. The final concentration of primers and probes should be approximately 0.4 µM and 0.2 µM, respectively.

Primers and probes sequences (Wernike *et al.,* 2012a)

PRRSV-1 (ORF6, ORF7)

EU-1 Forward primer 5'-GCA-CCA-CCT-CAC-CCR-RAC-3'

EU-2 Forward primer 5'-CAG-ATG-CAG-AYT-GTG-TTG-CCT-3'

EU-1 Reverse primer 5'-CAG-TTC-CTG-CRC-CYT-GAT-3'

EU-2 Reverse primer 5'-TGG-AGD-CCT-GCA-GCA-CTT-TC-3'

Probe EU-1 5'-(6-FAM)-CCT-CTG-YYT-GCA-ATC-GAT-CCA-GAC-(BHQ1)-

Probe EU-2 6-FAM-ATA-CAT-TCT-GGC-CCC-TGC-CCA-YCA-CGT-BHQ1

PRRSV-2 (ORF7, 3'UTR)

NA Forward primer 5'-ATR-ATG-RGC-TGG-CAT-TC-3'

NA Reverse primer 5'-ACA-CGG-TCG-CCC-TAA-TTG-3'

NA Probe 5'-(TEX)-TGT-GGT-GAA-TGG-CAC-TGA-TTG-ACA-(BHQ2)-3'

iv) Example thermal protocol for one-step real-time RT-PCR reaction

Reverse transcription	50°C for 30 minutes
Reverse transcriptase inactivation/polymerase activation: 95°C for 15 minutes	
45 cycles of	
Denaturation	94°C for 15 seconds
Annealing	60°C for 60 seconds
Extension	72°C for 10 seconds
Final extension	72°C for 10 minutes

v) Interpretation of results

The results may be considered positive if:

a) Fluorescence curves show a typical sigmoidal shape,

b) Fluorescence in the channels dedicated to PRRSV-1 and PRRSV-2 exceeds the level of background fluorescence indicated by the threshold line (note that unspecific fluorescence may occur in several last cycles due to the probes degradation). The number of cycles required for the fluorescent signal to cross the threshold line is expressed as a Ct value.

c) Positive controls give a positive signal in the designated channel and no increase of fluorescence in negative controls are observed.

If the above-mentioned conditions are not met, the test should be repeated.

1.3. Other methods

Although seldom used for diagnostic purposes, *in-situ* hybridisation is capable of detecting and differentiating PRRSV-1 and -2 in formalin-fixed tissues. The sensitivity and specificity of this method for detection of PRRSV genome can be compromised by the very high genetic diversity of PRRSV, especially within PRRSV-1. Immunohistochemistry can be used to identify viral proteins and when performed on formalin-fixed tissues enables the visualisation of antigen together with histological lesions (Zimmerman *et al.,* 2019).

Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified products was developed and used for the differentiation of field and vaccine PRRSV isolates (Zimmerman *et al.,* 2019). However, this method has proven to be of limited value in characterising genetic relationships between strains and their pathogenicity. Although RFLP remains in use, for example for identifying epidemiological links among herds in close proximity, it has been increasingly replaced by sequencing, which allows the study of molecular epidemiology and genetic relatedness between PRRSV strains. Sequencing can also contribute to intervention strategies as a tool for monitoring herd level viral changes over time and differentiation of wild-type and vaccine strains (Zimmerman *et al.,* 2019).

The most common target used for sequencing is the highly variable ORF5 encoding the major envelope glycoprotein (GP5) associated with virus neutralisation. However, with 603/606 nt, it only covers 4% of the viral genome and 12% of structural genes. No correlation was demonstrated between phylogenetic grouping based on ORF5 sequences and pathogenicity, or cross-protection. Therefore this approach cannot be used for prediction of strain virulence or selection of the most efficacious vaccine. Additionally, the high rate of recombination events observed in the field may influence the results of phylogenetic analysis based on such short genome fragments and lead to misclassification of recombinant strains (Martin-Valls *et al*., 2014).

Despite those concerns, ORF5 sequencing is widely used to gain insight into the genetic structure of PRRSV strains via comparison to the large library of ORF5 sequences deposited in public databases. Most of the reported PRRSV phylogenetic studies are based on data from the ORF5 region, but comparative analyses proved that whole genome sequencing allows for better understanding of viral evolution, improved epidemiological monitoring and classification of strains (Lalonde *et al*., 2020; Martin-Valls *et al*., 2014). Currently limited data on PRRSV full-length genome sequences are available, but the rapid development of high throughput sequencing technology stimulates a dynamic progress in this field.

131. ORF5 sequencing PCR

i) Primers:

Multiple primer sequences suitable for ORF5 sequencing are available in the literature (Wernike *et al*., 2012a). Due to the high genetic diversity of PRRSV and accumulation of point mutations, some primers may not be optimal for certain strains; when false negative results are obtained from PCR sequencing, an alternative set of primers should be applied.

The following protocol is based on a set of primers used by Balka *et al*. (2018):

PRRSV-1:	Forward primer	5'-TTC-ACA-GAT-TAT-GTG-GCC-CAT-GTG-AC-3'
	(alternatively	5'-GCG-TYA-CRG-ATT-ATG-TGG-CYC-AYG-T-3')
	Reverse primer	5'-CGY-GAC-ACC-TTR-AGG-GCR-TAT-ATC-AT-3'
PRRSV-2:	Forward primer	5'-GTC-AAY-TTT-ACC-AGY-TAY-GTC-CAA-CA-3'
	Reverse primer	5'-AGR-GCA-TAT-ATC-ATY-ACY-GGC-GTG-TA-3'

ii) Isolate RNA from body fluids or tissue samples with high to moderate amounts of PRRSV.

iii) Prepare the reaction mix using an appropriate buffer and one-step RT-PCR enzyme mix (alternatively perform separate RT and PCR reactions). Use 10 pmol of forward and reverse primer per reaction. If not premixed in the buffer, add dNTPs to the final concentration of 400 μM. Optionally add RNAse inhibitor in recommended concentration. Supplement the reaction mix with RNase-free water up to 25 μl volume per reaction.

iv) Aliquot 22 µl of the prepared reaction mix to the PCR tubes, add 3 µl of sample RNA. Add positive and negative reaction controls to separate tubes.

v) The example protocol for one-step RT-PCR reaction includes the following steps: 50°C for 30 minutes; 95°C for 15 minutes; 35× (94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute), 72°C for 10 minutes (the temperatures may need to be adjusted according to the enzymes and buffers used).

vi) Check if the obtained product has expected size by comparing to positive control and marker bands after an electrophoresis on a 1.5% agarose gel.

vii) Submit the PCR product for further purification, sequencing and phylogenetic analysis.

2. Serological tests

A variety of assays for the detection of serum antibodies to PRRSV has been described. Serological diagnosis is, in general, easy to perform, with good specificity and sensitivity on a herd basis. Sera collected at a single time point may have limited diagnostic value as results can be influenced by maternal antibodies or previous vaccination. To diagnose active infection in an individual animal, acute and convalescent serum samples can be tested to demonstrate seroconversion. Serology is generally performed with a binding assay, such as the immunoperoxidase monolayer assay (IPMA), immunofluorescence assay (IFA), or the enzyme-linked immunosorbent assay (ELISA) – of which many varieties are described (Diaz *et al*., 2012; Jusa *et al.,* 1996; Sorensen *et al.,* 1998; Venteo *et al*., 2012; Yoon *et al.,* 1992). These tests are often performed with viral antigen of one species, which means that antibodies directed against the other, heterologous species may be detected with less sensitivity. A blocking ELISA has been used extensively in Denmark and has been described as a double ELISA set-up using both PRRSV-1 and -2 viruses as antigens and thus it can distinguish between serological reaction to both species (Sorensen *et al.,* 1998). This is of high importance as PRRSV-2 strains circulate in Europe following PRRSV-2-based modified live vaccine use and independent introduction (Stadejek *et al*., 2013). The identification of PRRSV-1 strains in North America and Asia has also been reported (Kleiboeker *et al*., 2005; Zimmerman *et al.,* 2019). The prevalence of PRRSV-2 infections in Europe and PRRSV-1 infections in North America and Asia is not well documented. As both types of PRRSV are globally spread, serological tests should contain antigens from both species. Commercial ELISAs with good sensitivity and specificity are available and have been compared (Biernacka *et al.,* 2018; Diaz *et al*., 2012, Venteo *et al*., 2012).

Antibodies to the virus can be detected by antibody-binding assays as early as 7–14 days after infection, and antibody levels reach maximal titres by 30–50 days. Some pigs may become seronegative within 3–6 months, but others remain seropositive for much longer. Antibodies to PRRSV have also been detected in muscle transudate and oral fluid. Neutralising antibodies develop slowly and do not reach high titres. They can appear from 3 to 4 weeks after infection and persist for 1 year or more, or remain undetected. The use of complement to increase the sensitivity of the serum virus neutralisation test has been reported (Jusa *et al*., 1996). Extensive research into the duration of antibody titres after infection has not yet been done, and the results probably depend on the test used. Maternal antibodies have a half-life of 12–14 days, and maternal antibody titre can, in general, be detected until 4–8 weeks after birth, depending on the antibody titre of the sow at birth and the test used. In an infected environment, pigs born from seropositive females can seroconvert actively from the age of 3–6 weeks.

This chapter describes the IPMA in detail as this test can easily be performed in laboratories where virus isolation procedures using macrophages have been established, and can be used with virus of both species. This assay can also be adapted to the MARC-145 cell line for both species (Jusa *et al.,* 1996). An indirect immunofluorescence assay (IFA) using MARC-145 cells can also be performed for PRRSV serology and is included in the present chapter.

2.1. Detection of antibodies with the immunoperoxidase monolayer assay

Alveolar macrophages are seeded in the wells of microtitre plates. After attachment, the macrophages are infected with PRRSV. The objective is to infect approximately 30–50% of the macrophages in a well so as to be able to distinguish nonspecific sera. After an incubation period, the macrophages are fixed and used as a cell substrate for serology. An alternative method is to use MARC 145 cells instead of macrophage cells. On each plate, 11 sera can be tested in duplicates. Tested sera are diluted and incubated on the cell substrate. If antibodies are present in the tested serum, they will bind to the antigen in the cytoplasm of the macrophages. In the next incubation step, the bound antibodies will be detected

by an anti-species horseradish-peroxidase (HRPO) conjugate. Finally, the cell substrate is incubated with a chromogen/substrate[1] solution. Reading of the test is done with an inverted microscope.

2.1.1. Seeding macrophages in the microtitre plates

i) Defrost one vial containing 6×10^7 macrophages/1.5 ml.

ii) Wash the cells once with 50 ml of PBS and centrifuge the cell suspension for 10 minutes at 300 ***g*** (room temperature).

iii) Collect the cells in 40 ml RPMI 1640 medium supplemented with 1% glutamine, 10% FBS, 100 IU (International Units) penicillin and 100 µg streptomycin per ml (growth medium).

iv) Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of 10^5 cells in each well of the plates).

v) Incubate the plates for 18–24 hours at 37°C in a 5% CO_2 incubator, under humid conditions. Alternatively, use HEPES buffer (N-2-hydroxyethylpiperazine, N-2-ethane-sulphonic acid) in the medium.

2.1.2. Infection of cells with PRRSV

i) Add to each well 50 µl of a virus suspension containing 10^5 $TCID_{50}$/ml, but leave two wells uninfected to act as controls.

ii) Incubate the plates for 18–24 hours at 37°C in a 5% CO_2 incubator.

2.1.3. Fixation of the cells

i) Discard the growth medium and rinse the plates once in saline.

ii) Knock the plates gently on a towel to remove excess liquid and then dry them (without lid) for 45 minutes at 37°C.

iii) Freeze the plates (without a lid) for 45 minutes at –20°C. (Plates that are not used immediately for testing must be sealed and stored at –20°C.)

iv) Incubate the cells for 10 minutes at room temperature with cold 4% paraformaldehyde (in PBS). Alternatively the cells could be fixed in ice-cold absolute ethanol for 45 minutes at 5°C or in ice-cold 80% acetone for 45 minutes.

v) Discard the paraformaldehyde and rinse the plates once in saline.

2.1.4. Preparation of serum dilutions in a dilution plate

i) Dispense 180 µl of 0.5 M NaCl with 4% horse serum and 0.5% Tween 80, pH 7.2 (dilution buffer), to the wells of rows A and E of the dummy plate(s).

ii) Dispense 120 µl of dilution buffer to all other wells.

iii) Add 20 µl of the test serum or control sera to the wells of rows A and E (= 1/10 dilution), and shake.

iv) Dilute the sera four-fold by transferring 40 µl from rows A and E to rows B and F, and so on to provide further dilutions of 1/40, 1/160 and 1/640.

1 *Preparation of chromogen solution*
Stock solution of chromogen (3-amino-9-ethyl-carbazole [AEC]): (a) 4 mg AEC; (b) 1 ml N,N-dimethyl-formamide. Dissolve (a) in (b) and store the AEC stock solution at 4°C in the dark.
Preparation of chromogen/substrate solution (*prepare shortly before use*)
Prepare 0.05 M sodium acetate buffer, pH 5.0, as follows: Dissolve 4.1 g sodium acetate in 1 litre distilled water. Adjust the pH to 5.00 with 100% acetic acid. Add 1 ml AEC stock solution to 19 ml of 0.05 M sodium acetate buffer. Add 10 µl 30% H_2O_2 for each 20 ml of chromogen/substrate solution. Filter the solution through a 5 µm filter.

2.1.5. Incubation of sera in the plate with fixed macrophages

i) Transfer 50 µl from each of the wells of the dummy plate(s) to the corresponding wells of the plate with the fixed macrophages. Seal the plate(s) and incubate for 1 hour at 37°C.

ii) Discard the serum dilutions and rinse the plate(s) three times in 0.15 M NaCl + 0.5% Tween 80.

2.1.6. Incubation with conjugate

i) Dilute the rabbit-anti-swine (or anti-mouse, if staining isolation plate with MAb) HRPO conjugate to a predetermined dilution in 0.15 M NaCl + 0.5% Tween 80. Add 50 µl of the conjugate dilution to all wells of the plate(s). Seal the plate(s) and incubate for 1 hour at 37°C. Rinse the plates three times.

2.1.7. Staining procedure

i) Dispense 50 µl of the filtered chromogen/substrate (AEC) solution to all wells of the plate(s) (see footnote 3).

ii) Incubate the AEC for at least 30 minutes at room temperature.

iii) Replace the AEC with 50 µl of 0.05 M sodium acetate, pH 5.0 (see footnote 3).

2.1.8. Reading and interpreting the results

If antibodies are present in the test serum, the cytoplasm of approximately 30–50% of the cells in a well are stained deeply red by the chromogen. A negative test serum is recognised by cytoplasm that remains unstained. A serum that reacts nonspecifically might stain all cells in a well (compared with a positive control serum). The titre of a serum is expressed as the reciprocal of the highest dilution that stains 50% or more of the wells. A serum with a titre of <10 is considered to be negative. A serum with a titre of 10 or 40 is considered to be a weak positive. Often nonspecific staining is detected in these dilutions. A serum with a titre of ≥160 is considered to be positive.

2.2. Detection of antibodies with the indirect immunofluorescence assay

Although there is no single standard accepted immunofluorescence assay in use at this time, several protocols have been developed and are used by different laboratories in North America. The IFA can be performed in microtitre plates or eight-chamber slides using the MARC-145 cell line and a MARC-145 cell-line-adapted PRRSV isolate. To prevent cross-reactivity with pestivirus, it is recommended that cells and FBS, to supplement culture medium, be pestivirus free. After an incubation period, PRRSV-infected cells are fixed and used as a cell substrate for serology. Serum samples are tested at a single screening dilution of 1/20 and samples are reported as being negative or positive at this dilution. Each porcine serum to be tested is added to wells or chambers containing PRRSV-infected cells. Antibodies to PRRSV, if present in the serum, will bind to antigens in the cytoplasm of infected cells. Following this step, an anti-porcine-IgG conjugated to fluorescein is added, which will bind to the porcine antibodies that have bound to PRRSV antigens in the infected cells. The results are read using a fluorescence microscope. Microtitre plates may also be prepared for serum titration purposes (see Section B.2.3 below).

2.2.1. Seeding and infection of MARC-145 cells in microtitre plates

i) Add 50 µl of cell culture medium (e.g. Minimal Essential Medium [MEM] containing 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100/ml µg streptomycin) without FBS to each well of columns 2, 4, 6, 8, 10 and 12 of a 96-well plate using a multichannel pipettor.

ii) Trypsinise confluent MARC-145 cells (grown in culture flasks) to be used for seeding 96-well microtitre plates and resuspend cells in cell culture medium containing 8% FBS at a concentration of 100,000–125,000 cells/ml. The MARC-145 cells are trypsinised from culture flasks for IFA once a week using trypsin/EDTA (ethylene diamine tetra-acetic acid) and are seeded in culture flasks at a concentration of 250,000 cells/ml. After 4 days in culture flasks, new cell culture medium containing 2% FBS is added for 3 additional days.

iii) Using a multichannel pipettor, add 150 µl of the cell suspension to each well of the 96-well plate.

iv) Dilute PRRSV preparation in MEM without FBS to $10^{2.2}$ $TCID_{50}$/50 µl and distribute 50 µl in each well of columns 1, 3, 5, 7, 9 and 11.

v) Incubate the plates for approximately 48–72 hours at 37°C in a humidified 5% CO_2 incubator to obtain a monolayer with approximately 40–50% of the cells infected as determined by indirect immuno-fluorescence. Alternatively, microtitre plates may first be seeded with MARC-145 cell suspensions (e.g. concentration of 100,000 cells/ml in medium supplemented with 5–10% FBS) and incubated for up to 72 hours until they are confluent. Then volumes of 50 µl of PRRSV preparations (e.g. 10^5 $TCID_{50}$/ml) are added per well and the plates are incubated for an additional 48–72 hours prior to fixation. The use of organic buffers such as HEPES in medium has been suggested to stabilise the pH when CO_2 incubators are not available.

2.2.2. Seeding and infection of MARC-145 cells in eight-chamber glass slides

i) Add 500 µl of a MARC-145 cell suspension (e.g. in MEM supplemented with 10% FBS) at a concentration of 100,000 cells/ml to each chamber of eight-chamber glass slides.

ii) Incubate the cells for approximately 48–72 hours at 37°C in a humidified 5% CO_2 incubator until they are confluent.

iii) Add to each chamber 50 µl of PRRSV suspension containing 10^5 $TCID_{50}$/ml and further incubate cells for approximately 18 hours at 37°C in a humidified 5% CO_2 incubator . At this time 15–20 infected cells per field of view may be observed by indirect immunofluorescence.

2.2.3. Fixation of the cells

i) Discard the medium, rinse once with PBS and discard the PBS. For chamber slides, remove the plastic chamber walls, leaving the gasket intact.

ii) Add volumes of 150 µl cold (4°C) acetone (80% in water) to each well of the 96-well plate. Incubate the plates at 4°C for 30 minutes. For chamber slides, acetone (80–100%) at room temperature is used to fix the cells for 10–15 minutes at room temperature. Some manufactured brands of acetone will degrade the chamber slide gasket leaving a film on the slide. It is recommended to check the acetone before using for routine fixation.

iii) Discard the acetone and dry the plates and slides at room temperature.

iv) The plates can then be placed in a plastic bag, sealed and stored at –70°C until use. Chamber slides can be kept similarly in slide cases.

2.2.4. Preparation of serum dilutions

i) Dilute serum samples to a 1/20 dilution in PBS (0.01 M; pH 7.2) in separate 96-well plates (e.g. add 190 µl of PBS using a multichannel pipettor followed by 10 µl of the sera to be tested).

ii) Include as controls reference PRRSV antibody positive and negative sera of known titre.

2.2.5. Incubation of sera with fixed MARC-145 cells

i) Stored plates are removed from the –70°C freezer and when the plates reach room temperature rehydrate the cells with 150 µl PBS for a few minutes. Discard the PBS by inverting the plates and blotting dry on paper towels. Cells of eight-chamber slides are not rehydrated.

ii) Add volumes of 50 µl of each diluted serum to one well containing the fixed noninfected cells and to one well containing the fixed infected cells. Add similar volumes for each serum to a single chamber.

iii) Add volumes of 50 µl of the negative control serum and positive control serum dilutions in the same manner.

iv) Incubate the plates with their lids on at 37°C for 30 minutes in a humid atmosphere. Slides should be incubated similarly in boxes or slide trays with a cover.

v) Remove the serum samples and blot the plates dry on paper towels. A total of six washes using 200 µl of PBS are performed. The PBS is added to each well, followed by inversion of the plates to remove the PBS. After removing serum samples, slides are rinsed in PBS followed by a 10-minute wash.

2.2.6. Incubation with conjugate

i) Add volumes of 50 µl of appropriately diluted (in freshly prepared PBS) rabbit, mouse or goat anti-swine IgG (heavy and light chains) conjugated with FITC (fluorescein isothiocyanate) to each well using a multichannel pipettor. Similar volumes are added to individual chambers.

ii) Incubate plates or slides with their lids on at 37°C for 30 minutes in a humid atmosphere.

iii) Remove the conjugate from the plates and blot the plates dry on paper towels. A total of four washes using PBS are performed as described above. Discard the conjugate from the slides, rinse in PBS, wash for 10 minutes in PBS and rinse in distilled water. Tap the slides on an absorbent pad to remove excessive water.

iv) The plates and the slides are read using a fluorescence microscope.

2.2.7. Reading and interpreting the results

The presence of a green cytoplasmic fluorescence in infected cells combined with the absence of such a signal in noninfected cells is indicative of the presence of antibodies to PRRSV in the serum at the dilution tested. The degree of intensity of fluorescence may vary according to the amount of PRRSV-specific antibody present in the serum tested.

Absence of specific green fluorescence in both infected and noninfected cells is interpreted as absence of antibody to PRRSV in that serum at the dilution tested. The test should be repeated if the fluorescence is not seen with the use of the positive control sera on infected cells or if fluorescence is seen using the negative control serum on infected cells. No fluorescence should be seen on noninfected cells with any of the control sera. Any test serum giving suspicious results should be retested at a 1/20 dilution and if results are still unclear, a new serum sample from the same animal is requested for further testing.

2.3. Evaluation of sera for antibody titres by IFA

Microtitre plates and IFA may also be used for serum titration purposes. Up to 16 sera may be titred per 96-well microtitre plate.

2.3.1. Test procedure

i) Seed 96-well microtitre plates with MARC-145 cells (at a concentration 10^4 cells per a well) or PAM cells (approximately 10^5 cells per a well) and incubate at 37°C in a humidified 5% CO_2 incubator until they are confluent.

ii) Inoculate all wells with the PRRSV suspension at a concentration adjusted to produce approximately 100 foci of infected cells per a well (to facilitate correct reading of the results) except the wells of columns 1, 6 and 11. Incubate the plates at 37°C in a humidified 5% CO_2 incubator for 48–72 hours.

iii) Discard culture medium and rinse the monolayers once with PBS (0.01 M, pH 7.2). Fix the monolayers with cold acetone (80% aqueous solution) for 10 minutes at ambient temperature. Discard the acetone, air-dry the plates and keep the plates with lids at –20°C for short-term storage or –70°C for long-term storage, until use.

iv) Serially dilute sera including a PRRSV-positive control serum using a four-fold dilution in PBS, beginning at 1/16 or 1/20. Dilute a negative control serum at 1/16 or 1/20 dilution. Dispense 50 µl of each dilution (1/16, 1/64, 1/256, 1/1024 or 1/20, 1/80, 1/320, 1/1280) in wells containing viral antigen of columns 2, 3, 4, 5 or 7, 8, 9, 10. For each serum, also dispense 50 µl of dilution 1/16 or 1/20 in control wells of columns 1 and 6. Similarly dispense dilutions of positive and negative control sera in wells of columns 11 and 12.

v) Incubate the plates at 37°C for 30 minutes in a humid chamber. Discard the sera and rinse the plates three times using PBS.

vi) Add 50 µl of appropriately diluted anti-swine IgG conjugated with FITC and incubate plates at 37°C for 30 minutes in a humid chamber. Discard conjugate, rinse plates several times and tap the plates on absorbent material to remove excessive liquid.

2.3.2. Reading and interpreting the results

Following examination with a fluorescence microscope, the titre of a serum is recorded as the reciprocal of the highest serum dilution in which typical cytoplasmic fluorescence is observed. For paired serum samples, a four-fold increase in titre with a 2-week interval is indicative of active infection in an individual animal. No specific fluorescence should be observed with test sera or positive and negative control sera on noninfected control cells. No fluorescence should be seen on infected cells with negative control serum. Specific fluorescence should be observed on infected cells with positive control serum at appropriate dilutions. The IFA end-point may vary among laboratories. Test results may also vary depending on the PRRSV isolate used in the test because of antigenic diversity.

2.4. Detection of antibodies with the enzyme-linked immunosorbent assay

The ELISA is one of the most commonly used techniques for detection of antibodies specific to PRRSV, allowing fast, specific and sensitive confirmation of exposure to the virus. Several laboratories have developed ELISAs (indirect or blocking) for serological testing (Diaz *et al*., 2012; Sorensen *et al.,* 1998; Venteo *et al*., 2012). A double-blocking ELISA format that can distinguish between serological reactions to PRRSV-1 and PRRSV-2 species has been described (Sorensen *et al.,* 1998). Another study reported the development of an ELISA that allows differentiation of highly pathogenic PRRSV-2 from classical PRRSV-2 infections (Xiao *et al*., 2014). ELISA kits are available commercially to determine the serological status of swine towards PRRSV, also in the oral fluids as a diagnostic matrix (Kittawornrat *et al*., 2010; Venteo *et al*., 2012). These kits use as antigens either one of the two species separately or combined antigens of both. Their main advantage is the rapid handling of a large number of samples. Commercial ELISAs are available that use mainly recombinant N proteins of both PRRSV species as antigens. The potential application of ELISAs based on the nonstructural proteins NSP1, NSP2 and NSP7 was also suggested. The performance of NSP7 ELISA was reported to be comparable to a commercial ELISA kit. Moreover, it allowed the differentiation of species-specific humoral response and resolved 98% of false-positive results of a commercial assay (Brown *et al*., 2009). The ELISA method should be applied at the population level.

2.4.1. Antigen preparation and plate coating

For ELISAs directed at both species, mixed antigens derived from both PRRSV-1 and PRRSV-2 strains should be used. Preparation of purified recombinant antigens (e.g. recombinant nucleocapsid protein) is preferable and has been described (Chu *et al*., 2009; Seuberlich *et al*., 2002). Optionally, a high-quality antigen may be prepared by condensing an infected cell culture supernatant by a series of ultracentrifugation steps followed by solubilisation of the antigen pellet in 0.2% Triton X-100 (Cho *et al*., 1996). A simpler and less expensive version of the ELISA based on antigens directly collected from a virus-infected primary culture of porcine alveolar macrophages (PAMs) is described here (Albina *et al*., 1992). For PRRSV-2 strains MARC-145 or MA-104 continuous cell lines may also be used (Cho *et al*., 1996).

i) Prepare PAM culture as described in Sections B.1.1.1 and B.1.1.2.

ii) Seed 15×10^6 macrophages onto a 25 cm^2 cell culture flask and incubate for 5 hours in RPMI 1640 medium supplemented with 10% FBS and 1% of an antibiotic-antimycotic solution under a humidified 5% CO_2 atmosphere at 37°C. Replace the medium to eliminate the non-attached cells and incubate for 20 hours.

iii) Add PRRSV isolate at an infection ratio of one viral particle for 40–80 cells.

iv) When the virus-induced cytopathic effect reaches a maximum, usually 3–4 days after the PRRSV infection, submit the flask to two or three freeze–thaw cycles.

v) Centrifuge at 800 ***g*** for 10 minutes, aliquot supernatant and freeze at –70°C until use.

vi) Mock antigen consisting of non-infected macrophage cell culture should be prepared in parallel.

vii) Determine the appropriate dilution of an antigen (previously inactivated by b-propiolactone treatment) to be used in ELISA by checkerboard titration: the positive antigen should be titrated in comparison with the mock antigen using the positive and negative control sera in order to determine the dilution giving the highest signal with the positive control and the lowest signal with the negative control.

viii) Determine the appropriate dilution of samples to be tested and the threshold value by preliminary testing of samples of known characteristics. Sera are diluted in saturation buffer (e.g. PBS supplemented with 10% FBS and 2% skim milk).

ix) Prepare the plates adding the positive and mock antigens appropriately diluted in PBS to alternative columns of a polystyrene 96-well microtiter plate, coat for 1 hour at 37°C and then overnight at 4°C.

2.4.2. Test procedure

i) Wash plates three times with PBS-Tween 20 (0.1%) for removal of the excess protein.

ii) Add 100 µl of the appropriate dilution of the sera to be tested to one positive and one mock antigen well.

iii) Add 100 µl of positive and negative control sera in duplicates to positive and mock antigen wells in parallel.

iv) Incubate plates at 37°C for 1 hour.

v) Wash three times in PBS-Tween 20.

vi) Add 100 µl of a rabbit anti-swine horseradish peroxidase conjugate to each well at the appropriate dilution, incubate for 30 minutes at 37°C.

vii) Wash plates 3 times in PBS-Tween 20.

viii) Add 100 µl of substrate solution (e.g. tetramethyl benzidine) to each well, incubate at room temperature for 15 minutes.

ix) Stop reaction by adding 50 µl of 1 N sulphuric acid.

x) Read optical density (OD) at a wavelength of 450 nm.

xi) Plate validation: Calculate ΔOD value for each sample, based on the difference between the positive antigen well OD minus the mock antigen well OD. ΔOD of the positive control serum minus the ΔOD of the negative control serum should be approximately > 0.5. Calculate the average ΔOD value for positive and negative controls. Depending on the accepted format, the results may be expressed as P/N ratio (positive antigen well OD/mock antigen well OD) or S/P value (ΔOD sample/ΔOD positive control).

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Modified-live (MLV) and inactivated (killed) PRRSV vaccines are approved and commercially available in many countries for the control of the reproductive and/or respiratory forms of PRRS (Murtaugh & Genzow, 2011). It is assumed the most benefit from vaccination occurs when the vaccine virus is closely related antigenically to the field virus (Scortti *et al.,* 2006). However, there are no methods available to predict the vaccine efficacy. Although vaccination of pigs does not prevent PRRSV infection, it may be helpful in herds experiencing problems with PRRS. The killed vaccines are approved to be used as an aid in the reduction of abortions and weak piglets caused by the reproductive form of PRRS. MLV vaccines are intended to be used in sows and gilts 3–6 weeks prior to breeding and in piglets from 3 weeks of age or older (or in some cases down to one day old) as an aid in the reduction of diseases caused by PRRS.

MLV vaccines are not intended to be used in naive herds or boars of breeding age. Vaccine virus can persist in boars and be disseminated through semen (Christopher-Hennings *et al.,* 1997). MLV vaccine virus may be shed and transmitted to non-vaccinated contact pigs or vertically to offspring (Zimmerman *et al.,* 2019). Recombination events between vaccine strains or vaccine and wild-type strains have also been observed in the field (Kvisgaard *et al*., 2020). Vaccines based on biotechnology are under development but not available on the market yet. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The isolate of PRRSV used for vaccine production must be accompanied by a history describing its origin and passage history. The master seed virus (MSV) must be safe in swine at the intended age of vaccination and provide protection against challenge. Isolates for a MLV vaccine must be shown not to revert to virulence after passage in host animals. It is recommended to determine the full genome sequence of the PRRS MSV. This reference sequence could then be used to control the genetic stability of the PRRS MLV during the production process or during the serial in vivo passages.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and free from extraneous viruses, including transmissible gastroenteritis virus, porcine respiratory coronavirus, porcine epidemic diarrhoea virus, porcine adenovirus, porcine circovirus type 1 and 2, porcine haemagglutinating encephalitis virus, porcine parvovirus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic swine cell type. As a complement to culture, PCR could also be used to detect extraneous virus.

2.2. Method of manufacture

2.2.1. Procedure

PRRSV is propagated in a continuous cell line such as MARC-145 (clone of MA-104) cells. Viral propagation should not exceed five passages from the master seed virus (MSV) unless further passages prove to provide protection in swine.

The cell line is seeded into suitable vessels. MEM supplemented with FBS is used as the medium for production. Cell cultures are inoculated directly with PRRS working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant. MLV vaccines are generally mixed with a stabiliser before bottling and lyophilisation. If formalin is used as an inactivant, the final product should be tested for residual formaldehyde concentration, which should not exceed 0.74 g/litre.

2.2.2. Requirements for substrates and media

The FBS must be free from pestivirus and antibodies to pestivirus and free from bovine spongiform encephalopathy risk.

2.2.3. In-process control

Production lots of PRRSV for MLV and for inactivated (killed) virus vaccines must be titrated in tissue culture for standardisation of the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

2.2.4. Final product batch tests

Final container samples are tested for purity, safety and potency. MLV vials are also tested for the maximum allowable moisture content.

i) Sterility and purity

Samples are examined for bacterial, fungal and pestivirus contamination. To test for bacteria in a MLV vaccine, ten vessels, each containing 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth. Killed vaccines require 1.0 ml from ten final container samples be inoculated into the appropriate ten vessels of media. Pestivirus contamination should be evaluated according to the guidelines given in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and Chapter 3.9.3. *Classical swine fever*.

ii) Safety

Target animal batch release safety tests or laboratory animal batch release safety tests should be avoided wherever possible. However, if animal-based batch release safety testing cannot be avoided, the testing should conform to the 3R principles. If regulatory authorities require such tests they can be conducted in a combination of guinea-pigs, mice or pigs.

iii) Batch potency

Final container samples of an MLV vaccine are titrated (log_{10}) in microtitre plates for determination of the titre.

a) Test procedure

i) Prepare tenfold dilutions from 10^{-1} through 10^{-5} by using 0.2 ml of rehydrated test vaccine and 1.8 ml of MEM. An internal positive control PRRSV should be titrated in the appropriate range.

ii) Inoculate 0.1 ml/well from each dilution into five wells of a 96-well plate containing MARC-145 monolayers.

iii) Incubate the plate at 37°C in a CO_2 atmosphere for 5–7 days.

iv) Read the plates microscopically for CPE. The internal positive control PRRSV should give a titre within 0.3 log_{10} $TCID_{50}$ from its predetermined mean.

v) Determine the $TCID_{50}$/dose by the Spearman–Kärber method. The release titre must be at least 1.2 logs higher than the titre used in the immunogenicity trial. The 1.2 logs include 0.5 logs for stability throughout the shelf life of the product and 0.7 logs for potency test variability.

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. Parallel-line assays using ELISA antigen-quantifying techniques to compare a standard with the final product are acceptable in determining the relative potency of a product. The standard should be shown to be protective in the host animal.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Field trial studies should be conducted to determine the safety of the vaccine. Non-vaccinated sentinel pigs should be included at each site for monitoring the shed of the attenuated virus.

ii) Reversion-to-virulence for attenuated/live vaccines

MSV must be shown not to revert to virulence after several passages in host animals, although the definition of virulence with such a virus is difficult. Attenuated PRRSV isolates are known to cause viraemia and will transmit to susceptible animals. The MSV should be shown to be avirulent in weaned piglets and pregnant animals by five serial passages (up to ten passages depending on country) of the MSV through susceptible swine using the most natural route of infection.

iii) Environmental consideration

Not applicable

2.3.2. Efficacy requirements

i) For animal production

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible swine against a virulent, unrelated challenge strain. For the respiratory form, 3-week-old piglets are vaccinated with the highest passage level of MSV. The piglets are challenged with approximately 10^5 $TCID_{50}$ a virulent isolate of PRRSV 2–16 weeks later to determine protection from respiratory clinical signs of PRRS. To determine protection from the losses caused by the reproductive form of PRRS, vaccinated animals are challenged at approximately 85 days' gestation. A prevented fraction, the proportion of potential PRRS disease occurrence reduced due to vaccination, is calculated to determine if there is acceptable protection, based on the proposed label claims, in the vaccinates from the clinical signs of reproductive disease, including fetal mummification, stillborn piglets or weak piglets, when compared with the controls. As MLV vaccines are frequently used in piglets bearing PRRSV specific maternally derived antibody (MDA), the interference of MDA with the efficacy of MLV vaccine should be evaluated.

Duration of immunity studies are conducted before the vaccine receives final approval. For the respiratory form of PRRS, duration should be shown up to the market age in pigs. Duration of immunity for the reproductive form should be shown through weaning of the piglets.

ii) For control and eradication

Not applicable

2.3.3. Stability

All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date.

Multiple batches of MLV vaccines should be re-titrated periodically throughout the shelf-life to determine vaccine variability. The release value should be adjusted if the titres are insufficient or highly variable.

Killed vaccines using *in-vivo* potency tests should be retested at expiry to demonstrate stability. Parallel-line assays using ELISA antigen-quantifying techniques should demonstrate the stability of the standard.

3. Vaccines based on biotechnology

Biotechnology-based vaccines are under development but not yet available on the market.

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NB: There are WOAH Reference Laboratories for porcine reproductive and respiratory syndrome (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for porcine reproductive and respiratory syndrome

NB: FIRST ADOPTED IN 1996; MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.9.7.

INFLUENZA A VIRUSES OF SWINE

SUMMARY

Description and importance of disease: *Influenza A viruses of swine (IAV-S) cause a highly contagious respiratory disease characterised by coughing, sneezing, nasal discharge, fever, lethargy, breathing difficulty, and depressed appetite. In some instances, IAV-S infections are associated with reproductive disorders such as abortion. Clinical signs and nasal shedding of virus can occur within 24 hours of infection. Morbidity rates can reach 100% with IAV-S infections, while mortality rates are generally low. Secondary bacterial infections can exacerbate the clinical signs following infection with IAV-S. Transmission is through contact with IAV-S-containing secretions such as nasal discharges and aerosols created by coughing or sneezing.*

Detection and identification of the agent: *Samples should be collected within 24–72 hours after development of clinical signs. The animal of choice is an untreated, acutely ill pig with compatible clinical signs. Antigen enzyme-linked immunosorbent assays (ELISA) are commercially available for detection of type A influenza viruses and are typically directed against the conserved nucleoprotein (NP). Viral RNA can readily be detected by reverse-transcription polymerase chain reaction (RT-PCR) assays targeting the matrix (M) or the hemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) encoding genes in samples from respiratory tissues, such as trachea or lung and nasal swabs. Immunohistochemistry can be conducted on formalin-fixed tissue. Real-time RT-PCR is increasingly used as it is highly sensitive and can be performed on scale at relatively low unit cost. Oral fluids collected from cotton ropes hung in a pig pen may also be useful as a group or population sample. Virus isolation can be achieved using embryonated fowls' eggs, continuous cell lines or primary cell cultures. Viruses can be subtyped (HxNy) by identification of the hemagglutinin (HA) and neuraminidase (NA) viral envelope proteins using the haemagglutination inhibition (HI) and the neuraminidase inhibition tests on virus isolates, or by RT-PCR assays direct on clinical material or on isolates. Gene sequencing is now widely applied and can be used to determine virus subtype and, importantly, genotype, the latter providing invaluable data associated with genetic diversity of IAV-S driven by both genetic reassortment and continual genetic drift especially in the genes encoding HA and NA.*

Serological tests: *Historically, the primary serological test for detection of IAV-S antibodies is the HI test conducted on paired sera. The HI test is HA subtype specific. The sera are ideally collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent IAV-S infection. Additional serological tests that have been described are the agar gel immunodiffusion test, indirect fluorescent antibody test, virus neutralisation, and ELISA. Due to the increasing amount of antigenic diversity in influenza A viruses of swine and the need to use multiple H subtypes in HI assays, there is a general trend towards use of commercially available ELISAs that are not subtype specific.*

Requirements for vaccines: *Inactivated, adjuvanted IAV-S vaccines are commercially available, some based on autologous strains. Vaccines may include antigens from a single IAV-S subtype or multiple IAV-S subtypes. Vaccines should ideally reflect the current antigenic profile of field viruses, containing subtypes and strains that are changed as needed to assure protection.*

A. INTRODUCTION

Influenza A viruses of swine (IAV-S) cause a highly contagious viral infection of pigs that can have significant economic impact on an affected herd. IAV-S is an enveloped virus with a segmented RNA genome. It belongs to the

Influenzavirus A genus of the family *Orthomyxoviridae.* The type A viruses are further subdivided based on the haemagglutinin (HA) and neuraminidase (NA) proteins in the viral envelope that contain the immunodominant epitopes. Subtypes (HxNy) of IAV-S that are most frequently identified in pigs include classical and avian (av) H1N1, human (hu) H1N1 and H1N2, reassortant (r) H3N2, and rH1N2. Other subtypes that have been infrequently identified in pig populations (although not necessarily maintained) include rH1N7, rH3N1, H2N3, avH4N6, avH3N3, and avH9N2. The H1N1, H1N2 and H3N2 viruses found in Europe are antigenically and genetically different from those found in America or Asia (Anderson *et al.,* 2016; Brown, 2013; Karasin *et al*., 2000; 2002; Lewis *et al.,* 2016; Olsen, 2002; Vincent *et al*., 2009; Watson *et al.,* 2015; Webby *et al*., 2004; Zhou *et al*., 1999). Due to the substantial genetic diversity in H1 viruses circulating in swine among geographical regions, haemagglutinin H1 can be classified according to genetic characteristics into clades; 1A classical lineage derived from the 1918 human pandemic viruses, 1B human seasonal lineage associated with 1990s human-to-swine transmission episodes, and 1C Eurasian avian lineage associated with viruses introduced to swine in Europe and Asia from avian species (Anderson *et al.,* 2016).

Pig cells have receptors in their respiratory tract that allow attachment and infection by influenza A viruses of swine, humans, and avian species. Consequently, pigs have been called 'mixing vessels' for the development of novel influenza viruses through genetic reassortment in pigs. IAV-S infections cause respiratory disease characterised by coughing, sneezing, nasal discharge, fever, lethargy, breathing difficulty and depressed appetite. Other agents that may cause respiratory disease in pigs include porcine reproductive and respiratory syndrome virus, Aujeszky's disease (pseudorabies) virus, porcine respiratory coronavirus, *Actinobacillus pleuro-pneumoniae, Mycoplasma hyopneumoniae* and other bacterial agents (Mollett *et al.,* 2023). Thus, laboratory investigations are necessary to determine infection with IAV-S. Clinical signs and nasal shedding of influenza A virus can occur within 24 hours of infection and shedding typically ceases by day 7–10 after infection. Two forms of disease occur in swine herds, epidemic or endemic (Li & Robertson, 2021). In the epidemic form, the virus quickly moves through all phases of a swine unit with rapid recovery, provided there are no complicating factors such as secondary bacterial infections. In the endemic form, clinical signs may be less obvious and not all pigs may demonstrate typical clinical signs of infection. In some cases, the endemic form may affect successive batches of pigs at a given physiological stage (Rose *et al.,* 2013). In all cases, the endemic form is responsible for IAV-S persistence at the herd level. Morbidity rates can reach 100% with epidemic IAV-S infections, while mortality rates are generally low. The primary economic impact is related to retarded weight gain resulting in an increase in the number of days to reach market weight. Transmission is through contact with IAV-S containing secretions such as nasal discharge, droplets and aerosols created by coughing or sneezing. Human infections with IAV-S so-called variant (v) strains can occur and a limited number of deaths have been reported (Lindstrom *et al.,* 2012; Myers *et al*., 2007; Pulit-Penaloza *et al.,* 2019). Precautions should be taken to prevent human infection as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Conversely, influenza A viruses can occasionally be transmitted from people to pigs, so called reverse zoonoses or anthroponoses, creating opportunity for further virus diversity. Influenza A viruses can also be transmitted occasionally from poultry to pigs as well as from pigs to poultry, especially domestic turkeys. In the spring of 2009 a newly identified H1N1 virus (H1N1pdm09) was detected in people in the western hemisphere. This novel virus was composed entirely of genes from IAV-S, but with a complicated evolutionary history. The matrix (M) and neuraminidase (NA) genes were from European H1N1 IAV-S of 1C avian lineage and the remaining genes were from North American IAV-S of a triple reassortant (swine, avian, and human) lineage (Zhou *et al.,* 1999). The H1N1pdm09 spread rapidly throughout the world through human-to-human transmission. In addition to continued independent circulation in humans, reverse zoonoses of swine cases in both Northern and Southern hemispheres occurred simultaneously and the virus became endemic in many swine populations worldwide. The H1N1pdm09 subsequently reassorted with other IAV-S and contributed to newly identified genomic constellations of viruses around the globe (Anderson *et al.,* Lewis et al., 2016; 2021; Watson *et al.,* 2015).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of* IAV-S *and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	+	+	++	+++	+	–
Real-time RT-PCR	+++	+++	+++	+++	++	–
Conventional PCR	–	–	–	++	–	–
Detection of immune response						
HI	+	+	+	++	++	+++
ELISA	+++	+	++	+	+++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.RT-PCR = reverse-transcription polymerase chain reaction; HI = haemagglutination inhibition; ELISA = enzyme-linked immunosorbent assay; Note that antigen ELISA assays are designed for use in clinically ill animals. Their reliability in clinically healthy animals is questionable.
[(a)]A combination of agent identification methods applied on the same clinical sample may be needed in some situations

1. Identification of the agent

Clinical specimens and cultures of IAV-S should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Because IAV-S is a potential human pathogen, all work with infectious virus, potentially infectious diagnostic samples, embryonated eggs, and cell cultures should be done in a class II microbiological safety cabinet (MSC). Additional safety precautions (personal protective equipment) should be used when working with infected pigs such as respiratory personal protective equipment (RPE) and eye protection.

1.1. Culture

1.1.1. Sample processing

Respiratory tissues can be processed for virus isolation in a variety of ways, for example by lavage with sterile media listed below, or with tissue maceration by mortar and pestle, stomacher, homogeniser, or mincing with a scalpel blade or scissors. Processing of the tissue is done in a physiological solution such as cell culture medium with antibiotic supplement (e.g. 10 × working strength), at a final concentration of 10–20% weight to volume. Nasal swabs should be collected in cell culture medium (e.g. 1% fetal bovine serum [FBS] or FBS-free medium) or phosphate-buffered saline (PBS), supplemented with antibiotics and bovine serum albumin (5 mg/ml). Oral fluids may require adjustments to sample processing method used for nasal swabs due to the viscous nature of the specimen and increased propensity for bacterial contamination. Samples should ideally be shipped to a diagnostic laboratory overnight on wet ice, not frozen (see http://offlu.net for guidance on specimen collection and shipment). Upon receipt at the laboratory, the nasal swabs are vigorously agitated by hand or on a vortex mixer. The nasal swab and tissue specimens are centrifuged at 1500–1900 ***g*** for 15–30 minutes at 4°C. The supernatant is collected and maintained at 4°C until inoculation. If supernatant is to be held for longer than 24 hours before inoculation, it should be stored at –70°C or colder. The clarified tissue suspension is inoculated without further dilution. Swab and oral fluid supernatant can also be inoculated

without dilution or diluted 1/3 in cell culture medium. Antibiotics are added to the cell culture medium used for processing and/or the supernatant can be filtered to reduce bacterial contamination, but this may decrease virus titre. For filtration, low protein adsorption membrane, such as PVDF (polyvinylidene fluoride) membrane, is recommended to minimise virus loss. As an alternative, the virus preparation may be treated with antibiotics such as gentamicin (100 μg/ml) or penicillin (10,000 units/ml: streptomycin (10,000 units/ml) and 2% fungizone (250 mg/ml) for 30–60 minutes at 4°C prior to inoculating the embryos or cell culture.

1.1.2. Cell culture virus isolation

i) Virus isolation can be conducted in cell lines and primary cells susceptible to influenza A virus infection (Feng *et al.,* 2011; Karakus *et al.,* 2018). Madin–Darby canine kidney (MDCK) cells are broadly permissive for various subtypes and strains of IAV-S and are therefore often a preferred cell line, but primary swine kidney, swine testicle, swine lung, or swine tracheal cells can also be used.

ii) Wash confluent cell monolayers (48–72 hours post-seeding) three times with cell culture medium without FBS and containing a final concentration of 1 μg/ml of TPCK[1]-treated trypsin. The concentration will depend on the type of trypsin and the cells used (0.3–10 μg/ml may be used). The cell culture medium can be supplemented with antibiotics.

iii) Inoculate cell cultures with an appropriate amount of lavage fluid, tissue suspension, oral fluids, or swab supernatant. Note: The volume of inoculum will vary with the size of the cell culture container. In general, 100–200 μl are inoculated in each well of a 24-well culture plate, or 0.5–2 ml into a 25 cm^2 flask.

iv) Incubate inoculated cell cultures for 1–2 hours at 37°C with occasional rocking. When using cell culture containers that are open to the environment, such as culture plates, incubation should be done in a humidified incubator with 5% CO_2.

v) Remove the inoculum and wash the cell monolayer three times with the cell culture medium containing trypsin.

vi) Add an appropriate volume of the cell culture maintenance medium to all containers and incubate at 37°C for 3–7 days with periodic examination for cytopathic effect (CPE). If CPE is not observed at the end of the incubation period, the cell culture container can be frozen at –70°C or colder, thawed, and blind passaged as described above (step iii). If CPE is observed, an aliquot of the cell culture medium can be tested for haemagglutinating viruses or by reverse transcription-polymerase chain reaction (RT-PCR) for conserved influenza virus genes such as nucleoprotein (NP) or matrix (M) encoding genes, which have largely replaced antibody based confirmatory tests. However all culture medium can be used in an HA assay to detect viral particles. Alternatively, the culture supernatant can be used as inoculum for virus detection by ELISA (see Section B.1.5) or by the fluorescent antibody or immunohistochemistry (IHC) techniques (see Section B.1.3 and 1.4 below). Usually monolayer cultures (i.e. 24-well cell culture plate) with MDCK (or other appropriate cell) can be inoculated for this purpose. The isolation procedure is as described above (step iii). In some instances, it may be necessary to make tenfold dilutions of the cell culture virus in order to produce appropriate CPE on the monolayer. Influenza subtypes can be determined by the haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests, or by real-time or conventional RT-PCR with primers validated for sensitive and specific amplification of individual HA and NA genes (for example Bonin *et al*., 2018; Chiapponi *et al*., 2012; Henritzi *et al.*, 2016; Nagarajan *et al.,* 2010; Ryt-Hansen *et al*., 2020) or by gene sequencing and comparing data obtained with those of previously characterised swIAV-s. Validation using endemically circulating strains in the region should be done to ensure fitness for purpose of tests since endemic strains of IAV-S may vary genetically between regions.

1.1.3. Egg inoculation

i) Use 9- to 11-day-old embryonated fowls' eggs (Senne, 1998).

ii) Inoculate 0.1–0.3 ml of inoculum into the allantoic cavity and amniotic sac; many laboratories only inoculate via the allantoic route with similar sensitivity however for primary

1 TPCK: tosylphenylalanylchloromethane

isolation, amniotic inoculation can increase sensitivity. Generally, 3–4 eggs are inoculated per specimen.

iii) Incubate eggs at 35–37°C for up to 5 days and candle daily. Eggs with embryos that die within 24 hours of inoculation are discarded (assumed to be intercurrent deaths associated with the inoculation process).

iv) Refrigerate eggs with embryos that died more than 24 hours after inoculation or at the end of the incubation period. All egg materials should be considered to be potentially infectious and should be treated accordingly to prevent IAV-S exposure to the laboratory worker.

v) Harvest amniotic and allantoic fluids, centrifuging if necessary at 1500–1900 ***g*** for 10–20 minutes at 4°C. Transfer the supernatant to another tube for testing.

vi) Fluids are evaluated for the presence of IAV-S with the haemagglutination (HA) test, or by ELISA (see Section B.1.5) or by (M- or NP-gene) RT-PCR (see Section B.1.6).

vii) Repass (up to 1–2 passages) fluids negative for haemagglutinating activity (negative for IAV-S) in eggs or on cell lines as described above. Isolation may be improved by making tenfold dilutions of the fluid in cell culture medium.

1.1.4. Haemagglutination test

i) Prepare a 0.5% erythrocyte suspension from male turkey or chicken or guinea-pig blood (Takemae *et al.,* 2010). Dispense whole blood into a tube and add PBS. For example, 10–20 ml whole blood in a 50 ml centrifuge tube to which PBS is added to fill the tube. Gently invert the tube several times to wash the erythrocytes. Centrifuge at 800 ***g*** for 10 minutes in a refrigerated centrifuge. Aspirate PBS and buffy coat (white blood cell layer) from the tube. Refill the tube with fresh PBS and resuspend erythrocytes thoroughly. Repeat the washing and centrifugation cycle two additional times. Once washing is complete, add sufficient erythrocytes to PBS to make a 0.5% solution. Certain virus strains agglutinate turkey or guinea-pig rather than chicken erythrocytes to greater or lesser degrees. Therefore, it may be necessary to choose the species of erythrocytes based on the strains circulating in a given area. Washed erythrocytes and 0.5% suspensions of erythrocytes can be stored at 4°C for up to 1 week. Discard if haemolysis is observed.

ii) Dispense 50 µl PBS in a row of 8–12 wells of a 96-well V- or U-bottom microtitre plate for each unknown virus. One additional row of wells should be included for a positive control.

iii) Add 50 µl of undiluted isolate to the first well of each corresponding row.

iv) Complete serial two-fold dilutions of the isolate with a micropipette set to deliver 50 µl. The resulting dilutions will range from 1/2 (well 1) to 1/2048 (well 11). Well 12 contains PBS only and serves as a cell control.

v) Add 50 µl of 0.5% erythrocyte suspension to each well and agitate the plate to mix thoroughly. Note: keep erythrocytes thoroughly suspended during the dispensing process.

vi) Cover the plate with sealing tape and incubate at room temperature (24°C) or 4°C until a distinct button has formed (30–60 minutes) in the negative control well.

vii) Wells with complete haemagglutination (positive HA, IAV-S present) will have erythrocytes spread throughout the well in a diffuse 'mat' type appearance. Wells with a distinct button of erythrocytes at the bottom of the well are negative for haemagglutinating activity (negative for IAV-S). Incomplete HA activity is demonstrated by partial buttons characterised by fuzzy margins or 'donut-like' appearance. When interpretation between negative and incomplete inhibition is doubtful, tilt the microtitre plate to about a 45-degree angle for 20–30 seconds and look for streaming, which produces a tear-drop appearance and translucency around the cells in wells with negative hemagglutination. Wells with partial inhibition will not produce a tear drop.

1.2. Typing influenza A viruses of swine (IAV-S) isolates

1.2.1. Haemagglutination inhibition test

i) Dilute reference HA antigens (H1, H3, etc.) to a concentration of 8 HA units (HAU) per 50 µl (4 HAU/25 µl) in 0.01 M PBS, pH 7.2–7.4. Reference antigens should represent what is actively circulating in the region where the pigs are located. For guidance, the WOAH Reference Laboratory in the region should be consulted regarding reference antigens.

ii) Standardise unknown influenza A viruses to contain 8 HAU in 50 µl.

iii) Conduct a back titration (HA test) for all unknown isolates and the H subtype antigens to ensure that the correct HAUs are present. The back titration is performed as described in the HA procedures except that six well dilutions are used instead of eleven.

iv) Treat each reference serum (specific for an individual HA subtype noting multiple sera for same subtype may be required to take account of antigenic diversity, and representative of actively circulating viruses in the region) with RDE (receptor-destroying enzyme); add 50 µl serum to 200 µl RDE (1/10 dilution in calcium saline solution equalling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 µl 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes. Combine 200 µl treated sample and 25 µl PBS. Note: RDE treatment is highly recommended as it will reduce nonspecific reactions and will enhance the identification of H1N2 and H3N2 isolates.

v) Remove natural serum agglutinins from the reference sera by treating diluted sera with 0.1 ml packed, washed erythrocytes per 1 ml diluted serum. Incubate for 30 minutes at room temperature with occasional mixing to keep the erythrocytes suspended. Centrifuge the treated serum at 800 ***g*** for 10 minutes and then retain the serum.

vi) Dispense 25 µl of standardised antigen (unknown isolate or positive control antigen) into three wells of a 96-well V- or U-bottom microtitre plate. Add 50 µl of PBS to several wells to serve as an erythrocyte cell control. Note: 25 µl of PBS can be used in place of the 25 µl of standardised antigen in cell control wells.

vii) Add 25 µl of the appropriate reference serum to the first well of the H subtype being evaluated. Serially twofold dilute the antiserum in 25 µl volumes in the antigen wells with a pipette set to deliver 25 µl. Repeat this procedure for each H subtype being evaluated. Note: If 25 µl of PBS was used in place of the 25 µl of standardised antigen in step vi, add 25 µl of standardised antigen to each well containing the reference serum.

viii) Cover plate(s) and incubate at room temperature for 10–30 minutes.

ix) Add 50 µl 0.5% erythrocyte suspension to each well and shake/agitate the plate(s) to mix thoroughly. Keep the erythrocytes thoroughly suspended during the dispensing process.

x) Cover the plate(s) with sealing tape and incubate at room temperature (24°C) or 4°C until a distinct button has formed in the positive serum control wells (usually 30–60 minutes). Observe the plates after about 20 minutes' incubation for evidence of haemagglutination as some isolates may begin to elute (detach from erythrocytes) in 30 minutes.

xi) Read test results as described above for the HA test. A sample is considered positive for a specific H subtype if haemagglutination is inhibited. The test is considered valid if the positive reference antigen and its homologous antiserum demonstrate the expected HI titre (within twofold) and the back titration of each antigen (unknown and positive control) is 4 or 8 HAUs. If these conditions are not met, the test should be repeated.

xii) If erythrocytes in the cell control wells do not settle into a well-defined button, check the following as possible causes: incorrect formulation of PBS, excessive evaporation from the plates, erythrocytes too old, or incorrect concentration of erythrocytes.

1.2.2. Neuraminidase inhibition test

Reliable subtype identification based on the NI test is beyond the scope of many laboratories. Reference laboratories can be consulted for N typing of isolates (Kaplan & Vincent, 2020). Increasingly PCR or NA gene sequencing methodologies are replacing such tests.

1.3. Fluorescent antibody test

1.3.1. Test procedure

i) This technique can be used for tissue sections, cover-slips/slides, or 96-well plates of infected cell monolayers (Vincent *et al*., 1997). Positive and negative controls should be included with all staining procedures.

ii) Note this technique is highly dependent on use of reference reagents representative of circulating viruses in the region and on skilled operators who can differentiate between positive results and background staining (specificity). This method of virus detection is of lower sensitivity compared with other available assays such as RT-PCR.

iii) Inoculated cells are incubated for an appropriate length of time to allow 10–25% of the cells to become productively infected with virus. Rinse the cover-slip or slide once in PBS, place in a fixative (i.e. 4% paraformaldehyde, 100% acetone or 10% normal buffered formalin) for 5–10 minutes and air-dry. Many fixatives should be used in a vented hood.

iv) Prepare frozen tissue sections on glass slides. Fix the glass slides in acetone for 5–10 minutes and air-dry.

v) Apply conjugate (fluorescein-labelled IAV-S antibody) and incubate in a humid chamber at 37°C for 30 minutes. Preferably the conjugate contains Evans blue for counter staining. Note it is important to use an antibody that recognises all possible viruses circulating in the area (e.g. a pan-anti-influenza A nucleoprotein antibody).

vi) Rinse in PBS, pH 7.2, soak for 5–10 minutes in fresh PBS, rinse in distilled water, and air-dry.

vii) Place cover-slips on glass slides, cell side down, with mounting fluid. Remove the rubber gasket from chamber slides and add mounting fluid followed by a glass cover-slip. Mounting fluid followed by a glass cover-slip is also placed over tissue sections on the slide. If 96-well plates are used, mounting medium and cover-slips are not required.

viii) Observe stained slides in a darkened room with the use of an ultraviolet microscope. Cells infected IAV-S are identified by the presence of bright apple-green fluorescence. It is recommended that the person examining the slides receive training in reading fluorescein-labelled slides as they can be difficult to interpret. Known positive and negative slides should be included when testing unknowns to verify the test procedure worked and to use as a basis for differentiating between positive (IAV-S) staining and negative (background) staining.

1.4. Immunohistochemistry

1.4.1. Test procedure (Vincent *et al*., 1997)

i) Slice formalin-fixed, paraffin-embedded lung in 4-µm thick sections and place on poly-L-lysine-coated slides (alternatively, commercially available charged slides can be used). Positive and negative control tissues should be included with all tests.

ii) Heat slides at 60°C for 15 minutes, deparaffinise, and rehydrate through immersions in decreasing concentrations of ethanol and then in distilled water.

iii) Treat samples with 3% hydrogen peroxide for 10 minutes and rinse twice in distilled water.

iv) Digest samples with 0.05% protease for 2 minutes and rinse twice for 2 minutes in 0.1 M Tris/PBS buffer, pH 7.2, at room temperature.

v) Apply primary mouse anti- IAV-S monoclonal antibody (directed against the viral nucleoprotein) at a predetermined dilution to each slide and incubate at room temperature for 1 hour or overnight at 4°C. Rinse slides with Tris/PBS buffer.

vi) Apply secondary antibody (biotinylated goat anti-mouse antibody) for 10 minutes at room temperature. Rinse with Tris/PBS buffer.

vii) Apply tertiary development reagent (peroxidase-conjugated streptavidin) for 10 minutes at room temperature. Rinse with Tris/PBS buffer.

viii) Apply diaminobenzidine tetrahydrochloride solution for 5 minutes at room temperature. Rinse twice in distilled water.

ix) Counterstain slides in Gill's haematoxylin for 10–30 seconds, wash in water for 2 minutes, dehydrate, clear, and add cover-slips.

x) IAV-S-infected tissues are identified by the presence of brown staining.

1.5. Antigen-capture enzyme-linked immunosorbent assays

Type A antigen-capture enzyme-linked immunosorbent assays (ELISAs) and membrane immunoassays are commercially available for detection of human and animal influenza viruses. These types of assays have been used for detection of IAV-S in lung tissue and nasal swabs (Swenson *et al*., 2001). The assays are generally available through human health and animal health care companies. These assays tend to be of lower sensitivity compared with other assays such as RT-PCR.

1.6. Reverse-transcription polymerase chain reaction

At present, conventional virus isolation and other characterisation techniques for the diagnosis of IAV-S infections remain a key method and provide virus for more detailed analyses including *in-vivo* assessment and gene sequencing. Furthermore, they may be invaluable in confirming or disproving the presence of infectious virus when other test results including conventional and real-time RT-PCR are all weakly positive. However, conventional methods tend to be costly, labour intensive and slow. There have been enormous developments and improvements in molecular and other diagnostic techniques, many of which are now routinely applied as a first choice for the diagnosis of IAV-S infections.

Molecular techniques have been used preferentially for diagnosis for some time now. Furthermore, there have recently been developments in their application to the detection and characterisation of IAV-S directly from clinical samples from infected pigs. It is imperative that when using highly sensitive molecular detection methods that allow rapid direct detection of viral RNA for confirmatory laboratory diagnosis of IAV-S infections, stringent protocols are in place to prevent the risk of cross-contamination between clinical samples. In addition, RNA detection test methodologies should be validated to the WOAH standard (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*) using clinical material to demonstrate the tests as being 'fit for purpose' for application in a field diagnostic setting, which may include the use of internal test standards. The control reactions enable greater confidence in the integrity of the molecular reactions, clinical samples and results.

Furthermore, these evaluations enable the appropriate setting of test thresholds for interpretation between positive and negative samples. The increased sensitivity of real-time RT-PCR leads to the detection of viral RNA in samples in the absence of infectious virus and care should be taken when interpreting outputs with small detection limits that may not be indicative of active infection. This problem can be overcome, through the testing of multiple samples from the same cohort of infected pigs.

In settings with more limited facilities, RT-PCR techniques on clinical samples can, with the correctly defined primers, result in rapid detection and subtype identification including a cDNA or PCR product that can be used for nucleotide sequencing. Real-time RT-PCR, usually based around the hydrolysis probe method for generation of the target-specific fluorescence signal, has become the method of choice in many laboratories for at least partial diagnosis directly from clinical samples. The method offers rapid results, with sensitivity and specificity comparable with virus isolation. These are ideal qualities for IAV-S disease management, where the period of time in which an unequivocal diagnosis can be obtained is crucial for decision making. The approach to diagnosis using real-time RT-PCR adopted in most laboratories has been based on initial generic detection IAV-S in clinical samples, primarily by initially targeting the matrix (M) or the nucleoprotein (NP) encoding genes, which are highly conserved for all influenza A viruses and the best targets for screening for infection with IAV-S by RT-PCR, followed by specific real-time RT-PCR testing for H1 and H3 subtype viruses. Genomic sequencing is required to further classify the H1 clades (e.g. gamma vs beta H1) and H3 clusters (e.g. cluster IV or 2010.1).

Due to the high diversity of swine HA and NA gene sequences, it can be difficult to use real-time RT-PCR to differentiate between subtypes and specially to differentiate between different lineages within a subtype. Therefore, sequencing is often more precise.

Numerous assays have been reported for highly sensitive detection of M (or NP) gene fulfilling the criteria for a suitable screening test. Following the identification of the novel (pandemic) H1N1 in 2009, molecular assays based on an avian influenza M gene real-time RT-PCR (Spackman *et al*., 2002) were adapted for use in swine (Slomka *et al.,* 2010). Modifications to the assay vary by country and a swine influenza Reference Laboratory may be consulted for the most suitable matrix RT-PCR assay for the region in which it is being applied.

The IAV-S real-time RT-PCR procedure described in this chapter targets the matrix (M) gene of Influenza A viruses. The matrix primer/probe set is a quasi-multiplex real-time RT-PCR that uses a single forward primer, probe and two reverse primers. The two reverse primers can generically detect the Eurasian, North American and pandemic 2009 H1N1 matrix lineages.

For this procedure, it is critical to have separate preparation areas and equipment for nucleic acid extraction, RNA transfer, and master mix preparation. A "clean" area is needed to prepare reagents used for RT-PCR that is free of amplified c-DNA or sample RNA.

Table 2. IAV-S matrix hydrolysis probe and primer sequences

Specificity	Description	Sequence (5' → 3')
Matrix (any influenza A virus)	M+25* 5' Primer	AgA TgA gTC TTC TAA CCg Agg TCg
	M+64* Probe	FAM-TCA ggC CCC CTC AAA gCC gA-BHQ-1
	M-124* 3' Primer	TgC AAA AAC ATC TTC AAg TCT CTg
	M-124* SIV 3' Primer**	TgC AAA gAC ACT TTC CAg TCT CTg

*Refers to the nucleotide position where the 5' end of the probe or primer anneals to the genome
**Primer detects the 2009 H1N1 pandemic matrix

i) Extract nucleic acid from sample. A positive and negative extraction control (PEC and NEC, respectively) will need to be used to confirm that the extraction was successful.

ii) Prepare RT-PCR master mix in a "clean" PCR room (Table 3).

iii) Aliquot 17 µl of reaction mix to each well in a 96-well plate. Transfer 8 µl of RNA template to each reaction in a designated RNA transfer room. When using a 96-well plate, use a support base to protect the bottom of the plate from scratches, finger prints, or picking up particles that could interfere with the optical system and alter the background fluorescence.

    a) The following controls will need to be included in the PCR run to verify that the PCR and RNA extraction were successful: positive extraction control (PEC), negative extraction control (NEC), positive amplification (template) control (PAC), and negative amplification (template) control (NAC). PACs are diluted by each diagnostic laboratory and must have a C_t value in the range of 21–30 for the run to be valid.

iv) Place samples in thermocycler and run at appropriate parameters.

v) Analyse results. The PCR run will be valid if:

    a) The PAC C_t value is at a predetermined level typically in the range 25–30

    b) The PEC is positive

    c) Both NEC and NAC are negative

    d) All samples and controls that are positive have "sigmoidal curve"

    e) If the above conditions are not met, the test will need to be repeated.

Table 3. Example real-time RT-PCR master mix for a one step reaction

Component	Final Concentration	Volume per reaction (µl)
H_2O	–	0.83
2× RT-PCR buffer	1×	12.5
M+25 5' primer (20 µM)	200 nM	0.25
M-124 3' primer (20 µM)	200 nM	0.25
M-124 SIV 3' primer (20 µM)	200 nM	0.25
25× RT-PCR enzyme mix	1×	1
M+64 probe (6 µM)	60 nM	0.25
Detection enhancer (15×)	1×	1.67
Template	–	8
Total Reaction Volume	**–**	**25**

Table 4. Example thermocycler parameters

Stage	Cycles	Step	Time	Temperature
1	1		10 minutes	45°C
2	1		10 minutes	95°C
3	45	denaturation	1 second	94°C
		annealing*	30 seconds	60°C
		extension	15 seconds	72°C

*Collection of fluorescence

Viral isolates (or from clinical samples dependent on C_t value) can be subtyped using conventional methods or by real-time PCR assays that can differentiate the genetically distinct HxNy viruses (Bonin *et al.,* 2018; Chiapponi *et al.,* 2012; Henritzi *et al.,* 2016; Larsen *et al.,* 2020). Increasingly, differential HA and NA real-time PCRs are being used in many regions. Matrix RT-PCR positive diagnostic samples can also be subtyped through use of HA and NA subtyping RT-PCRs. Samples with high matrix C_t's may not be detectable by subtyping RT-PCRs and it may be necessary to attempt virus isolation prior to identifying the subtype. Screening and subtyping RT-PCR reagents are commercially available; however, laboratories need to ensure they will detect currently circulating influenza viruses in their area. In many instances, due to the increased genetic variability of HA and NA genes, it is necessary to conduct partial or complete gene sequencing of one or more of the IAV-S genes (i.e. neuraminidase, haemagglutinin) to ascertain the subtype of detected virus. Furthermore, virus genotyping based on sequencing of several or all gene segments is increasingly being used to determine and monitor virus diversity. A recently developed universal nomenclature web-based tool provides an accurate method to assign clade designations to H1-HA sequences; http://www.fludb.org (Anderson *et al.,* 2016). High throughput sequencing can be applied to obtain genomic information on the isolate or directly from field samples to speed up characterisation of the influenza virus (Lee *et al.,* 2016).

Tests should be validated for the region in which they are to be applied given the worldwide genetic and antigenic diversity of IAV-S.

1.7. Gene sequencing

In 2023, real-time RT-PCR is the preferred method of virus surveillance because the test provides rapid sensitive diagnostics for IAV-S with high throughputs. However, greater use of sequencing technologies particularly as unit costs reduce with improvement in technology, offer powerful opportunities to simultaneously detect and sequence from clinical samples in a laboratory or field setting, for example applying nanopore technology.

Increasingly gene sequencing is being applied not only to detailed characterisation of viruses for use in molecular epidemiology but also in virus subtyping and defining markers for host range including zoonotic risk (Chauhan & Gordon, 2022). Sanger sequencing methodology has been widely used for decades and enables the rapid determination of typically a single (HA) target gene in 24–36 hours and still has widespread utility. However, as genomic data can be rapidly determined using whole genome sequencing technology it enables a broader analysis using a range of bioinformatics tools (Zhang *et al.,* 2017). For example, with the advent of greater access to sequencing methodology either through specialised laboratories or commercial providers it is now possible to determine the genomic sequences of IAV-S to provide a level of characterisation important in rapid pathogen identification and disease investigations. Conventionally, nucleotide sequences have been used in outbreak epidemiology to infer virus origin and precise relationships between different viruses associated within the same event (by phylogeny) to support outbreak management. Virus gene sequences of haemagglutinin and neuraminidase can rapidly be compared to known sequences of all subtypes in gene databases and used to reveal the closest match thereby identifying the virus subtype and phylogenetic relationships. This often avoids the need to culture the virus for rapid identification although reliability and quality of data reduces with increasing cycle threshold values in samples from real-time RT-PCR testing.

Increasingly such analyses are being applied at the whole genome level to reveal virus genotypes and provide greater analytical specificity to the analyses. Such approaches are especially valuable to track as virus evolution can be more precisely mapped including change through genetic reassortment, a key mechanism associated with virus diversity and fitness for pigs. This approach is especially valuable for early or first incursions in a new event as it enables greater precision in determining virus origin and the mechanisms leading to the emergence of virus. Translation of nucleotide sequences of all genomic segments into amino acid sequences enables data mining for other virus characteristics or traits such as tropism, host range markers including zoonotic and predicted antiviral drug susceptibility, which are invaluable for informing outbreak management (Garrido-Mantilla *et al.,* 2019; Noronha *et al.,* 2012; Pulit-Penaloza *et al.,* 2019).

2. Serological tests

The primary serological test for detection of IAV-S antibodies is the HI test and it is HA subtype specific. Reference antigens should reflect the contemporary virus genetic lineages circulating in the region and as broadly cross reactive as possible with the specific subtype. It should be conducted on paired sera collected 10–21 days apart. A four-fold or greater increase in titre between the first and second samples is suggestive of a recent IAV-S. Additional serological tests that have been described but not commonly used are the virus neutralisation (Gauger & Vincent, 2020), agar gel immunodiffusion test, and indirect fluorescent antibody test. ELISA technology for detection of IAV-S antibodies is well described in the literature and commercial kits are available (Barbe *et al.,* 2009; Ciacci-Zanella *et al.,* 2010).

2.1. Haemagglutination inhibition test

see Section B.1.2.1.

2.2. Enzyme-linked immunosorbent assay

ELISA technology for detection of (IAV-S) antibodies has been described in the literature and ELISAs are available as commercially produced kits (Barbe *et al.,* 2009; Ciacci-Zanella *et al.,* 2010).

C. REQUIREMENT FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1.1. Rationale and intended use of the product

IAV-S infections can cause significant economic impact for producers because of reduced feed intake during illness resulting in decreased weight gain, increased days to market, and decreased feed efficiency. Where vaccination is practiced, vaccine is used to reduce the economic impact of disease by reducing the severity and duration of clinical signs. In addition, vaccines can reduce the level and duration of viral shedding, an important aspect for reducing virus transmission while minimising the risk of exposure for pigs and people. Generally these vaccines are applied to breeding sows but piglet vaccination is becoming more widespread as IAV-S prevalence increases. The utility and efficacy of vaccination is highly variable at the global scale with many factors requiring consideration (Sandbulte *et al.,* 2015). To date, the majority of IAV-S vaccines used in pigs have been conventional inactivated whole virus vaccines prepared from infective allantoic fluid of embryonated chicken eggs or cell culture fluids, inactivated by beta-propiolactone or formalin and emulsified with mineral oil adjuvants. Because of the potential for reassortment leading to increased virulence, live conventional influenza vaccines have been used on a limited scale. Live attenuated vaccines tend to result in broader immune responses by eliciting both, humoral and cellular immune responses and some may also have the advantage of being effective in the presence of maternally derived antibodies. Biotechnology holds great potential for generating live IAV-S vaccines with altered gene segments that reduce the risk of reassortment, limit replication and abrogate negative aspects of live IAV-S vaccines.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Strains used in vaccine production should be antigenically matched to IAV-S strains circulating in the field where vaccination is to be applied. Haemagglutination inhibition and neutralisation tests demonstrating cross-reactivity between antisera from pigs vaccinated with the candidate vaccine strain and current field isolates can be used for the selection.

Identity of the seed should be well documented, including the source and passage history of the virus. Antigenic characteristics such as haemagglutinin (HA) and neuraminidase (NA) subtype should be established. Haemagglutination inhibition and neuraminidase inhibition by subtype-specific antisera or real-time RT-PCR and sequencing can be used to establish the HA and NA subtypes. Also, aliquots of the master seed virus (MSV) can be neutralised with specific antiserum (Gauger & Vincent, 2020), e.g. antiserum produced against H1 or H3 IAV-S, then inoculated into the allantoic sac of 10-day old embryonated chicken eggs or on susceptible cell lines such as the MDCK cell line. Allantoic fluid or cell culture supernatant is harvested 72–96 hours post-inoculation and tested for HA activity. Identity is demonstrated by the lack of HA activity in the neutralised seed, and the presence of HA activity in the non-neutralised seed. Significant antigenic differences present in a given strain that set it apart from other members of its subtype, and that purportedly have a beneficial impact on its use as a vaccine, should be confirmed.

Factors that may contribute to instability during production, such as replication on an unusual cell line, should be investigated. If production is approved for five passages from the master seed, then sequencing of the genes for HA and NA at the maximum passage may be warranted to confirm the stability of the viral seed.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The purity of the seed and cells to be used for vaccine production must be demonstrated. The MSV should be free from adventitious agents, bacteria, or *Mycoplasma*, using tests known to be sensitive for detection of these microorganisms. The test aliquot should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against IAV-S and the virus/antibody mixture is cultured on several types of cell line monolayers. Cultures are subpassaged at 7-day intervals for a total of at least 14 days, then tested for cytopathogenic and haemadsorbing agents.

2.2. Method of manufacture

2.2.1. Procedure

Once the vaccine is shown to be efficacious, and the proposed conditions for production are acceptable to regulatory authorities, approval may be granted to manufacture vaccine dependent on local requirements. IAV-S can be grown in eggs or in cell culture. Selection of a culture method is dependent on the degree of virus adaptation, growth in medium, rate of mutation, and viral yield in the specific culture system. IAV-S vaccine products should be limited to five passages from the MSV to avoid genetic/antigenic variation. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. When the virus has reached its maximum titre, as determined by HA, CPE, fluorescent antibody assay, or other approved technique, the virus is clarified, filtered, and inactivated. Several inactivating agents have been used successfully, including formalin, beta-propiolactone or binary ethylenimine. Typically, an approved adjuvant is added to enhance the immune response.

2.2.2. Requirements for substrates and media

Cells are examined for adventitious viruses that may have infected the cells or seed during previous passages. Potential contaminants include bovine viral diarrhoea virus, reovirus, rabies virus, Aujeszky's disease (pseudorabies) virus, transmissible gastroenteritis virus, porcine respiratory coronavirus, porcine parvovirus, porcine adenovirus, haemagglutinating encephalomyelitis virus, porcine rotavirus, porcine circovirus, and porcine reproductive and respiratory syndrome virus. Cell lines on which the seed is tested include: an African green monkey kidney (Vero) cell line (rabies and reoviruses), a porcine cell line, a cell line of the species of cells used to propagate the seed, if not of porcine origin, and cell lines for any other species through which the seed has been passaged. Additionally, a cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended. Bovine viral diarrhoea virus is a potential contaminant introduced through the use of FBS in cell culture systems.

2.2.3. In-process controls

Cell cultures should be checked macroscopically for abnormalities or signs of contamination and discarded if unsatisfactory. A lot is ready to harvest when viral CPE has reached 80–90%. Virus concentration can be assessed using antigenic mass or infectivity assays.

2.2.4. Final product batch tests

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.

i) Sterility and purity

During production, tests for bacteria, *Mycoplasma*, and fungal contamination should be conducted on both inactivated and live vaccine harvest lots and confirmed on the completed product (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

ii) Safety

An inactivation kinetics study should be conducted using the approved inactivating agent on a viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, and then inoculated on to a susceptible cell line or into the allantoic sac of embyonated eggs, should indicate a linear and complete loss of titre by the end of the inactivation process. This is represented as less than one infectious particle per 10^4 litres of fluids following inactivation.

iii) Batch potency

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation and prior to

further processing. Relative potency ELISA, HA, and HI are among the assays that can be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

The potency assay established at the time of the minimum antigen protection study should be used to evaluate new lots for release. The assay needs to be specific and reproducible. It must reliably detect vaccines that are not sufficiently potent. If laboratory animal serology is used instead of swine serology, it should first be demonstrated that vaccination of the laboratory animal induces a specific, sensitive, dose-dependent response as measured in the potency assay and is correlated to protection in swine. A standard alternative is HAU or guinea-pig neutralisation units or GMNU (geometric mean of neutralising units induced in guinea-pigs after two immunisations with 0.5 ml of vaccine).

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Final container samples of completed product from inactivated vaccines should be tested in young mice for safety. Generally, healthy pigs of weaning age or older and pregnant sows at any stage of gestation may be safely vaccinated with inactivated IAV-S vaccines. Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended.

ii) Reversion-to-virulence for attenuated/live vaccines

Reversion-to-virulence for live viral vaccines is often demonstrated by back passage through susceptible species. Virus is isolated from the vaccinated animal and the isolated virus is then used to inoculate additional animals. Sequential passage through animals should show that animals remain clinically healthy with no demonstration of typical IAV-S-S signs.

iii) Environmental consideration

Inactivated IAV-S vaccines present no particular danger to the user, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. Modified live virus vaccines may pose a hazard to the user depending on the level of inactivation of the virus and the susceptibility of humans to the swine-adapted virus.

Preservatives should be avoided if possible, and where not possible, should be limited to the lowest concentration possible. The most common preservative is thimerosol, at a final concentration not to exceed 0.01% (1/10,000). Antibiotics may be used as preservatives in IAV-S vaccines but are limited as to kinds and amounts. Also restricted are residual antibiotics from cell culture media that may be present in the final product. For example, the total amount of preservative and residual gentamicin is not to exceed 30 μg/ml of vaccine.

Vaccine bottles, syringes, and needles may pose an environmental hazard for vaccines using adjuvants or preservatives and for modified live virus vaccines. Instructions for disposal should be included within the vaccine packaging information and based on current environmental regulations in the country of use.

2.3.2. Efficacy requirements

i) For animal production

A vaccination/challenge study in swine, using homologous and heterologous challenge strains, will indicate the degree of protection afforded by the vaccine. Swine used in vaccination/challenge studies should be free of antibodies against IAV-S at the start of the experiments. Vaccination/challenge studies should be conducted using virus produced by

the intended production method, at the maximum viral passage permitted, and using swine of the minimum recommended age listed on the label. Initially, lots are formulated to contain varying amounts of viral antigen. The test lot containing the least amount of antigen that demonstrates protection becomes the standard against which future production lots are measured. The most valuable criterion for blind trial evaluations of treatment groups is a statistically significant reduction of virus (titres and duration of shedding) in the respiratory tract of vaccinated pigs. Differences in clinical observations and lung lesions are also among the criteria used in evaluation of a successful trial. If *in-vivo* or *in-vitro* test methods are to be used to determine the potency of each production lot of vaccine, those assays should be conducted concurrent with the minimum antigen studies in order to establish the release criteria. Combination vaccines containing more than one strain of IAV-S are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label. Once a suitable potency assay has been identified, should antigenic drift require replacement of strains within the vaccine, strains of the same subtype can be evaluated in either the host animal or a correlated laboratory animal model. Other factors that play a role include the adjuvant and the antigenic dose. Consequently, it would appear that the efficacy of a vaccine will always have to be evaluated in swine.

If the vaccine is to be used in swine destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

ii) For control and eradication

The same principles apply as for animal production usage. In addition, it should be noted that antibody responses in vaccinated animals may not be differentiated from animals exposed to field virus. Therefore, vaccinated animals will need to be clearly identified if serological methods will be used in conjunction with compatible clinical signs to assess field virus exposure.

2.3.3. Stability

Vaccines should be stored with minimal exposure to light at 4°C±2°C, or as approved by the designated regulatory authorities. The shelf life should be determined by use of the approved potency test over the proposed period of viability.

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NB: There are WOAH Reference Laboratories for swine influenza (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for swine influenza

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.9.8.

SWINE VESICULAR DISEASE

SUMMARY

***Description and importance of the disease**: Swine vesicular disease (SVD) is a contagious disease of pigs, caused by an enterovirus and characterised by vesicles on the coronary bands, heels of the feet and occasionally on the lips, tongue, snout and teats. Strains of SVD virus (SVDV) vary in virulence, and the disease may be subclinical, mild or severe, the latter usually only being seen when pigs are housed on abrasive floors in damp conditions. The main importance of SVD is that it can be clinically indistinguishable from foot and mouth disease (FMD), and any outbreaks of vesicular disease in pigs must be assumed to be FMD until investigated by laboratory tests and proven otherwise. However, subclinical infection has been the most frequent condition observed during recent years.*

***Identification of the agent:** Where a vesicular condition is seen in pigs, the demonstration by enzyme-linked immunosorbent assay (ELISA) of SVD viral antigen in a sample of lesion material or vesicular fluid is sufficient for a positive diagnosis. If the quantity of lesion material submitted is not sufficient (less than 0.5 g), or if the test results are negative or inconclusive, a more sensitive test, such as the reverse transcriptase polymerase chain reaction (RT-PCR) or isolation of virus (VI) in porcine cell cultures, may be used. If any inoculated cultures subsequently develop a cytopathic effect, the demonstration of SVD viral antigen by ELISA or viral RNA by RT-PCR will suffice to make a positive diagnosis. Subclinical infection may be detected by random sampling of pen-floor faeces tested for identification of SVD virus using RT-PCR or VI tests.*

***Serological tests:** Serological tests can be used to help confirm clinical cases as well as to identify subclinical infections. Specific antibody to SVDV can be identified using ELISA for screening and the microneutralisation test for confirmation. A small proportion (up to 0.1%) of normal, uninfected pigs will react positively in serological tests for SVD. The reactivity of these singleton reactors is transient, so that they can be differentiated from infected pigs by resampling of the positive animal and its cohorts.*

Diagnostic and standard reagents are available from reference laboratories.

***Requirements for vaccines:** There are currently no commercial vaccines available against SVD.*

A. INTRODUCTION

Swine vesicular disease (SVD) can be a subclinical, mild or severe vesicular condition depending on the strain of virus involved, the route and dose of infection, and the husbandry conditions under which the pigs are kept. When it occurs clinically, SVD can be indistinguishable from foot and mouth disease (FMD) and therefore a differential diagnosis and laboratory investigation is an urgent requirement. However, during the present century, outbreaks of SVD have been less severe or with no clinical signs and infection has been detected when samples are tested for serosurveillance programmes or for export certification.

After its first detection in 1966, the disease occurred with epidemics in eastern and western Europe (during the 1970s and 1980s), and was also detected in East Asia. Since then, SVD has only been sporadically reported, mainly from Italy where its circulation is investigated and controlled through a virological and serological surveillance plan.

The incubation period for SVD is between 2 and 7 days, after which a transient fever of up to 41°C may occur. Vesicles then develop on the coronary band, typically at the junction with the heel. These may affect the whole coronary band

resulting in loss of the hoof. More rarely, vesicles may also appear on the snout, particularly on the dorsal surface, on the lips, tongue and teats, and shallow erosions may be seen on the knees. Affected pigs may be lame and off their feed for a few days. Abortion is not a typical feature of SVD. Recovery is usually complete in 2–3 weeks (Loxam & Hedger, 1983). Affected pigs may excrete virus from the nose and mouth and in the faeces up to 48 hours before the onset of clinical signs. Most virus is produced in the first 7 days after infection, and virus excretion from the nose and mouth normally stops within 2 weeks. Virus may continue to be shed for up to 3 months in the faeces, though under usual circumstances virus is detectable in faeces only up to 1 month. The SVD virus (SVDV) is extremely resistant to inactivation in the environment, and is stable in the pH range 2.5–12.0 (Mann, 1981). This is in contrast to the FMD virus, which is very labile outside the pH range 6.0–8.0.

Because SVD may be mild or subclinical, it is essential when submitting samples from suspect clinical cases that serum samples from both the suspect pigs and other apparently unaffected animals in the group be included. It is possible for SVD to circulate unnoticed until it affects a particularly susceptible group. Therefore, in order to ascertain how long infection has been present, it is necessary to look for seroconversion to SVDV in apparently healthy animals. Also the identification of the isotype of the immunoglobulins (M or G) to SVDV may help to ascertain the time of exposure to infection.

SVDV has been classified as a porcine variant of a human coxsackievirus B5 (*Enterovirus B*), in the family *Picornaviridae*. All isolates are classified in a single serotype, with four distinguishable antigenic/genomic variants (Brocchi *et al*., 1997), which evolved sequentially in different time-periods, except for the third and fourth variants that were co-circulating in Italy during 1992–1993. All SVDVs occurring since then diverge from a common origin and cluster in a unique antigenic/genomic lineage corresponding to the fourth and most recent group; however, two genomic sub-lineages are distinguishable within it (Knowles *et al*., 2007). Antigenically and genetically, SVDV is closely related to the human virus coxsackievirus B5 and it has been suggested that it arose through recombination with another human enterovirus, coxsackievirus A9 (Bruhn *et al.,* 2015). A second adaptation of a human enterovirus to cause vesicular disease in pigs was reported in Russia in 1975 involving coxsackievirus B4, which is serologically distinct from SVDV/CV-B5 (Lomakina *et al.,* 2016).

There are reports of seroconversion to SVDV in laboratory workers handling the agent. However, clinical disease in humans is reported to be mild, with the exception of a single case of meningitis associated with SVDV infection, and there have been no reported cases of seroconversion or disease in farmers or veterinarians working with infected pigs. Under experimental conditions, it has not been possible to show transmission of coxsackievirus B5 between pigs. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of swine vesicular disease and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Virus isolation	–	+	+	+++	–	–
RT-PCR	–	+++	+++	+++	–	–
ELISA for antigen detection	–	–	–	+++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
Virus neutralisation	+	+++	+	+	+	–
Competitive ELISA for Ab screening	+++	+++	+++	+	+++	–
ELISA for IgG and IgM identification	+	+++	+++	+	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcriptase polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
NOTE: Selection of assays suited for different purposes should take into account the different kinetics of the diagnostic targets (agent and antibodies) during infection.

1. Identification of the agent

Any vesicular condition in pigs could represent an FMD infection and differential diagnosis between FMD and other vesicular conditions, including SVD, is necessary. The diagnosis of SVD requires the facilities of a specialised laboratory. Countries that lack such a facility should send samples for investigation to a WOAH Reference Laboratory for SVD[1].

For clinical cases, the detection of antigens or genome of SVDV by means of enzyme-linked immunosorbent assay (ELISA) and reverse-transcriptase polymerase chain reaction (RT-PCR) has the same diagnostic value as virus isolation. Due to their speed, ELISA and RT-PCR make suitable screening tests. However, virus isolation is the reference method and should be used if a positive ELISA or RT-PCR result is not associated with the detection of clinical signs of disease, the detection of seropositive pigs, or a direct epidemiological connection with a confirmed outbreak.

If there are clinical signs, investigation should start with the examination of a 10% suspension of lesion material in phosphate buffered saline (PBS) or tissue culture medium and antibiotics. Faecal samples are the specimen of choice for the detection of virus where subclinical SVD is suspected. Faecal samples can be collected from individual pigs or from the floor of premises suspected to contain, or to have contained, pigs infected with SVD. The level of virus in faeces is usually insufficient for detection by ELISA and the use of RT-PCR and/or virus isolation is required. A significant proportion of faecal samples inoculated into cell cultures will give rise to the growth of other enteroviruses. These can be differentiated from SVDV by ELISA or RT-PCR, but they may also outgrow SVDV that is present, and give rise to false negative results. Therefore, RT-PCR is more sensitive than virus isolation when applied to faecal samples.

1.1. Preparation of samples

1.1.1. Lesion material

Samples should be transported in phosphate buffered saline (PBS) with antibiotics, mixed with glycerol (1/1), pH 7.2–7.6. A suspension is prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of PBS or tissue culture medium and antibiotics.

1 Please consult the WOAH web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

Further medium should be added to obtain approximately a 10% suspension. This is clarified by centrifugation at 2000 ***g*** for 20–30 minutes and the supernatant is harvested.

1.1.2. Faecal samples

Faecal material (approximately 20 g) is resuspended in a minimal amount of tissue culture medium or phosphate buffer (0.04 M phosphate buffer or PBS). The suspension is homogenised by vortexing and clarified by centrifugation at 2000 ***g*** for 20–30 minutes; the supernatant is harvested and filtered through 0.45 μm filter.

1.2. Virus isolation

A portion of the clarified epithelial or faecal suspension is inoculated on to monolayers of IB-RS-2 cells or other susceptible porcine cell line, grown in appropriate containers (25 cm^2 flasks, rolling tubes, 24-, 12-, 6-well plates). For differential diagnosis (e.g. FMD) in case of clinical lesions bovine cell culture systems should also be employed in parallel. Generally SVDV will grow in cells of porcine origin only. Tissue culture medium is supplemented with 10% bovine serum for cell growth, with 1-3% bovine serum for maintenance, and with antibiotics.

Cultures are examined daily. If a cytopathic effect (CPE) is observed, the supernatant fluid is harvested and virus identification is performed by ELISA (or other appropriate test, e.g. RT-PCR). Negative cultures are blind-passaged after 48 or 72 hours, and observed for a further 2–3 days. If no CPE is evident after the second passage, the sample is recorded "NVD" (no virus detected). When isolating virus from faeces in which the amount of virus present may be low, a third tissue culture passage may be required.

1.3. Immunological methods

1.3.1. Enzyme-linked immunosorbent assay

The detection of SVD viral antigen by an indirect sandwich ELISA has replaced the complement fixation test as the method of choice. The test has the same format as that used for FMD diagnosis. Wells of ELISA plates are coated with rabbit antiserum to SVDV. This is the capture serum. Test sample suspensions are added and incubated. Appropriate controls are also included. Guinea-pig anti-SVD detection serum is added at the next stage followed by rabbit anti-guinea-pig serum conjugated to horseradish peroxidase. Extensive washing is carried out between each stage to remove unbound reagents. A positive reaction is indicated if there is a colour reaction on the addition of chromogen (for example orthophenylenediamine) and substrate (H_2O_2). With strong positive reactions this will be evident to the naked eye, but results can also be read spectrophotometrically at the appropriate wavelength, in which case an absorbance reading ≥0.1 above background indicates a positive reaction. As an alternative to guinea-pig and rabbit antisera, suitable monoclonal antibodies (MAbs) can be used, coated to the ELISA plate as the capture antibody and peroxidase conjugated as detector antibody. For example, a simple sandwich ELISA performed with MAb 5B7 as both catching and conjugated/detector antibody, that represents also the reference method for the serological competitive ELISA, is suited for the detection of SVD viral antigen.

A MAb-based ELISA can also be used to study antigenic variation among strains of SVDV. Tissue-culture grown viral strains are trapped by a rabbit hyperimmune antiserum to SVDV adsorbed to the solid phase. Appropriate panels of MAbs are then reacted and the binding of MAbs to field strains is compared with the binding of MAbs to the parental strains. Strong binding indicates the presence of epitopes shared between the parental and the field strains (Brocchi *et al*., 1997).

1.4. Nucleic acid recognition methods

1.4.1. Reverse-transcriptase-polymerase chain reaction

Reverse-transcriptase followed by the PCR (RT-PCR) is a useful method to detect SVD viral genome in a variety of samples from clinical and subclinical cases. Several methods have been described (Benedetti *et al*., 2010; Callens & De Clercq, 1999; Fallacara *et al*., 2000; Hakhverdyan *et al*., 2006; Lin *et al*., 1997; McMenamy *et al*., 2011; Nunez *et al*., 1998; Reid *et al*., 2004a; 2004b; Vangrysperre & De Clercq, 1996), employing different techniques for RNA extraction, targeting

different parts of the SVDV genome and using different approaches to detect the DNA products of amplification. However, in a comparative study on positive faecal samples from many different SVD outbreaks, the one step RT-PCR (Benedetti *et al.,* 2010) had the best diagnostic performance, with the capability to reveal all the circulating genomic sub-lineages, compared with two real-time RT-PCR assays targeting the 5'-untranslated region (Reid *et al.,* 2004a; 2004b), and an RT-loop-mediated isothermal amplification (LAMP) assay (Blomström *et al*., 2008).

The method reported below describes an RNA immune-extraction protocol and a one-step RT-PCR protocol targeting SVDV *3D* region, which codes for the RNA-polymerase.

To isolate RNA the immunocapture technique using a SVDV-specific MAb has been shown to be particularly effective in the case of faecal samples (Fallacara *et al*., 2000): it enables enrichment and purification of the SVDV, usually present in low concentration in faeces, with efficient removal of potential reaction inhibitors.

This method is suitable for laboratories without equipment for real-time detection of DNA amplification products, but where such facilities are available an approach such as that described by Reid *et al.* (2004a; 2004b) offers advantages in terms of ease of use and reduced risk of laboratory contamination by PCR products.

1.4.2. RNA immune-extraction

i) RNA Immune-extraction: Coat wells of an ELISA plate with a saturating solution of MAb 5B7 (200 µl/well, diluted in carbonate-bicarbonate buffer) by overnight incubation at 4°C. Wash plates three times with PBS. Use plates immediately or store at –20°C for up to 2–3 weeks, or more if stabilised.

ii) Distribute each sample (faeces suspension) into three wells of the 5B7-coated plate (200 µl/well, 600 µl of sample in total).

iii) After incubation for 1 hour at 37°C with very slow shaking, wash wells three times with PBS. Washing is performed manually, in order to avoid cross-contamination between wells.

iv) RNA is extracted from each sample by adding approximately 100 µl/well of lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7, 0.5% Sarkosyl). Incubate wells for 3–5 minutes and recover the sample from the three wells (300–350 µl total), and transfer into a single tube.

v) RNA is then precipitated by adding a mixture of 750 µl of absolute ethanol and 35 µl of 3 M sodium acetate (pH 5.2); vials are vortexed and incubated at –20°C for a minimum of 1 hour (prolonged overnight precipitation at –20°C may also be suitable).

vi) Centrifuge the sample at 15,500–16,000 ***g*** for 30 minutes at 4°C, after which a pellet should be visible which should be washed with 500 µl of 70% cold ethanol (centrifuged at 15,500–16,000 ***g*** for 10 minutes at 4°C) and dried.

vii) Resuspend the RNA pellet in 20 µl of DEPC water, or commercially available RNase-free water.

NOTE: As an alternative to immunocapture, RNA extraction can be performed using suitable commercially available kits based on chaotropic salt lysis and silica RNA affinity.

1.4.2.1. One step RT-PCR

The following protocol for the conventional RT-PCR may need to be adjusted to suit particular reagents used.

i) Assemble the reaction mix (20 µl is the final volume for each test sample)

Rnase free water	10.75 µl
5× RT-PCR buffer	5 µl
dNTP mix (10 mM each dNTP)	1 µl
pSVDV-SS4 Forward Primer 10 pmol/ul*	1 µl
pSVDV-SA2 Reverse Primer 10 pmol/ul*	1 µl

RNAse inhibitor	0.25 µl (equivalent to 5 U)
RT-PCR enzyme mix	1 µl
*Primers sequence:	pSVDV-SS4 5'-TTC-AGA-ATG-ATT-GCA-TAT-GGG-G-3' pSVDV-SA2 5'-TCA-CGT-TTG-TCC-AGG-TTA-CY-3'

ii) Add 5 µl of each template RNA to 20 µl reaction mix.

iii) Run the following program in a thermal cycler:

One cycle at 50°C for 30 minutes (reverse transcription step)

One cycle at 95°C for 15 minutes (initial activation step)

40 cycles of 94°C for 20 seconds (denaturation), 60°C for 20 seconds (annealing), 72°C for 45 seconds (extension)

One cycle at 72°C for 10 minutes (final extension).

iv) Mix a 20 µl aliquot of each sample with 4 µl of loading dye and load onto a 2% agarose gel containing an appropriate DNA intercalating dye. After electrophoresis, a positive result is indicated by the presence of a 154 bp fragment of the SVDV RNA polymerase (3D) encoding region in the gel. Alternatively, gel can be stained after electrophoresis to reduce contamination of equipment by soaking in a staining solution.

1.4.2.2. Real-time RT-PCR

This test can also be adapted to the format of real-time RT-PCR with dedicated reagents/kits, in the presence of a suitable DNA stain, using the following adjusted programme in a real-time PCR cycler:

i) 1 cycle at 50°C for 30 minutes (reverse transcription step)

ii) 1 cycle at 95°C for 15 minutes (initial activation step)

iii) 40 cycles, each composed of 94°C for 15 seconds (denaturation), 58°C for 30 seconds (annealing), 72°C for 30 seconds (extension), 77°C for 15 seconds (detection).

For the melting cycle:

iv) 1 cycle at 72°C for 1 minute and increasing temperature from 72°C to 95°C, by incremental steps of 0.5°C for 5 seconds each. Specific amplification products for SVDV generate melting curves with a peak within the temperature range 79.5–82.5°C.

1.4.3. Sequence analyses

Comparative analysis of sequences of the viral genome is useful to establish relationships between isolates of SVDV. By sequencing the 1D region, which codes for the major structural protein VP1, or the 3D region, it has been possible to group strains of SVDV according to their sequence homology, and epidemiologically to relate strains causing disease in different locations or at different times (Brocchi *et al*., 1997). The databases of *1D* and *3D* gene sequences of SVDVs are held at the WOAH Reference Laboratory, Pirbright, UK and the WOAH Reference Laboratory, Brescia, Italy, respectively. Further sequences (including those for complete SVDV genomes) are available via the International Nucleotide Sequence Database Collaboration (including GenBank, ENA, and DDBJ).

2. Serological tests

Serological assays are used in the laboratory confirmation of outbreaks, for serological surveillance and for export certification of pigs. SVD is often diagnosed solely on the evidence of serological tests. Because of the subclinical or mild nature of the disease, it is often first suspected following routine serology for disease surveillance or export certification. The virus neutralisation (VN) test, the double immunodiffusion test, the radial immunodiffusion test, the counter immunoelectrophoresis test and the ELISA have all been described for the detection of antibodies to SVDV (Brocchi *et al*., 1995; Donaldson *et al*., 1983; Golding *et al*., 1976). However, the VN test and the ELISA are the only techniques commonly used. The VN test is the accepted confirmatory test, but has the disadvantage that it

takes 2–3 days to complete and requires tissue culture facilities and handling of live virus in appropriate biosafety and containment facilities as determined by biorisk analysis (see chapter 1.1.4). The ELISA is quicker and simpler. A small proportion of sera from animals with no previous exposure to SVDV may react positively in serological tests for antibody to SVDV. The 5B7 MAb competitive ELISA (MAC-ELISA) is a reliable technique for detecting SVD antibody (Brocchi *et al*., 1995; Heckert *et al*., 1998) and similar results have been obtained with other ELISAs (Chenard *et al*., 1998; Ko *et al*., 2005). Results from a small proportion, 0.2%–0.4%, of sera from normal pigs are borderline or positive by the MAC-ELISA and should be retested by the VN test. Up to approximately 50% of these sera will also be positive by the VN test (i.e. 0.1–0.2% of the original population). Animals that test positive by ELISA but negative by VN test can be regarded as uninfected. Repeat samples should be collected from animals positive in both tests and from cohorts. A constant or declining titre in the positive animal and the absence of antibody to SVDV in cohorts confirms the status of the positive animal as a 'singleton reactor'. The factors responsible for 'singleton reactors' are unknown. Serological cross-reactivity with SVDV might arise due to infection with another, as yet unidentified, picornavirus or may be due to other non-specific factors present in the serum. Identification of the isotype of antibody present in positive sera (Brocchi *et al*., 1995) can be helpful as sera from 'singleton' reactors usually contain exclusively IgM and do not convert to IgG (De Clercq, 1998). IgM/IgG isotype-specific ELISAs are also helpful in assessing the time of infection in the pig or on the infected premises. The presence of IgM, alone or together with IgG, is evidence of recent infection and indicative of virus shedding, while detection of IgG alone suggests an older exposure to infection (Brocchi *et al*., 1995).

2.1. Virus neutralisation

The quantitative VN microtest for antibody to SVDV is performed using IB-RS-2 cells (or suitable susceptible porcine cells) in flat-bottomed tissue-culture grade microtitre plates.

Virus is grown on IB-RS-2 cell monolayers and stored at –20°C after the addition of an equal volume of glycerol. SVDV has been found to be stable under these conditions for at least 1 year. The sera are inactivated at 56°C for 30 minutes before testing. A suitable medium is Eagle's complete medium/LYH with antibiotics.

The test is an equal volume test in 50 µl volumes:

i) Starting from a 1/4 dilution, sera are diluted in a twofold dilution series across the plate, two rows of wells per serum and a volume of 50 µl.

ii) Previously titrated virus is added; each 50 µl unit volume of virus suspension contains about 100 $TCID_{50}$ (50% tissue culture infective dose).

iii) Controls include at least a weak positive serum and a negative serum, a cell control, a medium control and a virus titration used to calculate the actual virus titre used in the test.

iv) Incubate at 37°C for 1 hour with the plates covered.

v) A cell suspension at 10^6 cells/ml is prepared in medium containing 10% bovine serum for cell growth. 50 µl of cell suspension is added to each well.

vi) Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 5% carbon dioxide at 37°C for 2–3 days.

vii) Microscopic readings are feasible after 48–72 hours; the plates may be finally fixed and stained on the third day. Fixation is effected with 10% formalin/saline for 30 minutes; staining is done by immersion in 0.05% methylene blue in 10% formalin for 30 minutes. The plates are rinsed in tapwater. Positive readings are blue-stained cell sheets (where the virus has been neutralised and the cells remain intact), whilst empty wells (where virus has not been neutralised) are read as negative.

viii) Interpretation of the results

The test is considered to be valid when the amount of virus actually used per well is between $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$, and the positive standard sera are within twofold of their expected titre. Titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected. Laboratories should establish their own cut-off titres by reference to both results from a negative population and standard reagents available from the WOAH Reference Laboratories, in particular the low-positive serum defining the lowest level of antibodies that Laboratories should consistently score positive.

2.2. Enzyme-linked immunosorbent assay

Commercial kits are available for antibody detection in pig specimens. In the ELISA developed by Brocchi *et al.* (1995), the SVD viral antigen is trapped to the solid phase using the MAb 5B7. The ability of test sera to inhibit the binding of peroxidase-conjugated MAb 5B7 to the trapped antigen is then evaluated. Finally, the amount of conjugated MAb bound is detected by the addition of substrate and chromogen.

i) ELISA plates are coated with 50 µl/well of MAb 5B7 at a saturating dilution in carbonate/bicarbonate buffer, pH 9.6, by overnight incubation at 4°C.

ii) The plates are washed three times with PBS containing 0.05% Tween 20, and 50 µl of SVD antigen (SVDV grown in IB-RS-2 cells, clarified, filtered and BEI-inactivated) at a predetermined optimal dilution, is added to each well. The optimal dilution of antigen is determined by checkerboard titrations of antigen and conjugated MAb that define the working dilution giving an absorbance on the upper part of the linear region of the antigen titration curve (between 1.5 and 2.0 optical density units). Plates are then incubated for 1 hour at 37°C.

iii) After three additional washes, 50 µl of diluted test sera (inactivation is irrelevant) and control sera are incubated with the trapped antigen for 1 hour at 37°C. Sera can be tested at a single dilution (1/7.5) or titrated. In the latter case, three-fold dilutions of sera are obtained directly in ELISA wells by adding 10 µl of serum to 65 µl of buffer (1/7.5 dilution) then transferring 25 µl to sequential wells containing 50 µl of buffer, mixing, and finally discarding 25 µl. For spot-test, the screening dilution 1/7.5 is obtained by adding 7µl of each test serum (and control sera) to 45 µl of buffer previously distributed into wells.

iv) After incubation for 1 hour, 25 µl of an optimal dilution of peroxidase-conjugated MAb 5B7 (see step ii above) is added to each well and the plates are incubated at 37°C for a further 1 hour.

v) After a final series of washes, the colorimetric reaction is developed by distributing 50 µl per well of the substrate solution (for example 0.5 mg/ml orthophenylene-diamine in phosphate/citrate buffer, pH 5, containing 0.02% H_2O_2).

vi) The reaction is stopped after 10 minutes by adding 50 µl of 2N H_2SO_4. The absorbance is read at the appropriate wavelength using a microplate reader.

Antigen, sera and conjugate are diluted in PBS, pH 7.4, containing 0.05% Tween 20 and 1% yeast extract; the dilution buffer for sera contains, in addition, 1.0% mouse serum (or alternatively another source of murine immunoglobulins) to prevent nonspecific binding of pig serum to MAb 5B7 either coated to the plate or conjugated to peroxidase.

vii) *Controls:* Four wells on each plate containing all reactants except test serum confirm the maximum absorbance reading for the antigen; negative pig serum; a low positive standard pig serum; optionally, a strong positive pig serum at four dilutions, previously calibrated in order to give ≥50% inhibition (see step viii below) at the highest dilution.

viii) Interpretation of the results: Reactions are expressed as the percentage inhibition by each test serum of the MAb reaction with the SVD antigen. Sera are considered to be positive when producing an inhibition ≥80% at the 1/7.5 dilution; negative when producing an inhibition <70% at the 1/7.5 dilution; doubtful when producing an inhibition ≥70% and <80% at the 1/7.5 dilution. The second dilution (1/22.5) provides an indication of the level of antibodies: strongly positive sera show >80% inhibition at both 1/7.5 and 1/22.5 dilutions, while sera registering >80% inhibition at the 1/7.5 dilution but <50% inhibition at the 1/22.5 dilution are considered to be low positive or borderline. All positive, borderline and doubtful sera should be confirmed using the VN test.

STANDARD REFERENCE SERA FOR SVD SEROLOGY

The WOAH Reference Laboratory, Pirbright, UK maintains a panel of reference sera that have been extensively validated by the National SVD Reference Laboratories of the Member States of the European Union. This panel includes the low-positive serum defining the lowest level of antibodies that should consistently provide a positive

result by ELISA and Virus Neutralisation (RS01-04-94 or equivalent). Positive sera equivalent to these reference standards and Mab 5B7 are available at the WOAH Reference Laboratory, Brescia, Italy.

C. REQUIREMENTS FOR VACCINES

No commercial SVD vaccines are currently available.

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NB: There are WOAH Reference Laboratories for swine vesicular disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for swine vesicular disease

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 3.9.9.

TESCHOVIRUS ENCEPHALOMYELITIS

SUMMARY

Teschovirus encephalomyelitis was first described as a particularly virulent, highly fatal encephalomyelitis of pigs and was previously known as Teschen disease (or enterovirus encephalomyelitis). It is caused by strains of porcine teschovirus serotype 1 (PTV-1) of the genus Teschovirus, *family* Picornaviridae*. Less severe forms of the disease were first recognised in the United Kingdom, where it was called Talfan disease, and in mainland Europe, where it was called poliomyelitis suum or benign enzootic paresis. In addition to PTV-1 strains, the milder form of the disease can be caused by other PTV serotypes, including PTV-2, -3, -4, -5, -6, -9 and -10.*

The disease was first described in Teschen, Czechoslovakia in 1929. During the 1940s and 1950s it caused serious losses in European countries and was spread to other continents. More recently outbreaks have occurred in Haiti and the Dominican Republic. The clinical disease is otherwise rare and has not been reported in Western Europe since 1980. However, there has been serological evidence that virus variants, which are not pathogenic or of low pathogenicity, circulate in pig populations.

Identification of the agent: *The virus has affinity for the central nervous system and therefore suspensions of brain and spinal cord from affected pigs are used as inocula for virus isolation. The virus propagates successfully on monolayers derived from swine tissue, in particular from kidney. If PTV is present, it gives rise to specific cytopathic effects characterised by rounded refractile cells. For PTV identification and serotyping, suitable tests are employed using specific antisera or monoclonal antibodies against standard strains of PTV. Virus neutralisation tests and indirect fluorescent antibody tests are preferred. Reverse-transcription polymerase chain reaction amplification of parts of the viral genome is possible, but as yet no specific tests have formally been accepted for diagnosis.*

Serological tests: *As the seroprevalence of PTV-1 may exceed 60% in healthy pig populations in Central Europe, and identical clinical signs may be caused by other viruses, including other serotypes of PTV, a single serological test for PTV-1 giving positive results does not indicate that the neurological signs observed are actually caused by a PTV-1 infection. A four-fold rise in titre together with typical signs should be considered to be an indication that PTV-1 infection caused clinical disease. For screening for specific antibodies in pig populations, it is recommended to use the virus neutralisation test in microtitre plates or the enzyme-linked immunosorbent assay.*

Requirements for vaccines: *When clinical disease was common, vaccines were available and used; however, as the disease is now rare, vaccines are no longer available.*

A. INTRODUCTION

Teschovirus encephalomyelitis (previously Teschen/Talfan diseases, and later enterovirus encephalomyelitis) is an acute condition of pigs characterised by central nervous system (CNS) disorders. Teschen is the name of the town in the Czech Republic where the disease was first recognised in 1929 (Klobouk, 1931; 1933). In the 1950s, the disease spread throughout Europe and caused huge losses to the pig breeding industry. Less severe forms of the disease were first recognised in the United Kingdom, where it was called Talfan disease, and in Denmark, where it was called poliomyelitis suum; these were benign enzootics of swine. Teschovirus encephalomyelitis has not been reported in Western Europe since 1980 (Austria) and the disease is now considered rare. In the last 20 years (since 1996) disease was reported to WOAH by the following countries: Belarus (1996, 1999 and 2005), Japan (2002), Latvia (1997 and 2000–2002), Madagascar (1996–2000, 2002 and 2004–2005), Moldavia (2002–2004), Romania

(2002), Russia (2004), Uganda (2001) and Ukraine (1996–2005). In most of these cases it is not known if diagnosis was made purely on clinical grounds or in conjunction with laboratory tests; the exception being in Japan in 2002 (Yamada *et al*., 2004).

The causal agent of teschovirus encephalomyelitis is porcine teschovirus serotype 1 (PTV-1), which belongs to the species *Teschovirus A*, genus *Teschovirus*, family *Picornaviridae* (Betts, 1960; Klobouk, 1933; Knowles *et al*., 2012). Originally the PTVs were classified within the genus *Enterovirus* and the original 11 porcine enterovirus (PEV) serotypes, PEV-1 to PEV-11, were placed in three groups – I, II and III – on the basis of cytopathic effect (CPE) produced, serological assays and replication in different cell cultures (Knowles *et al*., 1979). PEV-1 to PEV-7 and PEV-11 to PEV-13 were identified as group I. Based on nucleotide sequencing and phylogenetic analysis, the PEV group I viruses have now been placed in the genus *Teschovirus*. PEV-1 to -7 have been renamed PTV-1 to -7, and PEV-11 to -13 were renamed PTV-8 to 10; additional types PTV-11 to PTV-13, have also been described (based on nucleotide sequence differences in VP1) (Boros *et al*., 2012; Cano-Gomez *et al*., 2011; Krumbholz *et al*., 2002). PEV group II contained PEV-8, which has now been reclassified as porcine sapelovirus 1, species *Sapelovirus A*, genus *Sapelovirus*. Group III consisted of PEV-9 and PEV-10, and has now been reclassified as enterovirus G1 and G2, species *Enterovirus G*, genus *Enterovirus*.

Outbreaks of severe teschovirus encephalomyelitis in pigs occurred in Haiti in February 2009, and PTV-1 was isolated from brain samples of sick pigs. Phylogenetic analyses on the polyprotein gene indicated that the Haitian isolate was closely related to other PTV-1 strains, including the strain Konratice that was isolated in Czech Republic from pigs with Teschen disease (Deng *et al.,* 2012). Surveillance studies indicated that infection was prevalent in multiple regions in Haiti and the Dominican Republic.

PTV-2, -3, -4, -5, -6, -9 and -10 have been isolated from pigs with milder forms of the disease (Witte von *et al*., 1994). PTV infections often do not produce clinical signs. Serotypes may be differentiated using a virus neutralisation (VN) test (Betts, 1960; Knowles *et al*., 1979), complement fixation test (Knowles & Buckley, 1980) or indirect fluorescent antibody (IFA) test (Auerbach *et al*., 1994; Romanenko *et al*., 1982).

PTV infections only occur in swine (including wild boar); other animal species, including humans, are not known to be susceptible.

Differential diagnoses include pseudorabies (Aujeszky's disease) and classical swine fever (acute form). In addition, Japanese encephalitis, *Streptococcus suis* and haemagglutinating encephalomyelitis may occasionally produce similar clinical signs. Non-infectious aetiologies, in particular toxicities, must also be considered.

PTV may be identified serologically using standard antisera that have been prepared by hyperimmunisation of guinea-pigs, rabbits, or colostrum-deprived piglets with standard strains of PTV serotypes 1–11; these reagents are not known to be available for types 12 and 13.

The virus enters the animal via the oral or nasal cavity. The incubation period is about 14 days. The main signs of the prodromal stage are fever up to 41.5°C, lassitude, anorexia and locomotor disturbances. This stage is followed by hypersensitivity, tremors, clonic spasms of the legs, flaccid paralysis, opisthotonos and nystagmus; convulsions may be observed in young pigs. In the final clinical stage, paralysis proceeding from the hind part through the loins to the fore part of the body is observed. Paralysis of the thermoregulatory centre results in hypothermy. When respiratory muscles are paralysed, the animal dies of suffocation.

For histological diagnosis, samples of cerebrum, cerebellum, diencephalon, medulla oblongata and cervical and lumbar spinal cord are collected. The samples are fixed in formaldehyde and sections are stained using conventional histological methods. The virus multiplies in the CNS causing a nonsuppurative polio-encephalomyelitis with lymphocytic perivascular cuffs, especially in the spinal cord (Klobouk, 1931). Pathological changes are observed in the grey matter of the diencephalon, cerebellum, medulla oblongata and in the ventral horns of the spinal cord, consistently including dorsal root ganglia and trigeminal ganglia (ganglioneurites) and to a lesser extent in the cerebral hemispheres. Lesions may involve the dorsal horns of the spinal cord in very young animals. Degeneration of neurons (swelling, chromatolysis, necrosis, neuronophagia, axonal degeneration) and their replacement by microgliosis (astrocytosis, astrogliosis) develops in the late stage of the disease (Cantile & Youssef, 2016).

Detection of teschovirus antigens by immunohistochemistry on fixed, paraffin-embedded CNS sections is very difficult and not consistently possible. If suitable specific antisera or monoclonal antibodies are available, as well

as specific detection techniques, correlation of pathological changes with the location of the agent may be possible on fixed, paraffin-embedded sections of the CNS.

Laboratory diagnosis of the disease is based on identification of the virus in the CNS of affected pigs, and on the detection of specific antibodies in the blood of convalescent animals.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of Teschovirus encephalomyelitis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Virus isolation	–	+++	–	+++	+	–
Antigen detection	–	–	–	++	++	–
Real-time RT-PCR	–	+	–	++	++	–
Detection of immune response						
AGID	–	–	–	+	–	–
CFT	–	–	–	++	–	–
ELISA	–	–	–	++	+	–
VN	–	–	–	+++	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; AGID = agar gel immunodiffusion;
CFT = complement fixation test; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.
[(a)] A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Isolation of the virus

Progress in diagnosis of teschovirus encephalomyelitis and vaccine production has been made possible by the propagation of virus in cell culture (Madr, 1959; Mayr & Schwoebel, 1957).

Samples of brain and spinal cord are collected from pigs slaughtered at an early clinical stage of the disease. When not processed immediately, the samples should be placed in a solution prepared from equal parts of phosphate buffered isotonic saline solution (PBS), pH 7.4, and glycerol. Pieces of tissue are minced to prepare a 10% (w/v) suspension in PBS. The suspension is centrifuged at 800 ***g*** for 10 minutes and the supernatant fluid is used for inoculation of cell cultures. Monolayer cultures of primary porcine kidney or established cell lines derived from porcine tissue are suitable for isolation of PTV.

1.1.1. Test procedure

i) Test tubes or tissue culture vessels with monolayer cell cultures are used. Growth medium is discarded and tubes or vessels are inoculated with 0.1 ml of suspect tissue homogenate.

ii) Inoculated test tubes are placed on a roller drum or tissue culture vessels are placed on a tray and incubated for 1 hour at 37°C.

iii) The inoculum is discarded; the tubes or tissue culture vessels are washed with PBS and replenished with 1–20 ml (depending on the type of tissue culture vessel used) of maintenance medium without calf serum.

iv) The tubes are examined microscopically each day. If the sample contains PTV, characteristic CPE will be seen after 3–4 days. The CPE is characterised by small foci of rounded refractile cells. After several passages the virus grows better and produces complete CPE. The identity of PTV can be confirmed by the use of specific antiserum or monoclonal antibodies. The VN or the IFA test is best suited to this purpose. Once an isolate has been identified serologically as PTV, piglet inoculation is the only certain means of determining that the given isolate is pathogenic.

1.2. Virus neutralisation test for porcine teschovirus identification

The virus harvested from cell cultures is diluted in cell culture maintenance medium over the range 10^{-1} to 10^{-6} in tenfold steps. For teschovirus serotyping, 12 rows of each dilution are prepared; 50 µl of standard antisera to PTV-1–11 diluted 1/10 is added to rows 1–11 and 50 µl of negative serum is added to the last row; if antisera to types 12 and 13 are available, a second plate would be required. Mixtures are incubated overnight at 4°C or for 1 hour at 37°C and thereafter inoculated into roller tube cultures or into wells of microtitration plates with confluent monolayer cell cultures. The inoculated cell cultures are incubated at 37°C. Assessment is carried out 72 hours later and every following day up to day 10, depending on when the CPE is seen. The identification of a PTV serotype is confirmed if the titre of the isolated virus in the presence of that antiserum is at least 10^3 lower than that virus incubated with negative serum.

1.3. Indirect fluorescent antibody test for the confirmation of porcine teschovirus antigen in cells

The IFA test is based on the reaction of the antigens in infected cells with specific antibodies in positive serum (Romanenko *et al*., 1982). The reaction is visualised by a fluorescein isothiocyanate (FITC)-conjugated antiglobulin, using a microscope with a UV or a blue light source. The antigen is detectable in cells 12 hours after the infection with PTV, i.e. before the development of CPE. Polyclonal antisera often show cross-reactivity with different PTV types, which can confuse the interpretation of results.

1.3.1. Test procedure

i) Monolayers of porcine kidney cells on cover-slips are inoculated with the suspected material. Positive and negative controls should be processed in parallel with the test specimens.

ii) After incubation for 12–16 hours, the cover-slips are removed, washed twice in PBS, air-dried and fixed in cold acetone for 5–15 minutes.

iii) The cover-slips are placed into a wet box and flooded with rabbit or pig hyperimmune anti-PTV serum optimally diluted 1/10 with PBS or with PTV-specific monoclonal antibody at working dilution.

iv) The wet box is closed and incubated at 37°C for 60 minutes.

v) The cover-slips are removed and washed three times in PBS, then flooded with FITC-conjugated anti-rabbit or anti-pig goat serum, at a previously assessed working dilution, and incubated at 37°C for 30 minutes.

vi) The cover-slips are then washed three times with PBS, air-dried and mounted in 0.1 M Tris-buffered glycerol, pH 8.6.

After processing, the cover-slips are examined microscopically. The control slides are examined first to confirm that the fluorescence observed is specific. The fluorescence is apple green in colour and occurs in the cell cytoplasm and at the periphery of the nucleus. Instead of cover-slips, multispot slides or multiwell plates can also be used.

1.4. Reverse-transcription polymerase chain reaction

The reverse-transcription polymerase chain reaction (RT-PCR) provides a method for detection and differentiation of specific gene regions of porcine teschoviruses (Palmquist *et al*., 2002; Zell *et al*., 2000). The nested RT-PCR with specific primer sets has been used to differentiate between PTVs and PEVs (Zell *et al*., 2000). PCR is more rapid and less laborious than virus isolation by tissue culture technique and serotyping. However, the PCR technique is currently restricted to specialised laboratories.

2. Serological tests

As the seroprevalence of PTV-1 may exceed 60% in healthy pig populations in some countries of Central Europe, and identical clinical signs may be caused by other viruses – including other serotypes of PTV – a single serological test for PTV-1 giving positive results does not indicate that neurological signs observed are actually caused by PTV-1. A four-fold rise in titre together with typical signs should be considered to be an indication that PTV-1 infection caused clinical disease. Another reason that paired serum samples are needed for confirmation of the significance of titres is that cross-reactions have been reported with orphan teschoviruses.

Pigs that have recovered from disease, or those with inapparent disease, produce specific antibodies. Several serological methods are available for their detection, of which the microtitre VN test using pig kidney cell cultures is the most useful (Mayr & Bibrack, 1971). An ELISA has been developed that is more sensitive and rapid (Hubschle et al., 1983).

For serological diagnosis it is necessary to have standard strains of PTV serotypes propagated in cell cultures and hyperimmune serum monospecific for PTV types.

2.1. Standard strains of porcine teschoviruses

2.1.1. Characteristics

Following long experience, the strain ‘Zabreh’, isolated in Czechoslovakia during the period of peak incidence of the disease, was selected as the standard strain to generate the severe form of teschovirus encephalomyelitis. The pathogenicity of the strain is maintained by intracerebral passages in healthy, colostrum-deprived piglets. The virus produces typical signs of teschovirus encephalomyelitis after an incubation period of 5–7 days. For serological diagnosis, the following strains of PTV serotypes should be used as standard strains: type 1: Talfan, type 2: T80, type 3: O2b, type 4: PS36, type 5: F26, type 6: PS37, type 7: F43, type 8: UKG/173/74, type 9: Ger-2899/84, type 10: Ger-460/88, type 11: Dresden, type 12: CC25/SPA/2006, type 13: wild boar/WB2C-TV/2011/HUN.

2.1.2. Stock virus

Standard strains are propagated on monolayers of cell culture either from primary porcine kidney or testes or on an established cell line, for example PK-15. A 10% suspension in PBS, pH 7.4, is prepared from the brain and spinal cord of piglets infected experimentally with PTV. Some types are isolated from faeces. The suspension is centrifuged and the supernatant is used for the inoculation of cell cultures. The procedure for the cultivation of PTV in cell cultures is as follows:

The growth medium is removed from the cell culture and after rinsing with buffered saline, cells are inoculated with the virus suspension at 37°C. The size of the inoculum should be equal to 10% of the growth medium. After 1 hour of incubation at 37°C, the inoculum is decanted, the culture vessel is rinsed with buffered saline, and the cells are overlayed with the appropriate volume of serum-free medium supplemented with antibiotics. CPE is apparent within 48 hours, and the monolayer disintegrates more or less completely during the next 48–72 hours. In the subsequent three to five passages in cell culture, the development of the CPE accelerates and

the concentration of virions increases. Titration of the virus is performed in tube cultures or on microtitre plates. A cell-adapted strain usually reaches $TCID_{50}$ (50% tissue culture infective dose) titres of 10^6–10^7 per ml.

The fluid harvest is checked for specificity using known specific hyperimmune antiserum. Treatment with 5% chloroform and cultivation in human and bovine cell cultures and chicken embryos is used to exclude contamination with other viruses. PTV is chloroform resistant and multiplies only in cultures of swine origin. Immunofluorescent antibody staining is useful to detect possible contaminants that are also chloroform resistant and propagate on cells of swine origin (e.g. parvovirus), or that are non-cytopathic. The stock virus should be dispensed into small aliquots and preserved at –60°C. Frozen virus retains its properties for several years. For stock virus that is to be used in the neutralisation test, a constant dose of 100 $TCID_{50}$ is recommended.

2.2. Specific hyperimmune serum

Specific hyperimmune serum is obtained by repeated immunisation of guinea-pigs, rabbits or colostrum-deprived piglets with PTV. Although the animals are selected from specific pathogen free breeds, they are nonetheless tested before immunisation for absence of antibodies against PTV. The standard strains should be used. Rabbits are immunised either intravenously, using virus suspension alone, or subcutaneously or intraperitoneally, using the virus suspension with 10% oil adjuvant. Good results may be obtained by administering three doses of 2 ml of virus suspension plus 0.2 ml oil adjuvant, at intervals of 2 weeks. The rabbits are bled 10 days after the last immunisation. Piglets are immunised in the same way. The harvested sera are clarified by centrifugation and stored in small aliquots at –20°C. The sera are titrated using a neutralisation test and constant antigen. Only sera with an antibody titre of at least 1/256 can be used for the identification of the virus.

2.3. Virus neutralisation test in microtitre plates

The test is performed in flat-bottomed cell culture microtitre plates, using low passage porcine kidney or testes cells or cell lines derived from porcine cells. Stock virus is grown in cell monolayers. The virus harvested from cell cultures is clarified by centrifugation and stored in aliquots at –70°C. Culture medium, such as Eagle's complete medium or LYH (Hanks balanced salt solution with yeast extract, lactalbumin and antibiotics), is used as diluent. The virus harvested from cell cultures is clarified by centrifugation and stored in aliquots at –70°C or as 50/50 mixture with glycerol and can be stored at –20°C.

2.3.1. Test procedure

i) Inactivate swine sera for 30 minutes at 56°C.

ii) The sera to be tested are diluted in cell culture medium in twofold steps from 1/2 to 1/64, four wells per dilution and 50 µl volumes per well.

iii) Controls include positive and negative sera, cells and medium control.

iv) Add to each well 50 µl of virus stock previously diluted in culture medium to provide 100 $TCID_{50}$.

v) Incubate for 1 hour at 37°C with the plates covered. The residual virus stock is also incubated.

vi) Make back titrations of the residual virus stock in four tenfold dilution steps using 50 µl per well and four wells per dilution.

vii) Add 50 µl of porcine kidney cell suspension at 5×10^5 cells per ml.

viii) After further shaking, lids are put on and the plates are incubated at 37°C in a 5% CO_2 atmosphere for 2–3 days or longer, to a maximum of 8 days.

ix) Examine the plates microscopically for CPE. The test should be validated by checking the back titration of virus and titration of positive control serum. Virus should give a value of 100 $TCID_{50}$ with a permissible range of 30–300. The standard positive serum should give a titre within 0.3 $\log_{10}$ units from its predetermined mean. A negative serum should give no neutralisation at the lowest dilution tested, i.e. 1/2.

x) The VN results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of the wells.

xi) Virus neutralisation titres are regarded as positive if the corresponding serum neutralises the virus at an initial serum dilution of 1/8 or higher.

2.4. Enzyme-linked immunosorbent assay

An alternative method for the detection and titration of specific antibodies against PTV is the ELISA technique (Hubschle *et al*., 1983). The test is performed in microtitre plates using PTV grown on cell cultures as antigen. The technique can be carried out using the following steps.

2.4.1. Antigen preparation

i) Virus is propagated on monolayers of cell culture either from primary porcine kidney or testes or on an established cell line, for example PK-15. The growth medium is removed from the cell culture and after rinsing with buffered saline, cells are inoculated with virus suspension at a low multiplicity of infection. After 30 min of incubation at 37°C, cells are overlayed with the appropriate volume of serum-free medium supplemented with antibiotics. Incubation at 37°C is continued with daily microscopic observations. CPE should be apparent within 48 hours, and the monolayer disintegrates more or less completely during the next 48–72 hours. A cell-adapted strain usually reaches $TCID_{50}$ (50% tissue culture infective dose) titres of 10^6–10^7 per ml.

ii) The harvested virus is clarified by centrifugation at 200 ***g*** for 15 minutes, and then precipitated with a final 50% saturated $(NH_4)_2SO_4$ for 120 minutes at 4°C.

iii) After centrifugation at 2000 ***g***, the resulting precipitate is suspended in TEN buffer (Tris-hydroxymethyl-methylamine [0.01 M], ethylene diamine tetra-acetic acid [1 mM] and NaCl [0.15 M]), pH 7.4, to 1/100 of the initial volume.

iv) The concentrated viral suspension is extracted by shaking with freon 3/1 for 10 minutes at 4°C.

v) Following further centrifugation, the supernatant is divided into two separate phases. The upper aqueous phase, containing the viral antigen, is desalinated by passage through a 2.5 × 40 cm cylinder packed with sephadex G 25.

vi) The viral solution is finally concentrated by ultracentrifugation at 160,000 ***g*** for 3 hours.

vii) The pellet is suspended in TEN buffer, pH 7.4, in approximately 1000th the initial volume of virus.

viii) Insoluble proteins are separated by light centrifugation, and the supernatant is used as the positive antigen in the ELISA.

2.4.2. Test procedure

i) Plates are sensitised with pre-diluted antigen in phosphate buffered saline (PBS), pH 7.2, by adding 100 µl to each well. The adsorption of antigen to the surface of the plate takes place overnight at 4°C. Parallel rows of the plate should be treated with negative antigen.

ii) The plate is washed five times in PBS to remove excess antigen.

iii) Test sera are diluted 1/20 with PBST (PBS solution containing 0.05% Tween 20). 50 µl of the diluted sera is placed into each of two wells with positive antigen and into two wells with negative antigen. (Negative antigen is prepared as described above except that the tissue culture is not inoculated with virus and cells are disrupted by freezing.) The plate is incubated for 1 hour at 37°C.

iv) The plates are washed five times with PBST.

v) A predetermined dilution of horseradish peroxidase conjugated with anti-swine immunoglobulin prepared in rabbits is added in 50 µl quantities to each well. The plates are further incubated for a further 1 hour at room temperature.

vi) The plates are washed five times in PBS.

vii) Substrate solution (0.1% ortho-phenylendiamine with 0.03% hydrogen peroxide in PBS, pH 6.0) is added in 100 µl quantities to each well.

viii) After the addition of substrate, positive samples change colour to dark brown. When a sufficient degree of colour reaction is seen in the wells of known positive sera, the reaction is stopped by addition of 50 µl of 2 M sulphuric acid to each well. The absorbance of the wells is measured at a wavelength of 492 nm, preferably using an automatic multi-channel spectrophotometer with print-out mechanism. Positive and negative sera and non-infected cells should be processed as controls in parallel with the test specimens.

ix) The absorbance of a serum is the mean reading of two wells with positive antigen minus the mean reading of two wells with negative antigen. Absorbance readings of test sera that exceed by more than twofold the mean reading of standard negative sera are regarded as positive.

C. REQUIREMENTS FOR VACCINES

1. Vaccines against teschovirus encephalomyelitis

During the period of highest incidence of the disease in central Europe and Madagascar, active immunoprophylaxis was an important means for the control of this infection (Traub, 1942). As severe clinical disease has largely disappeared, vaccination has been discontinued and the vaccine is no longer being produced or used anywhere in the world.

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NB: At the time of publication (2024) there were no WOAH Reference Laboratories for Teschovirus encephalomyelitis (please consult the WOAH Web site for the current list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991 AS TESCHEN DISEASE. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 3.9.10.

TRANSMISSIBLE GASTROENTERITIS

SUMMARY

Transmissible gastroenteritis (TGE) is an enteric disease of pigs caused by TGE virus (TGEV), a member of the Coronaviridae. Since 1984, a distinct respiratory variant (porcine respiratory coronavirus or PRCV) has spread throughout many parts of the world. This virus is probably a deletion mutant of TGEV. PRCV does not appear to be an important primary pathogen, but it contributes to the porcine respiratory disease complex and it has greatly complicated the diagnosis of TGE, particularly by serological means.

Laboratory diagnosis is made by demonstrating the presence of virus, viral antigens or viral nucleic acid in material from suspected cases, or by demonstrating virus-specific humoral antibodies.

Identification of the agent: *Virus may be identified by virus isolation in tissue culture, electron microscopy, various immunodiagnostic assays, and more recently by specific detection of viral RNA. The most commonly employed rapid assays are probably the immunodiagnostic ones, particularly enzyme-linked immunosorbent assays (ELISAs) on faeces and fluorescent antibody tests on cryostat sections of intestine. Another enteric disease, porcine epidemic diarrhoea, is caused by a serologically distinct coronavirus that nevertheless has an identical appearance under the electron microscope. Diagnostically, immune electron microscopy circumvents this problem.*

Serological tests: *The most widely used methods are virus neutralisation tests and ELISAs. Only in the latter case is differentiation from PRCV possible, as TGEV and PRCV antibodies show complete cross-neutralisation.*

Requirements for vaccines and diagnostic biologicals: *There are no commercial biological products available internationally. However, several countries practise vaccination, and in the United States of America, licences have been issued authorising the production and distribution of monovalent and combined vaccines.*

A. INTRODUCTION

Transmissible gastroenteritis (TGE) is an enteric disease of pigs caused by TGE virus (TGEV), a member of the *Coronaviridae*. Since 1984, a distinct respiratory variant (porcine respiratory coronavirus or PRCV) has spread throughout many parts of the world and is now found in most countries where surveys for it have been conducted, one exception being Oceania. Occurrences of TGE have become more sporadic. The disease is still reported on an occasional basis from parts of Europe, North America and Asia. TGEV multiplies in and damages the enterocytes lining the small intestine, producing villous atrophy and enteritis. Diarrhoea and vomiting occur in pigs of all ages; mortality is highest in neonates. Extra-intestinal sites of virus multiplication include the respiratory tract and mammary tissues (Kemeny *et al*., 1975), but the virus is most readily isolated from the intestinal tract and from faeces. By contrast, PRCV is most readily isolated from the upper respiratory tract, the trachea, tonsils or the lungs, and little enteric multiplication of virus occurs (Cox *et al*., 1990; O'Toole *et al*., 1989; Pensaert *et al*., 1986) although PRCV can be detected by nested reverse-transcription polymerase chain reaction (RT-PCR) in nasal swabs and faeces of PRCV-infected swine (Costantini *et al*., 2004). PRCV is probably a deletion mutant of TGEV (Rasschaert *et al*., 1990) as confirmed by recent data comparing the complete 30 Kb genome sequences of TGEV and PRCV strains (Zhang *et al*., 2007).

As TGE is a contagious disease that can occur as explosive epizootics, rapid diagnostic methods for its confirmation are particularly important. The disease can also take the form of a low-level endemic problem of post-weaning diarrhoea, which is more difficult to diagnose. The occurrence of TGEV in PRCV-immune herds also

leads to milder and sporadic clinical cases of TGEV, further complicating TGEV diagnosis in such scenarios (Kim *et al*., 2000b).

Possible wild and domestic animal reservoirs for TGEV have been suggested. Wild and domestic carnivores (foxes, dogs, possibly mink) and cats seroconvert to TGEV and are suggested as potential subclinical carriers of TGEV, serving as reservoirs between seasonal (winter) epidemics. However only virus excreted by serially TGEV-infected dogs has been confirmed as infectious for pigs (Saif & Sestak, 2006). Based on genetic and antigenic similarities, it has been proposed that TGEV, PRCV, feline and canine coronaviruses represent host-range mutants of an ancestral coronavirus. Wild birds (*Sturnus vulgaris*) and flies (*Musca domestica*) have been proposed as mechanical vectors for TGEV, excreting virus for 32–72 hours, respectively (Saif & Sestak, 2006).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Virus may be identified by virus isolation in tissue culture (Dulac *et al*., 1977), immunofluorescence, reversed passive hemagglutination, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassay (RIA), hybridisation with DNA probes, electron microscopy, and, more recently, by specific detection of viral RNA (Enjuanes & Van der Zeijst, 1995; Kim *et al*., 2000a; Paton *et al*., 1997; Saif & Sestak, 2006; Sirinarumitr *et al*., 1996; Woods, 1997). Molecular techniques such as RT-PCR and nested RT-PCR developed in the past few years have increased the sensitivity and specificity of detection and differentiation of TGEV and PRCV directly from field samples (Costantini *et al*., 2004; Kim *et al*., 2000a; 2000b; Paton *et al*., 1997). An alternative diagnostic method that has been recommended for laboratories lacking facilities for specialised tests is the oral dosing of susceptible TGEV/PRCV seronegative piglets with suspect intestinal contents. However, laboratory tests are still required to confirm susceptibility of the pigs prior to inoculation and to show that any illness induced in these animals is due to TGE. The most commonly employed rapid assays are probably the immunodiagnostic ones, particularly enzyme-linked immunosorbent assays (ELISAs) on faeces (Bernard *et al*., 1986; Lanza *et al*., 1995; Van Nieuwstadt *et al*., 1988b), fluorescent antibody tests (FAT) on cryostat sections of intestine (Pensaert *et al*., 1968) and immunohistochemistry (IHC) on formalin-fixed, paraffin sections (Shoup *et al*., 1996). Detection of virus by reversed passive hemagglutination has also been described (Asagi *et al*., 1986). Another enteric disease, porcine epidemic diarrhoea (PED), is caused by a serologically distinct coronavirus that nevertheless has an identical appearance under the electron microscope. Diagnostically, immune electron microscopy circumvents this problem (Saif *et al*., 1977; Van Nieuwstadt *et al*., 1988a) as does the application of PED virus-specific detection assays (Kim *et al*., 2001).

1.1. Virus isolation in tissue culture

Apart from the inoculation of live piglets (Dulac *et al*., 1977), this is the most definitive method of diagnosis. However, for routine use it is slow and laborious. TGEV does not grow well in cell culture, making this technique impractical as a routine diagnostic procedure. Moreover isolation of TGEV from pigs in PRCV seropositive herds is also problematic and often requires placement of TGEV/PRCV seronegative pigs in the suspect herd to serve as sentinels, followed by collection of samples from the sentinel pigs for TGEV isolation or detection (Costantini *et al*., 2004; Kim *et al*., 2000b). PRCV can be isolated in tissue culture using similar cell types and techniques as for TGEV, but using nasal cells or fluids and tracheal, tonsil or lung tissues or homogenates as the optimal specimens (Costantini *et al*., 2004; Pensaert *et al*., 1986).

For TGEV, isolation is usually attempted ante-mortem from faeces or post-mortem from the small intestine. Loops of affected small intestine, ligated at each end to retain the contents, or mucosal impression smears of the small intestinal luminal surface are the preferred specimens. As the virus is heat labile, all samples should be fresh or chilled.

Sample material is homogenised in cell culture medium or phosphate buffered saline (PBS), pH 7.2, containing antibiotics, e.g. penicillin (1000 U/ml), dihydrostreptomycin (1000 μg/ml), and mycostatin (20 U/ml), to produce a 10% suspension. This is allowed to stand out of direct sunlight for 30 minutes at room temperature. The suspension is then sonicated and clarified by low-speed centrifugation. The supernatant fluid may be mixed with an equal volume of heat-inactivated bovine serum in order to reduce the cytotoxic effect of the material and it is then used to inoculate susceptible tissue cultures, such as 3- to 4-day-old primary or secondary pig kidney monolayers. Other low passage porcine

cultures (such as thyroid or testis) and some cell lines (Honda *et al*., 1990; McClurkin & Norman, 1966) may also be used for primary virus isolation. After incubation at 37°C for 1 hour, the cell sheets are overlaid with a medium, such as Earle's yeast lactalbumin (EYL) balanced salt solution, containing sodium bicarbonate and antibiotics, e.g. penicillin (100 U/ml), dihydrostreptomycin (100 μg/ml), mycostatin (20 U/ml), and 1% fetal calf serum. Incorporation of trypsin into the culture medium may enhance primary viral recovery (Bohl, 1979; Honda *et al*., 1990). Uninoculated control cultures are established concurrently and all cultures are incubated at 37°C.

Viral cytopathic effect (CPE) may be observed after 3–7 days, characterised by cells rounding, enlarging, forming syncytia and detaching into the medium. Plaque formation is sometimes more reliable and easier to recognise. A suitable plaquing overlay is 1.6% noble agar in 2 × minimal essential medium with 1% $NaCO_3$, antibiotics (as above), 0.7% neutral red and 1% DEAE (diethylaminoethyl) (100 μg/ml). Wild-type TGEV does not grow readily in tissue culture, so several subpassages may be necessary before these distinctive changes become apparent. Cytopathic isolates must be confirmed as TGEV by immunostaining or by *in-vitro* neutralisation tests using appropriate TGEV-specific antisera (Bohl, 1979). If suitable monoclonal antibodies (MAbs) are available they can be used to distinguish between TGEV and PRCV by immunostaining methods (Garwes *et al*., 1988; Simkins *et al*., 1992). Differentiation of TGEV from PRCV can also be accomplished by TGEV-specific cDNA probes (Bae *et al*., 1991) or by discriminatory RT-PCR or nested RT-PCR (Costantini *et al*., 2004; Kim *et al*., 2000a; 2000b; Paton *et al*., 1997).

1.2. Fluorescent antibody test for viral antigens

The fluorescent antibody test is a rapid, sensitive and specific means of identifying TGE viral antigens in cryostat sections of intestine. A freshly dead pig is required, and the ideal animal should be under 4 weeks of age (preferably less than 1 week) and just starting to show clinical signs of the disease (that is, within 24–28 hours of infection). Within 30 minutes of death, 2 cm lengths from four different regions of the posterior part of the small intestine should be removed. Lengths of 5–10 mm are cut from these for snap freezing with solid CO_2. Correct orientation of the material is important to ensure that subsequent cutting by cryostat yields true transverse sections. Sections are cut 6 μm thick, mounted on cover-slips, air-dried and fixed in acetone. An alternative and faster procedure is to excise and longitudinally cut open a piece of the distal small intestine, gently wash the mucosal surface with PBS and prepare impression smears of the luminal intestinal surface on ethanol-cleaned microscope slides followed by air drying and acetone fixation (Bohl, 1979). The slides are then processed and stained like the cryostat sections as follows. Fixed positive and negative control sections or smears are stored at -20°C for staining in parallel. After washing with Tris buffer, pH 8.7, or PBS, the sections are stained with a diluted solution of fluorescein isothiocyanate (FITC)-conjugated TGEV antibody, and placed in a humid incubator at 37°C for 30 minutes. Any unbound stain is removed by washing in Tris buffer. If desired, the sections are counterstained with a 10^{-5} dilution of Evans blue in Tris buffer and mounted in glycerol.

Stained sections or smears should be examined by ultraviolet light microscopy as soon as possible. The quality of the staining is assessed by reference to the controls. An accurate interpretation depends on the preservation of the villous architecture, the epithelial cells of which are examined for intracytoplasmic fluorescence.

A peroxidase–antiperoxidase IHC method for the demonstration of TGEV has been developed for detection of TGEV and PRCV in both frozen and formalin-fixed, paraffin-embedded tissues (Jean *et al*., 1987; Shoup *et al*., 1996). The IHC applied to formalin-fixed tissues is advantageous because it can be done prospectively or retrospectively on the same formalin-fixed tissues used for histopathology and the fixed tissues or slides can be more readily shipped as they are stable and they do not contain live virus (Shoup *et al*., 1996).

1.3. Enzyme-linked immunosorbent assay detection of faecal virus antigens

A double antibody-sandwich system may be used, for instance with a capture MAb and a polyclonal enzyme-linked detector antibody (Lanza *et al*., 1995; Sestak *et al*., 1996). This test is based on capture of the viral antigen from the faecal sample by three MAb, two specific for the S protein (site A and D) and one for the nucleoprotein N (Lanza *et al*., 1995; Sestak *et al*., 1996). A negative coating is used as control for the specificity of the test, consisting of antibodies purified from the ascitic fluid of mice inoculated

with SP2/0 myeloma cells that do not recognise TGEV. MAbs are applied to 96-well microplates in a bicarbonate buffer, pH 9.6, and incubated overnight at 37°C. All samples are tested in duplicate wells, one containing positive coating (TGEV MAbs) and one containing the negative coating. Faecal samples are diluted in cell culture medium (1/10), vortexed and centrifuged at low speed (2000 *g*) for 15 minutes. Then the supernatant is decanted into sterile tubes and tested or stored frozen. Plates are washed twice with washing buffer (PBS containing 0.05% Tween 20) before adding the prepared faecal samples. The plates are incubated overnight at 37°C. After washing four times, a biotinylated polyclonal anti-TGEV serum is added in PBS buffer containing 0.05% Tween 20. The plates are incubated at 37°C for 1 hour. The plates are washed four times before adding a horseradish peroxidase-labelled streptavidin conjugate and incubated at 37°C for 1 hour. The plates are washed six times before adding the enzyme substrate, which is ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) with 0.03% H_2O_2 in 0.1 M citrate buffer, pH 4.2. The reaction is stopped after 30 minutes at room temperature by the addition of 5% sodium dodecyl sulphate and the absorbance determined in an ELISA reader at 405 nm. TGEV negative and positive faecal samples are included on each plate.

1.4. Nucleic acid recognition methods

In-situ hybridisation (ISH) and RT-PCR methods have been described for the direct detection of TGEV in clinical samples, with differentiation from PRCV (Kim *et al*., 2000a; Paton *et al*., 1997; Sirinarumitr *et al*., 1996). A second round of nested PCR may significantly enhance the sensitivity (Costantini *et al*., 2004; Kim *et al*., 2000a; 2000b; Paton *et al*., 1998). Differentiation between TGE viruses may be achieved by analysing PCR products with restriction endonuclease enzymes (Woods, 1997) or by sequencing (Costantini *et al*., 2004; Kim *et al*., 2000b; McGoldrick *et al*., 1999; Paton & Lowings, 1997; Zhang *et al*., 2007). Duplex RT-PCR for the combined detection of TGEV and porcine epidemic diarrhoea virus has been described (Kim *et al*., 2001).

2. Serological tests

Serology may be diagnostic if a rising titre of antibody can be demonstrated. In addition, a single seropositive result has diagnostic value if collected from a population previously known to be seronegative. As the possibility of acquiring carrier virus status among pigs can be reduced by accepting only seronegative animals, serological testing is also a common precondition for importation.

Following infection with TGEV or PRCV, viral antibodies can be detected in serum from 6 or 7 days post-infection, and such antibodies persist at least for many months. Although PRCV and TGEV antibodies show complete neutralisation of either virus, there are differences in the specificities of some of the non-neutralising antibodies (Callebaut et al., 1988; Enjuanes & Van der Zeijst, 1995; Garwes et al., 1988; Saif & Sestak, 2006; Simkins et al., 1992), as PRCV lacks certain epitopes present on the TGEV. However, virus neutralisation (VN) is not a practical method to differentiate PRCV from TGEV infection. MAbs to such regions can be incorporated into competitive ELISAs to detect serum antibody that is entirely TGEV specific. While such tests are reliable in that they do not produce false-positive results with PRCV antisera, false negatives may occur because of a reduced sensitivity compared with neutralisation tests, and because of strain variation among TGE viruses, such that a single TGEV-specific MAb may not recognise all strains (Brown & Paton, 1991; Simkins et al., 1992). The problem of insensitivity can be reduced by using the tests on a group or herd basis. These MAb-based ELISAs are the method of choice for differentiating PRCV from TGEV to qualify animals for export.

In addition, using such tests for differential diagnosis less than 3 weeks after exposure to PRCV produced inconsistent and unreliable results (Sestak et al., 1999b). More accurate results were also achieved by testing paired serum samples (acute and convalescent) in the assays and by using the recombinant spike (S) protein of TGEV as the coating antigen in place of TGEV-infected, fixed swine testicular cells (Sestak et al., 1999b).

2.1. Transmissible gastroenteritis virus/porcine respiratory coronavirus tests

These tests detect antibody to both TGEV and PRCV, and include VN tests, indirect ELISAs (Hohdatsu *et al*., 1987; Huang *et al*., 1988; Liu *et al*., 2001; McGoldrick *et al*., 1999; Rukhadze *et al*., 1989) and competitive ELISAs based on TGEV/PRCV group-specific MAbs (Paton *et al*., 1991).

VN tests can be performed with a variety of cell types and viral strains. Commonly used cell lines include swine testes (McClurkin & Norman, 1966) or primary or continuous porcine kidney cells. Such tests have been very widely used for many years and are commonly regarded as standards against

which to assess new assays. A plaque reduction VN assay using swine testes cell monolayers in six-well plastic plates and the atttenuated Purdue strain of TGEV is commonly used (Bohl, 1979). A modification of the method of Witte (Witte, 1971) described below, uses flat-bottomed tissue-culture grade microtitre plates, a cell line of A72 cells derived from a dog rectal tumour, and a field strain of virus adapted to grow in such cells: 100 $TCID_{50}$ (50% tissue culture infective dose) of virus is incubated with heat-inactivated test sera, and neutralisation is indicated by absence of CPE after further incubation with A72 cells in Leibovitz 15 medium (Sigma, United Kingdom) with added antibiotics, 10% fetal calf serum and 1% L-glutamine. The total volume of reagents in all wells should be 150 µl.

2.1.1. Virus neutralisation: test procedure

i) Sera are inactivated for 30 minutes in a water bath at 56°C.

ii) Doubling dilutions of test sera are made in cell culture medium beginning with undiluted serum (this gives a neutralisation stage dilution of 1/2 when mixed with an equal volume of virus). The dilutions are prepared in a 96-well flat-bottomed cell-culture grade microtitre plate using, optimally, three wells per dilution and 25 µl volumes per well. Positive and negative control sera are also included in the test. No standard sera are available, but internal positive standards should be prepared and titrated in an appropriate range.

iii) 25 µl TGEV stock is added to each well at a dilution in culture medium calculated to provide 100 TCID50 per well. Virus should be added to two out of the three wells containing serum at each dilution. The third well serves as a serum-only control and should receive 25 µl per well of culture medium instead of virus.

iv) The residual virus is back titrated in four tenfold steps using 25 µl per well and at least four wells per dilution; 25 µl of culture medium is added to each of the back-titration wells to compensate for the absence of a test serum.

v) The plates are agitated briefly and then incubated for 1 hour in a 5% CO2 atmosphere at 37°C.

vi) 100 µl of, for example, A72 cell suspension at 2 × 105 cells per ml is added to each well.

vii) The plates are incubated for 3–7 days in a 5% CO2 atmosphere at 37°C; the test can be performed successfully, if the plates are incubated without CO2.

viii) The plates are read microscopically for CPE. The test is validated by checking the back titration of virus (which should give a value of 100 TCID50 with a permissible range of 50–200 TCID50) and the control sera. The standard positive serum should give a value within 0.3 log10 units either side of its predetermined mean. Readings of each test serum dilution should be made with reference to the appropriate serum-only control to distinguish viral CPE from serum-induced cytotoxicity or contamination.

ix) The test serum results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of wells.

x) A negative serum should give no neutralisation at the lowest dilution tested (i.e. undiluted serum, equivalent to a dilution of 1/2 at the neutralisation stage).

2.2. Transmissible gastroenteritis virus-specific tests to differentiate TGEV- from PRCV-infected pigs

TGEV-specific tests are blocking or competition ELISAs that use an MAb that recognises TGEV but not PRCV (Brown & Paton, 1991; Callebaut *et al*., 1989; Sestak *et al*., 1999b; Simkins *et al*., 1992; Van Nieuwstadt & Boonstra, 1991) and are the tests of choice for qualifying animals for export. Test sera from pigs previously infected with a strain of TGEV recognised by the MAb will contain antibodies of the same specificity that can compete with it for binding to TGEV antigen-coated ELISA plates. Pigs infected with PRCV that does not contain the TGEV unique epitope will not produce antibodies to this epitope; hence, PRCV antibodies will not compete with or block binding of the TGEV-specific MAb (Brown & Paton, 1991; Callebaut *et al*., 1989; Sestak *et al*., 1999b; Simkins *et al*., 1992; Van Nieuwstadt & Boonstra, 1991). ELISA antigens may be prepared from cell lysates of kidney cell lines that were either inoculated with tissue-culture-adapted strains of TGEV, or uninfected. Alternatively TGEV-infected or uninfected swine testes cells fixed in 80% acetone have been used as an antigen source, or antigens may be prepared from recombinant S (rec-S) protein harvested in soluble form from an insect (Sf9) cell

line infected with a recombinant baculovirus expressing a TGEV S protein containing the four major antigenic sites (Sestak *et al*., 1999b; Simkins *et al*., 1992). Positive and negative antigens are coated to alternate rows of microtitre plates using bicarbonate buffer, pH 9.6. Diluted test sera, including known TGEV positive and known TGEV/PRCV negative controls, as well as known PRCV positive (negative in this test, positive in VN test) are added to appropriate wells and incubated overnight before further addition of diluted MAb to all wells. Bound MAb is detected by a peroxidase-conjugated anti-mouse antibody that induces a colour reaction in the presence of an appropriate substrate. The colour changes are measured using spectrophotometer, and for each test sample the net result is the difference in absorbance between the positive and negative antigen wells, expressed as a percentage of the result obtained with the negative control serum. The negative–positive cut-off value for the test must be determined by previous testing of known negative and positive populations. There are several commercial kits available that are TGEV specific.

Haemagglutination-based tests described to date (Labadie *et al*., 1977; Noda *et al*., 1987; Shimizu & Shimizu, 1977) were validated before the appearance of PRCV. However, they may be TGEV specific as TGEV, but not PRCV, is haemagglutinating (Schultze *et al*., 1996).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccination against TGE is carried out in several countries.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Information on experimental work or field trials of TGEV vaccines licensed for use in the United States of America (USA) has been reviewed, including possible limitations in their field efficacy and concepts related to the design of optimal TGEV vaccines (Saif, 1993; Saif & Jackwood, 1990; Saif & Sestak, 2006). Several manufacturers are licensed to produce TGEV vaccines in the USA: the vaccines include modified live and inactivated vaccines. The modified live vaccines are used for oral administration to pregnant sows (to induce passive immunity) or have also been licensed for oral administration to nursing or weaned pigs (to induce active immunity). Inactivated TGEV vaccines are licensed for parenteral inoculation of pregnant sows by the intramuscular route or for intraperitoneal administration to nursing or weaned pigs. In general, these vaccines induced marginal passive protection against TGEV challenge of nursing piglets when evaluated under controlled experimental conditions or in the field in TGEV/PRCV herds. Although they fail to adequately protect against epizootic TGE, data suggest that these vaccines may provide some efficacy against enzootic TGEV by stimulating an anamnestic antibody response to TGEV in serum and milk (Saif & Jackwood, 1990; Saif & Sestak, 2006).

The main reason proposed for TGEV vaccine failures was their inability to stimulate high levels of secretory IgA (SIgA) antibodies in milk analogous to the SIgA antibody responses found in the milk of sows naturally infected with TGEV (Saif & Jackwood, 1990; Saif & Sestak, 2006). Furthermore, these vaccines did not adequately protect the seronegative sow against TGE, such that illness in the sow often resulted in anorexia, agalatica and failure to passively protect her piglets. Thus the modified live vaccines may fail to replicate to the extent required to induce protective immunity in the intestine, or if given to seronegative neonatal animals, concerns exist regarding their possible reversion to virulence. Killed vaccines given parenterally do not induce SIgA antibodies; cell-mediated immune responses are often poor and the duration of immunity may be short-lived. Although use of PRCV strains as vaccine candidates for TGE has been proposed, experimental studies regarding their efficacy against TGEV have shown a lack of efficacy (Paton & Brown, 1990) or only partial cross-protection (Bernard *et al*., 1989; Cox *et al*., 1993; Van Cott *et al*., 1994). However, the widespread prevalence of PRCV infections in the swine population in Europe appears to have dramatically reduced the incidence of epizootic TGE in Europe (Pensaert *et al*., 1986). Newer recombinant DNA strategies for the development of TGEV vaccines include the possible use of an S protein subunit vaccine (contingent upon the development of mucosal delivery systems and adjuvants) (Park *et al*., 1998; Sestak *et al*., 1999a; Shoup *et al*., 1997) or the use of live recombinant viral or bacterial vectors that express TGEV genes important for the induction of immunity (Enjuanes *et al*., 2001; Saif, 1993; Saif & Sestak, 2006; Smerdou *et al*., 1996; Torres *et al*., 1996; Yount *et al*., 2000).

There are a number of general requirements (e.g. produced in a licensed facility, label rules, tracking capability, etc.) that apply to all biological products including vaccines. A set of regulations exist (called standard requirements, or SRs) that describe testing to be done on the vaccine and parent materials. Detailed information

on SRs for vaccines in the USA are contained in the Code of Federal Regulations (CFR) Title 9, Volume 1, Part 113 (abbreviated below as 9 CFR, 113) (United States Department of Agriculture [USDA], 1995). The general European Pharmacopoeia monograph and EMEA (European Medicines Agency) guidelines are applicable to TGEV vaccines, even though no vaccines are currently used in the European Union.

1. Seed management

1.1. Characteristics of the seed

The seed virus must be tested for purity and identity. The purity includes freedom from bacteria and fungi (9 CFR 113:27), mycoplasmas (9 CFR 113:28), and extraneous viruses (9 CFR 113:55) (USDA, 1995). The demonstration of identity is usually accomplished by VN or FAT. Genetically engineered vaccines or naturally selected vaccines with claims of antigen-coding gene deletion/inactivation are required to provide evidence (genotypic and/or phenotypic) of that identity.

1.2. Method of culture

Culture must be carried out on proven uncontaminated (approved) cells, and the number of cell culture passages is limited (usually to five). It is not required that the species of origin of the cell line be that of the target species.

1.3. Validation as a vaccine

Vaccine validation takes two forms. The master seed is considered to be immunogenic if a vaccine made at the highest passage, and according to the outline of production, is shown to be protective. The lowest antigenic level (modified live virus titre or inactivated antigen mass) shown to be protective becomes the baseline for all future serials (lots) of the product. In the case of live products, factors for titration variation and the death curve over time would be added. These trial vaccines should be tested for purity, safety, and efficacy by the manufacturer. Protection must be shown against the natural disease with the virulent challenge virus. Virulent challenge virus is defined as the dose that causes disease in ≥95% of the susceptible controls. Three prelicense serials must subsequently be made and tested by the manufacturer and by the licensing authority, for potency, sterility and safety.

2. Method of manufacture

This is proprietary information for each manufacturer and hence not available.

3. In-process control

This is largely proprietary. Some in-process controls refer directly to production (e.g. O_2 concentration in the fermenter). Another category, however, includes tests similar to the final container potency test. For all vaccines, the simpler the final batch or container potency test, the more likely it is that it may be used as a monitoring/blending test: for example, virus titration on sub-batches may be used to predict final blended batch titre. Ingredients of animal origin must be sterilised or shown to be free from contamination.

4. Batch control

Batches must be blended to the final specifications and bottling specifications (e.g. fermentation runs may be pooled, or one run may be split and pooled with each of three others, etc.). In some countries, bulk and process control define the product and are the subject of intense regulation and scrutiny. The emphasis in the USA is on the final product. Batch control techniques must be detailed in the outline of production and must be meaningful, trackable, and the manufacturer must discard product that fails to meet specifications. If a batch is to be exported to another country for bottling or blending, then it is subjected to all the testing as though it were final product.

4.1. Sterility

All products must be tested for sterility. The manufacturer may also run sterility tests on batches for monitoring. Tests are similar to those described in Section C.1.1.

4.2. Safety

Safety tests are done before the licence is granted, and then on the final container (Sections C.5.1 and C.5.2).

4.3. Potency

Potency would normally only be done if the potency test were a simple test (e.g. ELISA) to confirm the blending calculations before bottling.

4.4. Duration of immunity

Duration of immunity is tested in the prelicence (efficacy) serial test, not the batch control. New products are required to support label claims for revaccination schedules with efficacy trials (challenge) at the specified time after vaccination.

4.5. Stability

Stability is established before the licence is granted. Usually accelerated ageing (37°C) is used to estimate the lifetime so that the products do not have to be kept at storage temperature (4°C) for the real-time period. This will be confirmed with real-time data later. The manufacturer is not required to do stability testing. Manufacturers are required to state the amount of antigenic material that will be in their product throughout the shelf life. Samples of product are selected (usually live) and tested within 30 days of expiration to see if, for example, the titre is at the level stated in the manufacturer's outline. Stability is also affected by moisture. Moisture left in a desiccated product can shorten its life, so this has to be tested in the final product or in-process.

4.6. Preservatives

There are restrictions on the maximum amounts of antibiotics that can be in a product. Restrictions on some vaccine components are related to their safety and to whether the stated withdrawal period is long enough for the component to have cleared before the animal is slaughtered. Preservatives used are proprietary.

4.7. Precautions (hazards)

Any risks to vaccinates need to be clearly stated on the label. This usually applies to pregnancy warnings for abortogenic live viruses, and the general anaphylaxis warning, but may also attempt to warn the user about soreness or swelling at the injection site, or transient fever or inappetence in some cases. No unusual label precautions apply to the TGE vaccines currently licensed.

5. Tests on the final product

5.1. Safety

Usually this will be a mouse and/or a guinea-pig or swine safety test (9 CFR 113:33, and Witte, 1971). Sterility tests are also carried out on the final product.

5.2. Potency

There is no single test for release potency. Whatever test is used must be correlated to protection in the host animal (the efficacy tests). The potency of live TGEV vaccines can be evaluated by *in-vitro* titration of the viral infectious dose in cell culture (Saif, 1993). This titre must be correlated with the minimum viral titre required to induce protective immunity against experimental challenge, and also against natural challenge under field conditions. The potency of killed vaccines is evaluated by vaccination and challenge tests using different doses of the vaccine. Titres of neutralising antibodies induced by inoculation of laboratory animals with the vaccine may be accepted if there is an established correlation with development of protective immunity.

Particular viral antigens associated with the induction of neutralising antibodies and protection against challenge can be quantified in killed vaccines using specific MAbs in ELISA, such as neutralising MAbs to the S protein of TGEV (Saif, 1993).

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NB: At the time of publication (2024) there were no WOAH Reference Laboratories for transmissible gastroenteritis (please consult the WOAH Web site for the current list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2008.

SECTION 3.10.

OTHER DISEASES[1]

CHAPTER 3.10.1.

BUNYAVIRAL DISEASES OF ANIMALS (EXCLUDING RIFT VALLEY FEVER AND CRIMEAN–CONGO HAEMORRHAGIC FEVER)*

SUMMARY

The order Bunyavirales *has hundreds of members distributed over 12 families with a large number of genera. Most viruses of the different families are transmitted to vertebrates by arthropods* (*arboviruses*). *Members of the family* Hantaviridae *are not arboviruses.*

The families of veterinary importance are Nairoviridae, Peribunyaviridae *and* Phenuiviridae. *The genus* Orthonairovirus *contains the zoonotic Crimean–Congo haemorrhagic fever virus* (*for which see Chapter 3.1.5*) *and the ruminant pathogen Nairobi sheep disease* (*NSD*) *virus* (*NSDV*). *The largest genus,* Orthobunyavirus, *is subdivided into 103 virus species and 48 serogroups including only a few significant pathogens of animals, among them Cache Valley virus* (*CVV*), *Akabane virus* (*AKAV*), *Schmallenberg virus* (*SBV*) and *Shuni virus* (SHUV). *These viruses have a tropism for fetal tissues and are responsible for prenatal losses and multiple congenital deformities in domestic ruminants. SBV is a novel* Orthobunyavirus *that emerged in 2011 in Europe. The virus was found in malformed lambs, kids and calves in different European countries and spread to most parts of Europe. Other members of the* order Bunyavirales *that are of veterinary importance are Rift Valley fever virus* (*RVFV*), *a member of the* Phenuiviridae *family* (*genus* Phlebovirus), *described in Chapter 3.1.18* Rift Valley fever.

Members of the Orthonairovirus *and* Orthobunyavirus *genera are enveloped spherical or pleomorphic RNA viruses, 80–110 nm in diameter, with three genome segments* (*S, M and L*) *of negative polarity.*

Detection and identification of the agent:

CVV, a member of the Bunyamwera virus serogroup of the Orthobunyavirus *genus, can be isolated from the blood of febrile or viraemic adult animals. Attempts to isolate from the fetus at birth are generally unsuccessful due to virus clearance by the fetal immune response. Cell lines derived from African green monkey kidney* (*Vero*) *or baby hamster kidney* (*BHK*) *are employed for isolation of the virus. Virus or antigen is identified by immunofluorescence* (*FA*), *immunohistochemistry* (*IHC*) *or neutralisation* (*VN*) *tests. Group- and virus-specific reverse transcription polymerase chain reaction* (*RT-PCR*) *techniques have been developed for the* Orthobunyaviruses.

AKAV can be isolated from the blood of viraemic animals and occasionally from fetal material. Vero, BHK and mosquito cell lines can be used. Virus or antigen is identified by FAT, IHC or VN tests. Different types of real-time RT-PCR techniques have been developed and validated for AKAV and related viruses.

1 The diseases in this section of the *Terrestrial Manual* that are marked with an asterisk (in the Table of Contents and the chapter title) are included in some individual species sections of the WOAH List, but these chapters cover several species and thus give a broader description.

SBV can be isolated from the blood of viraemic adults, and occasionally from different tissues of infected fetuses, especially from brain/CNS materials, using different cell lines: insect cells (KC; C6/36) or mammalian *BHK or Vero cells. However, isolation can be difficult and adaptation to cell culture is necessary for sufficient* in-vitro *growth of SBV. Several real-time RT-PCRs have been established and commercial PCR kits are available allowing highly sensitive and specific virus detection in blood of acutely infected ruminants as well as in organs and blood of infected fetuses, such as brain, placenta, amniotic fluid, and meconium. Nevertheless, detection of SBV genome is possible only in a proportion of the infected and malformed fetuses and not equally well in all tissues due to virus clearance during gestation.*

NSDV is best isolated from the plasma of febrile animals, mesenteric lymph nodes or spleen. BHK cells and lamb cell cultures are the most sensitive cells for isolation. Identification of the virus may be made by FAT on the inoculated tissue cultures. Infected tissue cultures may be used as sources of complement-fixing or enzyme-linked immunosorbent assay (ELISA) antigens. However, as for CVV, AKAV and SBV, real-time RT-PCR is the most sensitive and reliable detection technique and several protocols have been developed and validated.

Serological tests: *For CVV and AKAV, ELISA and VN tests are used to detect antibodies. A competitive ELISA specific for Akabane has been published and commercial kits are available. For SBV, ELISA (commercial indirect and blocking ELISAs are available), indirect immunofluorescence antibody (IFA) and VN tests are used to detect antibodies against SBV in serum samples. For NSDV a suitable test is the IFA, VN tests give equivocal results, a feature that also occurs with other members of the* Nairovirus *group. ELISAs are now also being developed and evaluated for NSD.*

Requirements for vaccines and diagnostic biologicals: *No vaccine is currently available for CVV. Vaccines against AKAV have been produced and has been used e.g. in Japan. For NSDV, an experimental attenuated live virus vaccine has been investigated, and a killed tissue culture vaccine has been shown to be immunogenic. Against SBV, several types of vaccines have been developed (modified live vaccines, vector vaccines, subunit vaccines and inactivated vaccines), and inactivated vaccines are authorised in Europe.*

A. INTRODUCTION

Bunyaviruses vary in their capability to infect humans, as indicated in the following description of each virus. Specific risk assessments as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities* should be carried out to determine both the biosafety and biocontainment measures required for handling infective materials in the laboratory.

1. Cache Valley virus

Cache Valley virus (CVV) is a teratogenic *Orthobunyavirus* (family *Peribunyaviridae*) of the Americas affecting mainly pregnant sheep and goats. Human illness has rarely been reported. Experimental infection of ovine fetuses has confirmed the role of CVV in causing malformation (Rodrigues Hoffman *et al.,* 2012). It is the most common of the *Orthobunyaviruses* of North America (Calisher *et al*., 1986). CVV was first isolated from a mosquito pool in Utah, United States of America (USA) in 1956, but was only linked to disease during an epizootic of neonatal loss and malformed lambs in a sheep flock in Texas in 1987 (Crandell *et al*., 1989). The virus has also been isolated from a horse and a clinically healthy cow.

Serological surveys have shown a widespread prevalence of CVV antibodies in domestic and wild ruminants and horses, for example Uehlinger *et al.* (2018) found positivity rates in Canada of 20% in cattle, 33% goats, 69% horses and 51% mule deer. The 1–3 day viraemia is sufficient to infect vectors allowing deer to act as amplifying hosts (Blackmore & Grimstad, 1998). Vectors include both *Culicoides* midges and mosquitoes of the *Aedes*, *Anopheles, Coquillettidia* and *Culiseta* groups.

CVV infection of adult animals is largely subclinical, and experimentally infected ewes show only a transient febrile response, but with a detectable viraemia. Human disease has been reported on two occasions (Campbell *et al*., 2006; Sexton *et al*., 1997).

CVV was the first North American orthobunyavirus to be linked to fetal arthrogryposis and hydranencephaly, however, other related viruses have been shown experimentally to have the same potential. The clinical outcome of fetal infection with CVV is age dependent. Malformations take place between 27 and 45 days of gestation, with infection at 28–36 days giving rise to central nervous system (CNS) and musculoskeletal defects, and infection at 37–42 days giving rise to musculoskeletal deformities only. Infection after 50 days gestation does not result in lesions and after 76 days the fetus is immunocompetent and antibodies are produced. Most CVV fetal deaths occur between 27 and 35 days of gestation. The fetus is, however, susceptible at any age, demonstrating the tropism of many orthobunyaviruses for fetal tissues (Chung *et al*., 1990).

Gross pathology of the musculoskeletal system includes arthrogryposis of one or more limbs, torticollis, scoliosis of the vertebral column and muscular hypoplasia. CNS lesions include hydranencephaly, hydrocephalus, porencephaly, microencephaly, cerebral and cerebellar hypoplasia and micromelia (Edwards *et al*., 1997). Dead embryos and stillborn or mummified lambs with no obvious defects are also found. Anasarca is seen, as is oligohydramnion. This reduction in amniotic fluid is thought to contribute to restriction of fetal movement and thus to the skeletal deformities seen. Limb defects are also due to neurodegenerative changes seen histopathologically as areas of necrosis and loss of paraventricular neutrophils in the brain together with a reduction in the number of motor neurons. Skeletal muscle changes involve poorly developed myotubular myocytes (Edwards *et al*., 1997).

2. Akabane virus

Akabane virus (AKAV) is a teratogenic *Orthobunyavirus* widely distributed across the world but not in the Americas. It affects mainly cattle, sheep and goats. Antibodies to AKAV have also been found in horses, donkeys, buffalo, deer, camels and pigs, but there are no reports of AKAV-associated illness in these species. It is a member of the Simbu serogroup[2], *Orthobunyavirus* genus, family *Peribunyaviridae*. Other potential pathogens in the Simbu serogroup include Aino, Peaton, Schmallenberg, Shuni, Shamonda and Tinaroo viruses. AKAV is a major cause of arthrogryposis and hydranencephaly. Experimental infections of neonatal calves and pregnant ewes demonstrated that Aino and Peaton viruses may also cause malformations in ruminants (Parsonson *et al.,* 1982; Tsuda *et al.,* 2004a). Aino virus has caused outbreaks of congenital abnormalities in ruminants in Japan and in Australia.

AKAV was first isolated in 1959, initially from a mosquito pool and then a pool of *Culicoides* midges. This was followed in 1972 by isolations from *Culicoides* in Australia and mosquito pools in Africa. AKAV antibodies have been demonstrated in sera from cattle, sheep, goats, horses, buffalo and camels. Many indigenous game species in Africa south of the Sahara have AKAV neutralising antibodies. The range of AKAV includes the Middle East, Asia, Cyprus, Africa and Australia. Epizootics of Akabane disease occur sporadically in countries such as Australia and Israel where vaccination is not routinely practised. Outbreaks usually occur when conditions are favourable for vectors and they move beyond the endemic range into populations of susceptible animals in early to mid-pregnancy or when the virus has been absent from an endemic area for one or more years, usually as a result of drought.

AKAV infection in adult animals is usually subclinical, but encephalomyelitis has been associated with AKAV infection in adult cattle (Kirkland, 2015). Ruminants seroconvert after a 3- to 7-day viraemia.

In endemic areas, females are infected prior to reaching breeding age and antibody in the female prevents fetal infection. Generally disease can be observed in the fetus of naïve dams following infection between 30 and 70 days gestation in the ewe or between 70 and 150 days gestation in the cow. At later stages of gestation, congenital defects are mild and uncommon although infection of the bovine fetus with some strains of AKAV close to term may result in the birth of calves with an encephalitis. AKAV has a predilection for brain, spinal cord and skeletal muscle cells where non-inflammatory necrosis interferes with morphogenesis.

AKAV infection has been studied experimentally in sheep and goats with the production of arthrogryposis/ hydranencephaly, kyphosis, scoliosis, micro- and porencephaly, stillbirths and abortions (Parsonson *et al*., 1975). Natural infection of the ovine and caprine fetus has been described where perinatal lamb mortality and congenital microencephaly were most often seen.

Experimental AKAV studies have been carried out in pregnant cattle and it was shown that the type of abnormality is dependent on the gestational age of the fetus with hydranencephaly seen at approximately 80–105 days and arthrogryposis at about 105–170 days gestation (Kirkland 2015). The time differential in appearance of abnormalities is clearly seen in bovine fetuses, whereas in sheep with a shorter gestation period, brain and skeletal

2 The current classification by the International Committee on Taxonomy of Viruses does not recognise the term "serogroup" for bunyaviruses. It is used in this chapter as a term of convenience.

lesions appear concurrently in the fetus. The sequence of events during an epizootic of AKAV-induced fetal loss are the birth of uncoordinated calves, followed by those with arthrogryposis and dysplastic muscle changes, and lastly those with hydranencephaly and other severe CNS lesions. These events may be preceded by stillbirths and abortions (Shepherd *et al*., 1978). AKAV is responsible for severe neural and muscular abnormalities and lesions are characterised by a nonpurulent encephalomyelitis, focal cerebral degenerative encephalomyelopathy, porencephaly, microencephaly, hydranencephaly, loss of ventral horn motor neurons and axons, depletion of myelin in spinal cord motor tracts, necrosis and polymyositis in the myotubules with parenchymal degeneration of skeletal muscles. Spinal cord abnormalities include scoliosis, and kyphosis and arthrogryposis may affect almost any skeletal joint.

3. Schmallenberg virus

SBV was first detected in November 2011 in Germany from samples collected in October 2011 from dairy cattle with fever and reduced milk yield. Similar clinical signs (including diarrhoea) were detected in dairy cows in the Netherlands, where the presence of SBV was also confirmed in December 2011. From early December 2011, congenital malformations were reported in newborn lambs in the Netherlands, and SBV was detected in and isolated from the brain tissue. Since then, SBV has been detected in many European and Western Asian countries. Suspicion of past infection has also been reported in Africa.

SBV belongs to the *Peribunyaviridae* family, within the *Orthobunyavirus* genus and is a member of the Simbu serogroup (Hoffman *et al.,* 2011). Of note, viruses from the Simbu serogroup had never been detected in Europe before 2011.

Like the genetically related viruses of the Simbu serogroup, SBV affects ruminants. Infection of cattle, sheep, goats, roe deer, mouflon and bison has been confirmed by real-time reverse-transcription polymerase chain reaction (RT-PCR) or virus isolation. Antibodies were found in various wild and captive ruminants and some zoo animals.

Experimental infection in non-pregnant cattle and sheep showed no clinical signs or mild signs at 3–5 days post-inoculation with an incubation period of 2–4 days and viraemia lasting for 2–5 days (Hoffmann *et al.,* 2012, Wernike *et al.,* 2013).

Transmission is by insect vectors and then vertically *in utero.* SBV genome was detected in several *Culicoides* species and vector competence was demonstrated. Vertical transmission across the placenta is proven but direct infection from animal to animal is very unlikely. Experimental infection was not successful via the oral route and animals that were in contact were not infected (Wernike *et al.,* 2013). Furthermore, re-infection of previously infected calves was not possible (Wernike *et al.,* 2013).

Manifestation of clinical signs varies by species: bovine adults have shown a mild form of acute disease during the vector season, congenital malformations have affected more species of ruminants (to date: cattle, sheep, goat and bison). Some dairy sheep and cow farms have also reported diarrhoea (Beer *et al.,* 2012; Hoffmann *et al.,* 2012).

The signs in fetuses or newborns can be summarised as arthrogryposis and hydranencephaly syndrome (AG/HE): in malformed animals and stillbirths (calves, lambs, kids) the pathological signs were arthrogryposis-hydranencephaly, brachygnathia inferior, ankylosis, torticollis, scoliosis, cerebellar hypoplasia and enlarged thymus. The rate of malformation varies depending on the stage of gestation at the time of infection.

Serological studies in humans did not show any evidence that it is a zoonotic agent (European Centre for Disease Prevention and Control, 2012; Reusken *et al.,* 2012).

4. Nairobi sheep disease virus

Nairobi sheep disease (NSD) is a disease of sheep and goats caused by NSD virus (NSDV), an *Orthonairovirus* in the *Nairoviridae* family. The disease has been identified primarily in countries of eastern Africa, where its distribution appears to be limited by the range of the tick vectors that carry NSDV. In Africa, the dominant vector is the *Ixodid* tick *Rhipicephalus appendiculatus*, although ticks of the species *Ambylomma variegatum* have also been found to carry the virus and to be competent for its transmission (Daubney & Hudson, 1934). Importantly, the virus is now known to be present in southern Asia and China (People's Rep. of). Molecular sequencing has shown that a virus previously isolated from *Haemaphysalis* ticks in India and Sri Lanka (where it was known as Ganjam virus) (Sudeep *et al.,* 2009) is also NSDV (Marczinke & Nichol, 2002), and RNA from the same virus has recently been found in *Haemaphysalis* ticks in northeast (Gong *et al.,* 2015) and central (Yang *et al.,* 2019) China. Despite this widespread

distribution of the virus, there is no recorded disease in small ruminants in Asia that can be ascribed to NSDV, apart from one outbreak in imported European sheep (Ghalsasi *et al.,* 1981).

NSD in Africa is characterised by a mortality rate that may range between 40% and 90%, and should always be suspected when animals have recently been moved from an area free from the disease into one where it is endemic. Outbreaks also follow incursions of ticks into previously free areas, particularly following heavy rains (Davies, 1997). The clinical signs are similar in both sheep and goats with sheep being more susceptible, although there are differences in susceptibility among the various breeds and strains in their response to infection with NSDV, some being more susceptible than others. Cattle and game are refractory to infection with NSDV (Zeller & Bouloy, 2000). The incubation period for the disease varies from 2 to 5 days, when a temperature reaction of 41–42°C develops. There is hyperventilation accompanied by severe depression, anorexia and a disinclination to move. Animals stand with lowered head, and show a conjunctivitis and sero-sanguinous nasal discharge. Some of the superficial lymph nodes, such as the prescapular and/or precrural, become palpable. Diarrhoea usually develops within 36–56 hours of the onset of the febrile reaction. This is at first profuse, watery and fetid, later haemorrhagic and mucoid, and accompanied by colicky pains and tenesmus. Abortion is a common sequela to the infection. Examination of the preferred sites for the attachment of ticks, such as the ears, head and body, is likely to reveal the presence of *Rhipicephalus appendiculatus.*

Death can occur in peracute cases within 12 hours of the onset of the fever and at any time during the febrile reaction, while the animal is acutely ill. Further deaths then follow the fall in temperature for a further 3–7 days, associated with severe diarrhoea and dehydration.

The gross pathology of NSD can be misleading, for most deaths are likely to occur during the period of viraemia, when the only signs are likely to be lymphadenitis with petechial and ecchymotic haemorrhages on the serous surfaces of the alimentary tract, spleen, heart and other organs. None of these signs allows a specific diagnosis of NSD to be made, for they are shared with many other febrile diseases of sheep in NSD-endemic areas. Diseases with which NSD may be confused include Rift Valley fever, peste des petits ruminants, salmonellosis and heartwater. Later in the course of the disease, a haemorrhagic gastroenteritis becomes more obvious, with haemorrhages on the mucosa of the abomasum, especially along the folds, in the region of the ileo-caecal valve, and most commonly in the colon and rectum. Zebra striping of the latter is often seen. The gall bladder is usually enlarged and haemorrhagic. Inflammatory lesions with haemorrhage may be seen in the female genital tract, if there has been abortion. However, in many animals dying from NSD, there may be none of these gastroenteric lesions, and a tentative diagnosis based on post-mortem signs can rarely be made. Common histopathological lesions are myocardial degeneration, nephritis and necrosis of the gall bladder.

NSDV is an apparently rare zoonotic agent in the field, causing a mild influenza-like disease in humans. Laboratory infection has been associated with fever and joint pains (Zeller & Bouloy, 2000).

B. DIAGNOSTIC TECHNIQUES

Table 1.1. Test methods available for the diagnosis of CVV and their purpose

[*in preparation*]

Table 1.2. Test methods available for the diagnosis of AKAV and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Virus isolation	–	+	–	+	–	–
RT-PCR	–	+++	–	+	++	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
ELISA	+++	+++	+++	+++[(a)]	+++	+++
VN	+++	+++	+++	+++[(a)]	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
VN = virus neutralisation.
[(a)]Appropriate for confirmation of clinical cases only when fetal samples are tested prior to colostrum intake.

Table 1.3. Test methods available for the diagnosis of SBV and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Virus isolation	–	+	–	+	–	–
RT-PCR	–	+++	–	+++	++	–
Detection of immune response						
ELISA	+++	+++	+++	+[(a)]	+++	+++
VN	+++	+++	+++	+[(a)]	+++	+++
IFAT	+++	+++	+++	+[(a)]	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
VN = virus neutralisation; IFAT = indirect fluorescent antibody test.
[(a)]Appropriate for confirmation of clinical cases only when fetal samples are tested prior to colostrum intake.

Table 1.4. Test methods available for the diagnosis of NSD and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Virus isolation in cell culture	–	–	–	++	–	–
Real-time RT-PCR	–	++	++	+++	+	–
Detection of immune response						
VN	+	–	–	++	++	++
Fluorescent staining (I)FAT)	–	–	–	+	–	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; VN = virus neutralisation
(I)FAT = direct or indirect fluorescent antibody test.

B1. Detection and identification of the agent

1. Cache Valley virus

At birth CVV cannot be isolated from the neonate but has been isolated from mosquito pools and the blood of viraemic adult animals. This has been done on tissue culture using cell lines such as baby hamster kidney (BHK), African green monkey kidney (Vero) and adult Rhesus monkey kidney (LLC-MK2). Virus can be isolated from a febrile animal using a 10% buffy coat suspension in minimal essential medium (MEM) and co-cultivation with Vero cells in MEM supplemented with 2% fetal bovine serum.

Many orthobunyaviruses have been sequenced as they are medically important pathogens associated with encephalitis in humans in both North and South America. Polymerase chain reaction (PCR) technology has been applied to mosquito-pool surveillance, instead of traditional isolation in infant mice, and sensitivity is reported to be one positive mosquito in a pool of 100, which is undetectable by traditional plaque titration in cell culture (Huang *et al*., 2001).

Group-specific and virus-specific primers have been designed, and using RT-PCR, the Bunyamwera (BUN) and California (CAL) serogroup viruses can be distinguished. Previously described nested RT-PCR techniques allow the CAL and most of the BUN serogroup viruses to be distinguished from other *Orthobunyavirus* genus members (Kuno *et al*., 1996; Moreli *et al*., 2001). A duplex real-time RT-PCR for the CAL serogroup and CVV has been reported (Wang *et al.,* 2009), and more recently a real-time PCR that amplifies the M-segment of glycoprotein 1 of CVV and other genes of interest (Hoffmann *et al.,* 2013). However, the tests have not yet been validated for veterinary applications.

CVV antigen can be detected by immunohistochemistry in infected tissue sections using purified rabbit hyperimmune polyclonal serum against CVV propagated in Vero cells (Hoffmann *et al.,* 2012).

See Waddell *et al.* (2019) for a review and gap analysis of published CVV studies.

2. Akabane virus

Diagnosis of infection is rarely made by virus isolation, but usually by serology (ELISA, VNT) undertaken on fetal fluids, occasionally by real-time RT-PCR and sometimes by histopathology. Virus can be readily isolated from viraemic sentinel animals using plasma or buffy coat suspensions, from vector pools and occasionally from fetal material. RT-PCRs have been described for the detection of AKAV.

Virus isolation in tissue culture is frequently undertaken using Vero, BHK-21 and HmLu-1 cell lines. If C6/36 mosquito or KC *Culiciodes* cells are used, cultures are left stationary for 7 days and material is repassaged onto a BHK or Vero cell line where cytopathic changes in the cultures become visible.

Methods employed for specific identification of AKAV using monospecific antibodies or monoclonal antibodies have included virus neutralisation (VN), and immunofluorescence (FA) (Blacksell *et al*., 1997; Gard *et al*., 1988). Antigen detection by immunoperoxidase staining can be used in formalin-fixed material of bovine and ovine fetal material, and in naturally infected newborn calves (Noda *et al*., 2001).

RT-PCR methods for the detection of AKAV nucleic acids have been developed, but additional RT-PCRs specific to Aino, Peaton and Tinaroo viruses or multiplex real-time RT-PCRs or sequencing must be performed to exclude cross reactions (Yang *et al.,* 2008; Yildirim *et al.,* 2015). Multiplex real-time RT-PCR methods (Ohasi *et al.,* 2004; Shirafuji *et al.,* 2015) have been developed for rapid, sensitive direct detection of multiple arboviruses, including AKAV.

3. Schmallenberg virus

Schmallenberg virus (SBV) can be detected by real-time RT-PCR (Bilk *et al.,* 2012; Vengust *et al.,* 2020). Several commercial PCR kits are also available. A one-step multiplex real-time RT-PCR (one-step real-time mRT-PCR) was developed for the simultaneous detection and differentiation of SBV, AKAV and AINV (Lee *et al.,* 2015) and a generic serogroup-specific RT-PCR followed by sequence analyses was described (Golender *et al.,* 2018).

Infectious virus can be isolated in cell culture. Insect cells (KC, C6/36), hamster cells (BHK), or monkey kidney cells (Vero) have been used. Samples for virus detection or isolation should be transported cooled or frozen. Serum or EDTA (ethylenediaminetetraacetic acid) treated blood are the usual source for the detection of acute infection in live animals during the short period of viraemia (2–6 days). For stillborns and malformed calves, lambs and kids, viral RNA can be detected in tissue samples of brain (cerebrum and brainstem) and amniotic fluid, and in amniotic fluid and placenta or meconium from live newborns. However, virus isolation is difficult and was only partially successful in trials.

For stillborns and malformed calves, lambs and kids diagnosis can be also performed by histopathology on fixed central nervous system specimens, including spinal cord. The lesions are characteristic of hydranencephaly, hypoplasia of the central nervous system, porencephaly and subcutaneous oedema (calves). However, sensitivity is lower than with RT-PCR methods and the changes are not specific to SBV.

As the signs are not specific, differential diagnosis should be performed. For the acute infection of the adults, all sources of high fever, diarrhoea and milk reduction should be taken into account. For the malformation of calves, lambs and kids, other orthobunyaviruses, bluetongue virus, pestiviruses, genetic factors and toxic substances should be considered.

3.1. Real-time reverse-transcription polymerase chain reaction

The method presented here targets the S-segment of SBV (Bilk *et al.,* 2012) and detects strains from different geographic locations and clinical presentations. The procedure given may require modification to accommodate individual laboratory or different RT-PCR kit requirements.

i) RNA extraction from blood, tissue samples, midges and semen

Commercial kits are widely available; the RNA extraction can be performed from the sample matrixes blood, serum, tissues and midges according to the procedures specified in each kit. For semen samples, the sensitivity depends on the application of optimized nucleic acid extraction methods combining Trizol(R) LS Reagent lysis with purification of the viral RNA with magnetic beads.

ii) Primers and probe sequences (5' → 3')

SBV-S-382F: TCA-GAT-TGT-CAT-GCC-CCT-TGC

SBV-S-469R: TTC-GGC-CCC-AGG-TGC-AAA-TC

SBV-S-408FAM: FAM-TTA-AGG-GAT-GCA-CCT-GGG-CCG-ATG-GT-BHQ1

iii) Preparation of reaction mixtures

For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC, reagents only), appropriate negative controls, e.g. 1 per 10 test samples, and a positive control should be included. The PCR amplifications are carried out in a volume of 25 µl. Prior to PCR, a master mix is made up, comprising all components except template RNA. This is then dispensed into PCR tubes or plates. Subsequently, test or control RNA (5 µl) is added to the tubes. This approach minimises pipetting errors when assaying large numbers of samples. The master mix comprises (per reaction): 12.5 µl 2 × RT-PCR buffer, 1.0 µl 25 × RT-PCR enzyme mix, 4.5 µl nuclease-free water and 2 µl SBV-specific primer-probe-mix (10 µM SBV-specific primers + 1.875 µM SBV-specific probe).

iv) Real-time reverse-transcription polymerase chain reaction

10 minutes at 45°C. 10 minutes at 95°C. 42 cycles of: 15 seconds at 95°C, 20 seconds at 55°C, and 30 seconds at 72°C. Fluorescence acquisition occurs at the 55°C step mode.

4. Nairobi sheep disease virus

Techniques for laboratory detection of NSDV have been reviewed recently (Hartlaub *et al.,* 2021). There are currently no commercial kits available for detecting the virus. The simplest method of confirming NSDV infection is one of the published methods for real-time RT-PCR (Bin Tarif *et al.*, 2012; Hartlaub *et al.*, 2021). As is the case for the other bunyaviruses, real-time RT-PCR is the standard for NSDV detection and confirmation. The live virus can also be detected using cell culture.

4.1. Real-time RT-PCR

RNA must first be purified from blood or tissue samples. Viral RNA can be detected in whole blood or serum, or in nasal or rectal swabs. The best tissues for detecting virus appear to be the spleen, liver, nasal epithelium and conjunctiva (Hartlaub *et al.*, 2021). Tissue samples should be extracted with acidified guanidine thiocyanate phenol using one of the commercial preparations available. Solid tissues (0.5–1.0 g) are minced and homogenised with 10 ml reagent. Swabs can be extracted, and whole blood, or serum homogenised with, the same reagent; RNA is then purified according to the manufacturer's instructions. For tissues, blood, serum or swabs, RNA extraction based on magnetic beads or spin columns is also suitable. The resulting RNA is stored at –70°C (or –20°C if –70°C not available) until required.

RT-PCR is based on amplification of a section of the S-segment of the virus. The published protocols use the same forward and reverse primers, and differ only in the use of SybrGreen (Bin Tarif *et al.*, 2012) or a TaqMan probe (Hartlaub *et al.*, 2021) for detection. The protocol using the TaqMan probe is given; the master mix recipe and thermal cycling conditions given are for a specific one-step RT-PCR kit, other reagents can also be used, but primer and probe concentrations and the thermocycling protocol should be optimised for other reagents.

i) Primers and probes used (5' → 3'):

Forward primer (F1):	TGA-CCA-TGC-AGA-ACC-AGA-TYG
Reverse primer (R1A):	GAA-ACA-AGC-CTC-ATG-CTA-ACC-T
Probe (P1):	FAM-CAA-GGA-TGC-CAT-CCT-TGC-ATG-GCA-BHQ1

ii) Reactions using the QuantiTect Probe RT-PCR Kit:

Add 5 µl of sample RNA to 20 µl reaction mix containing:

Reagent	Mix (1 reaction)	Final concentration
2× RT-PCR master mix	12.5 µl	
RT Mix	0.25 µl	
Probe P1 (1 µM)	0.5 µl	20 nM
Primer F1 (1 µM)	2 µl	80 nM
Primer R1A (1 µM)	2 µl	80 nM
Water	2.75 µl	
Final volume	20 µl	

iii) Thermocycling protocol

50°C for 30 minutes	1 cycle	Reverse transcription step
95°C for 15 minutes	1 cycle	Inactivates RT and activates polymerase
95°C for 15 seconds 63°C for 30 seconds 72°C for 30 seconds	48 cycles	PCR amplification of the cDNA

4.2. Virus isolation

NSDV may be isolated from material collected from field cases by the use of cell cultures (Davies *et al*., 1977a). Uncoagulated blood, mesenteric lymph nodes and spleen tissue submitted with frozen gel packs are the optimal samples to be collected from febrile or dead animals. The plasma can be used directly as inoculum, and the lymph nodes or spleen should be homogenised to make an approximate 10% (w/v) suspension in a transport medium. This medium can be Hanks' medium with 0.5% lactalbumin hydrolysate or 0.75% bovine serum albumin, and containing penicillin (500 International Units/ml), streptomycin sulphate (500 µg/ml), and nystatin (50 units/ml) or amphotericin B (2.5 µg/ml).

For virus isolation in cell culture, the BHK-21-Clone 13 cell line is especially valuable, but other BHK-21 clones, SW13 cells, Vero cells and primary and secondary lamb or hamster kidney cells have also been used (Shepherd *et al*., 1978). Most strains of NSDV produce a cytopathic effect (CPE) on first passage in BHK-21 cells, while others produce a more obvious CPE only after passage. CPE is not reliably produced on lamb testis or kidney cells, but may occur after multiple passages. The CPE is not specific for NSDV, which can be confirmed in RNA purified from infected cells by RT-PCR as in B1.4.1 or, in cover-slip cultures, by immunofluorescence if a specific anti-NSDV antiserum is available. If immunofluorescence is to be used for confirmation, cultures should be used both with and without flying cover-slips or microwell slide cultures should also be prepared. An appropriate volume of sample, depending on the size of the bottle, dish or well used, should be inoculated onto the cell monolayer and a period of 1–2 hours allowed for adsorption. The CPE becomes evident in BHK cell cultures as foci of granular rounded cells after 24–48 hours, and in a further 24–48 hours in other cell types. FA staining may be positive as early as 24–48 hours post-inoculation when no CPE has yet become evident. Antibodies for immunofluorescent staining may be prepared from hyperimmune mouse ascitic fluids, and from immune mouse, rabbit or sheep sera by standard methods. Such antibodies can be conjugated directly for FAT staining, or a species-specific secondary conjugate may be used (IFAT). Some cross-fluorescence may occur with other nairoviruses at low dilutions of the antibody, but these viruses are not normally associated with disease in sheep or goats.

B2. Serological tests

These include haemagglutination inhibition (HI), CF, IFAT, VN tests and ELISA, however HI and CF tests are rarely used.

1. Cache Valley virus

1.1. Virus neutralisation test

Virus neutralisation (VN) tests for CVV used to be done by a plaque reduction neutralisation method, which may exhibit some cross-reactivity to antibodies against related BUN-serogroup viruses (Beaty *et al.,* 1989), but are now usually performed using inhibition of CPE on Vero cells in microtitre plates (Chung *et al*., 1990).

1.1.1. Test procedure

i) Inactivate test sera at 56°C for 30 minutes in a water bath.

ii) Make serial twofold dilutions of the sera in MEM from 1/2 to 1/16 and incubate at 37°C for 60 minutes with an equal volume of 100 $TCID_{50}$ per ml of virus. Standard controls are prepared in a similar manner.

iii) Discard the medium in a 96-well flat-bottomed cell-culture grade microtitre plate containing a preformed 24-hour Vero monolayer.

iv) Add the serum/virus mixtures to the plate, 50 µl per well, using three wells per dilution.

v) Back titrate the virus used in the test, making three tenfold dilutions using 50 µl per well and four wells per dilution.

vi) Cover the plates and incubate for a further 60 minutes at 37°C.

vii) Add 50 µl MEM maintenance medium to each well.

viii) Incubate the plates at 37°C for 6 days in a humidified CO_2 incubator.

ix) Read the plates microscopically, evaluate the CPE and determine the 50% end points.

x) The virus control should give a value of 100 $TCID_{50}$ and there should be no neutralisation by the negative control serum at the lowest dilution tested. The positive control should give a titre within an expected range of its predetermined mean.

1.2. Enzyme-linked immunosorbent assay

An ELISA, modified and based on the one for Rift Valley fever described by Meegan *et al.* (1987), has been used for CVV serological surveys. Modifications included coating of the plates with mouse ascitic fluid, followed by addition of a sucrose/acetone mouse brain antigen in a sandwich ELISA format. However, alternative methods to produce the antigen (cell culture amplified or produced by recombinant technology) should now be used in place of the mouse brain extract). The diluent used is PBS with 0.5% Tween 20, 5% equine serum and 500 µg dextran sulphate per ml. A horseradish peroxidase conjugate detection system and an ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) substrate are used (Meegan *et al*., 1987).

2. Akabane virus

Serological assays can be used to test serum or plasma and, from stillborn or colostrum-deprived fetuses, detection of antibodies in pericardial and pleural fluids will also confirm *in utero* infection.

2.1. Virus neutralisation test

VN tests have been described using HmLu-1, Vero or BHK cells in flat-bottomed 96-well microtitre plates (Cybinski *et al*., 1978; Da Costa Mendes, 1984). Two techniques have been described with a serum/virus incubation period of 1 hour or incubation overnight before the addition of the cells.

2.1.1. Test procedure

i) Inactivate the test sera at 56°C for 30 minutes in a water bath.

ii) Prepare serial twofold dilutions of the sera in Eagles medium from 1/4 to 1/128 in a 96-well flat-bottomed microtitre plate using duplicate wells and 50 µl per well. Standard controls are prepared in a similar manner.

iii) Add 50 µl per well of virus in Eagles medium diluted to provide 100 $TCID_{50}$ per 50 µl.

iv) Cover and incubate at room temperature for 1 hour.

v) Include a back titration of virus in triplicate, making three tenfold dilutions using 50 µl per well.

vi) Add 100 µl per well cell suspension in Eagles medium with 2% serum at 5×10^5 cells/ml.

vii) Incubate the plates at 34–37°C for 5 days in a humidified CO_2 incubator.

viii) Read the plates microscopically and calculate the titre as the reciprocal of the highest serum dilution completely inhibiting the CPE.

ix) The virus and serum controls should give the expected results.

Where overnight incubation is used, duplicate twofold serial dilutions of inactivated serum are mixed with 100 $TCID_{50}$ of virus using 100-µl volumes in each case. Following incubation for 1 hour at 37°C and overnight at 4°C, 100 µl cell suspension is added to the test. The plate is examined at 3 and 5 days incubation at 37°C and checked for CPE.

2.2. Enzyme-linked immunosorbent assay

AKAV ELISAs, using both IgG and IgM, have been described. Coating antigen is 10^6 $TCID_{50}$ per ml of virus grown on HmLu-1 cells diluted in a 0.05 M carbonate/bicarbonate buffer, pH 9.6. The wash medium is PBS containing Tween 20 and alkaline phosphatase. Rabbit anti-bovine IgG and IgM conjugates are used (Ungar-Waron *et al*., 1989).

A similar ELISA using horseradish peroxidase rabbit anti-bovine IgG conjugate has also been described.

There are two assays commercially available as kits for use in serum and plasma samples of cattle, sheep and goats. One is a purified AKAV-based competition ELISA for detection of antibodies raised against AKAV, while the other is a purified SBV N-protein based indirect ELISA used for detection of antibodies against SBV and other Simbu serogroup viruses. The assays have different diagnostic sensitivities and specificities (Li *et al.,* 2019; Tsuda *et al.,* 2004b). In particular, the SBV N-protein-based assay has broad reactivity with antibodies to other members of the Simbu serogroup but some cross reactivity may also be encountered with the AKAV competitive ELISA.

3. Schmallenberg virus

Serological tests are performed on serum or plasma samples. The most currently used are i) ELISAs with in-house reagents or with the several kits (indirect or competition) that are now commercially available; ii) indirect Immunofluorescence test; and iii) the VN test.

3.1. Enzyme-linked immunosorbent assay

Several types of ELISAs have been developed, including commercially available kits.

The systems can be divided into indirect ELISAs on the basis of recombinant N-protein, Gc-protein or full-virus preparations, and competition ELISAs using nucleoprotein-specific monoclonal antibodies.

3.2. Virus neutralisation test

VN tests have been described using Vero or BHK cells in flat-bottomed 96-well microtitre plates (Loeffen *et al.,* 2012; Mansfield *et al.,* 2013).

The following protocol is based on Wernike *et al.* (2013). The neutralisation test is routinely performed with serum in 96-well microtitre plates using cell culture medium with antibiotics. Exceptionally, if no serum is available, the test can be performed with plasma, but in this case, dilutions <1/20 cannot be effectively evaluated.

3.2.1 Test procedure

i) Inactivate the test sera at 56°C for 30 minutes in a water bath.

ii) Prepare serial twofold dilutions of the sera in medium from 1/5 to 1/640 in a 96-well flat-bottomed microtitre plate using duplicate or quadruplicate wells and 50 µl per well.

Load the wells of the first row with 80 µl, the wells of the other rows with 50 µl culture medium. Add 20 µl serum sample to the first row. Take 50 µl of the first dilution step (1/5 dilution), add to the next row, mix, and continue the dilution series. Discard the last 50 µl. Each well now contains 50 µl of a serum-medium dilution.

Standard controls (positive and negative reference sera) are prepared in a similar manner.

iii) Add 50 µl per well of a virus preparation with 100 $TCID_{50}$/50µl (2000 $TCID_{50}$/ml). The required amount of test virus (approx. 5 ml per microtitre plate) should be prepared in one batch.

In addition to positive and negative serum controls, a cell control (100 µl culture medium without serum and virus), as well as a virus-free serum control (dilution 1/5) should be prepared.

A back titration of the test virus should be performed in each test. The virus dilution used in the test is diluted at log-2-steps in duplicate or quadruplicate, beginning with 1/10 to 1/1280. Wells are prefilled with 180 µl culture medium, and 20 µ test virus suspension at a dilution of 1/10 are added and finally diluted.

iv) Cover and incubate the microtitre plate for 2 hours at 37°C in a humid environment in a CO_2-cabinet. During this time the neutralisation process takes place.

v) After the incubation period, add 100 µl of the respective cell suspension to each well. Adjust the cell density, so that after 24 hours a confluent cell layer develops. The microtitre plate remains in a humid environment in the CO_2-cabinet for incubation.

vi) Incubate the plates at 34–37°C for 3–4 days in a humidified CO_2 incubator.

vii) Evaluation is done by assessment of the cytopathic effect. The final read-out is done at day 3 or 4 after preparation of the test.

The test is valid, if the back titration ranges between 30 and 300 $TCID_{50}$, and the positive control serum shows the indicated titre (± one log2-step).

The antibody titre is calculated as ND_{50} according to Behrens and Kärber.

Neutralisation titre = V–d × (S–0.5)

V: lg of the first 100% positive serum dilution

d: lg of the dilution factor (as a rule 0.3)

S: sum of the positive reagents from 0 to 100%/reagent number per dilution

4. Nairobi sheep disease virus

4.1. Virus neutralisation in cell culture

The titre of NSDV-neutralising antibodies may be assayed using either the inhibition of infection in 96-well microtitre plates or the reduction in plaque number in 6-well plates. BHK-21 cells and SW13 cells

have been used for these assays. In each case, sera are heated at 56°C for 30 minutes before use to inactivate complement.

4.1.1. Infection inhibition in microtitre plates

i) The procedure requires a stock of NSDV with a known titre (determined on the cell line to be used for the neutralisation assay) and a stock of those cells, trypsinised into suspension and adjusted to 2×10^5/ml (BHK-21) or 10^5/ml (SW13) (5ml of cells per 96-well plate).

ii) Each serum dilution is assayed in at least four replicate wells. Two-fold serial dilutions of serum (starting from 1/5 or 1/10, depending on the expected titre) are prepared in flat-bottomed 96-well microtitre plates (50 µl/well).

iii) Add 50 µl maintenance medium containing approximately 100 $TCID_{50}$ of NSDV, mix well by pipetting up and down, cover the plate and incubate for 1 hour at 37°C.

iv) Titrate the stock of diluted virus to ensure that the concentration is in the range 90–150 $TCID_{50}$ per 50 µl.

v) Add 50 µl of cell suspension per well, mix by tapping the sides of the plate, and incubate the plates at 37°C, 5% CO_2.

vi) The wells are scored directly for CPE at 5 dpi (BHK-21 cells) or fixed with neutral buffered formalin at 7 dpi and stained with 0.1% crystal violet to visualise lesions (SW13 cells).

vii) The neutralising titre is the serum dilution that inhibits infection in 50% of the replicate wells (ND_{50}), which can be calculated using the Spearman-Kärber equation. If X1 is the greatest dilution where no well shows CPE and ΣP is the sum of the proportions of uninfected wells from row X1 onwards, the ND_{50} is 10^m where m = (log[X1] – log[2] × [ΣP-0.5]). E.g. for X1=1/40 and ΣP=1.75, m = -1.6 – (0.301 × 1.25) = -1.97625, and ND_{50} = 1/94.68.

4.1.2. Plaque reduction

i) SW13 cells are plated in 6-well plates and should be sub-confluent when used.

ii) Two-fold dilutions of serum (600 µl) are mixed with approx. 50 $TCID_{50}$ of NSDV in 600 µl maintenance medium and incubated for 1 hour at 37°C.

iii) The medium is removed from cells in six-well plates and replaced with 500 µl aliquots of this mixture (in duplicate). The plates are incubated for 1 hour at 37°C, with gentle rocking every 15 minutes. Control wells are infected with virus that has been incubated without serum.

iv) Prepare an overlay by mixing equal volumes of an autoclaved solution of 1.8% Bacto-Agar and 2× MEM containing 4% FCS and 2× penicillin/streptomycin, and keep at ~40°C to prevent it solidifying.

v) The inocula are removed and cells overlaid with 2 ml per well Bacto-Agar/MEM, the overlay is allowed to set at room temperature (15–20 minutes) and then the plates are incubated at 37 ˚C in a CO_2 incubator.

vi) After 4 days, a second overlay consisting of 0.1% crystal violet in neutral buffered formalin is added. After a further 24 hours, the overlays are removed and plaques counted visually. The mean number of plaques in the control wells is set as 100%, and the neutralising titre is the greatest serum dilution that still reduces the plaque number by 80% ($PRNT_{80}$).

4.2. Indirect fluorescent antibody test

The indirect fluorescent antibody test (IFAT) can also be used with members of the Nairovirus serogroup. There are, however, some cross-reactions, particularly with Dugbe virus and also with other members of the group, such as Crimean–Congo haemorrhagic fever virus and Kupe virus (Davies *et al*., 1978). The NSDV antibody titres by this method range from 1/640 to 1/10,240, and such titres are not obtained with immune sera to other members of the group (Davies *et al*., 1976).

The method has been used in epidemiological studies and to study the response to experimental vaccines. There do not appear to be any serological differences among the 40–50 isolates that have

been examined. The NSDV I-34 strain[3] was the virus used to prepare antigen in the original studies, a strain that has been adapted to grow in BHK-21-Clone 13 cells, after a series of passages. Many other strains of NSDV have been found to grow in these cells, in BSR-T7 cells (a BHK cell derivative) or in Vero cells. CPE is greatly reduced in Vero cells, and this can be an advantage in the preparation of antigen (infected cell monolayers) for this assay.

The virus antigen in the cell substrate of choice may be prepared for the test on loose coverslips, on chambered multiwell slides, PTFE(Teflon)-printed multiwell slides or in flat-bottomed microtitre plates. If using loose coverslips, the 9 mm diameter size are suitable for 12-well multiwell plates. A method using PTFE-printed multiwell slides is described.

4.2.1. Preparation of antigen slides

i) Wash and sterilise the slides. This is done briefly with a hot detergent that is used for tissue culture glassware in the laboratory, followed by three rinses in tap water for 30 minutes, each followed by similar rinses in distilled/deionised water. Slides are then placed in 70% ethanol for 10 minutes, removed with sterile forceps and wrapped in greaseproof paper. They will then be found to be sterile, but further sterilisation in a microwave for two cycles of 5 minutes each is recommended.

ii) Place these slides in sterile dishes using sterile forceps.

iii) Prepare a suspension of BHK cells containing approximately 25,000 cells/ml in growth medium, and add 1000 $TCID_{50}$ of NSDV per ml. Mix by pipetting.

iv) Add the infected cells in 50 µl volumes (for the 12-well size) or as appropriate to the size of the wells on the PTFE slides. Replace the cover on the dishes and put into a humidified CO_2 incubator. Prepare negative control slides in the same way using uninfected cells.

v) Leave overnight for the monolayer to form. Then remove the plates from the incubator to a laminar flow cabinet, and flood with maintenance medium using a pipette to cover the slides to a depth of 2–3 mm. Return to the incubator.

vi) Harvest the antigen slides just as foci of CPE become detectable. This will be in 36–56 hours; individual laboratories should determine the optimal harvesting time for their cells and virus strain by fixing and staining slides after 24, 36 and 48 hours).

vii) The slides are washed three times in PBS and the cells fixed. This can be done using dry heat (minimum 80°C) or with ice-cold acetone or methanol:acetone (1:1) for 10 minutes. After fixing in acetone or methanol:acetone, wash the slides three times (5 minutes per wash) in PBS and then allow to air dry. The slides are wrapped and may be stored at 4°C for 2–3 months, or at –20°C for 1–2 years. Slides stored at –20°C must be brought to 4°C overnight before use.

Similar procedures may be followed to prepare antigen on loose coverslips or in multiwell plates or chambered multiwell slides. When using plastic tissue culture multiwell plates, however, fixation should not be with 100% acetone.

4.2.2. Test procedure

i) Hydrate the slides by adding a drop of PBS to each well with a Pasteur pipette. Number the slides according to the number of sera to be tested; at least 12 slides are needed per serum. Include positive and negative control sera, with infected and uninfected cell cultures, in every assay.

ii) Prepare a 2-fold dilution series for each serum, starting at 1/80 and finishing at 1/2560 (6 dilutions)

iii) Discard the PBS and add the serum dilutions in a predetermined manner to the wells. It is preferable to duplicate each dilution, on the same or another slide.

iv) Place the slides in dishes and hold at 37°C in a humid incubator for 40 minutes.

3 The I-34 strain was a virulent NSD isolate made in Kenya that was used extensively as a reference strain at the Kabete Laboratory – Kenya Agriculture Research Institute, P.O. Box 14733 – 00800 Nairobi, Kenya.

v) Wash the slides in racks in three changes of PBS, 5 minutes per wash.

vi) Add the fluorescein-conjugated anti-species conjugate (usually anti-sheep or anti-goat) at a predetermined working dilution (must be determined in each laboratory depending on the conjugated antibody used); one drop can be added to each well with a Pasteur or other pipette.

vii) Incubate as before for 30 minutes.

viii) Wash three times in PBS and dry the slides.

ix) Examine the slides by fluorescent microscopy. NSDV antigen is found in the cell cytoplasm, and foci of bundles of fluorescing BHK cells will be seen. The antigen is seen mainly in fine fluorescent particles, but larger irregularly shaped antigen clumps occur, often surrounding the nucleus, or in spindle-like masses filling the cytoplasm to the pole of the cells. These particles will not be seen with negative sera or in the uninfected control cells.

x) Sera that show this fluorescence at dilutions of 1/640 or 1/1280 are indicative of recent infection with NSDV (Davies *et al*., 1976).

4.3. Other tests

An ELISA using a partially purified tissue culture antigen has been described for antibody testing and is suitable for use in serological surveys (Hartlaub *et al.,* 2021). Virus neutralisation or the IFA test should, however, be used to check doubtful results (Munz *et al*., 1984). Furthermore, new ELISAs are under evaluation (Hartlaub *et al.,* 2021).

Monoclonal antibodies to the antigens of NSDVs strain I-34 have been developed and are being evaluated for their application as diagnostic reagents.

C. REQUIREMENTS FOR VACCINES

1. Cache Valley virus

Due to the sporadic nature of disease outbreaks, no vaccine has been developed.

2. Akabane virus

Major epizootics of Akabane disease have only been reported from Japan and Australia, albeit at irregular intervals, but vaccination is seen to have merit in preventing fetal loss.

An inactivated vaccine is used for immunising cattle and goats. Either formalin or beta propiolactone-inactivated intramuscular preparations with an aluminium phosphate gel adjuvant have been used. Two 3-ml doses are given at a 4-week interval just before mating, and yearly boosters are recommended. It is safe for use in pregnant animals. In field trials 88% of animals developed high VN antibodies after the first inoculation and there was a 100% response after the second dose (Kurogi *et al*., 1978). A high level of efficacy has been shown following natural challenge under field conditions.

In Japan, a live AKAV vaccine is commercially available. A 1-ml dose is administered subcutaneously to cattle before the haematophagous arthropod vectors become active. Pregnant cattle and calves have been inoculated subcutaneously, intramuscularly and intracerebrally; no leukopoenia, viraemia or pyrexia were observed and a good VN antibody response was produced. A live AKAV vaccine, safe in cattle, was tested in pregnant ewes. During the trials, some ewes became viraemic and virus was found in the organs of several fetuses. Although no fetal deformities were produced, the vaccine is deemed unsuitable for use in sheep.

3. Aino virus

Inactivated Aino virus vaccines have been developed and are commercially available in Japan. An inactivated trivalent vaccine (Aino, Akabane and Chuzan viruses) has been tested successfully in cattle (Kim *et al.,* 2011).

4. Schmallenberg virus

Three inactivated commercial vaccines to protect sheep and cattle from SBV infection have received regulatory approval in Europe. Several other forms of vaccines (recombinant modified live, vector, subunit vaccines) have been developed and are at different levels of evaluation (Wernike & Beer, 2020).

Most trials showed a high efficacy and safety of the different vaccine approaches to protect against SBV.

5. Nairobi sheep disease virus

Epidemiological investigations have shown that outbreaks of NSD do not occur in a state of enzootic stability. The disease arises from animal movements from free areas into endemic areas and can be avoided when such areas have been defined. Ecological changes that permit spread of the vector tick will result in extensions of these areas.

Experimental vaccines have been prepared for such situations. One vaccine consisted of virus attenuated by 35 passages in adult mice, but such vaccines can produce severe reactions in some breeds of sheep, and are not considered to be safe for general use. A similar vaccine was developed in Entebbe by further mouse brain passages, but this has not been further developed for use in the field in Uganda or elsewhere.

A tissue-culture-adapted strain of NSD virus has been grown to high titre in cultures grown in roller bottles. When precipitated with methanol, inactivated, and administered with an adjuvant, this was found to give good protection following two inoculations given at an interval of 14 days. Neither of these vaccines is routinely produced, for there has been little demand for their use from the field (Davies *et al*., 1974; 1977b).

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For further information, reference material and advice on Schmallenberg virus,
refer to the Friedrich-Loeffler-Institut, Insel Riems, Germany.

NB: FIRST ADOPTED IN 2004 AS BUNYAVIRAL DISEASES OF ANIMALS (EXCLUDING RIFT VALLEY FEVER);
MOST RECENT UPDATES ADOPTED IN 2023.

CHAPTER 3.10.2.

CRYPTOSPORIDIOSIS

SUMMARY

Description of the disease: *Cryptosporidiosis is the pathological condition caused by infection with the protozoan* Cryptosporidium. *Following infection, the* Cryptosporidium *life cycle, comprising asexual and sexual phases, is completed in a single host, producing sporulated oocysts. There are at least 46 'valid'* Cryptosporidium *species, some of which cause disease in humans, livestock, poultry and game birds, and companion animals.* Cryptosporidium parvum *infects mainly the gastrointestinal tract and is an important cause of diarrhoea in young, unweaned livestock. Mortality is generally low but severe outbreaks may occur occasionally. Weaned and adult animals do not normally exhibit signs of disease, but can excrete oocysts that may contaminate the environment facilitating onward transmission.* Cryptosporidium parvum *is one of the major causes of zoonotic human cryptosporidiosis.* Cryptosporidium andersoni *infects the digestive glands of the abomasum of older calves and adult cattle and also bactrian camels. Some infected cows exhibit reduced milk yields and poor weight gain, but do not develop diarrhoea.* Cryptosporidium baileyi *affects primarily the upper respiratory tract, bursa of Fabricius and cloaca, kidneys and eyes of gallinaceous birds, and has caused outbreaks and mortalities in game and poultry units.* Cryptosporidium meleagridis *affects primarily the ileum of turkey poults and game birds, and can cause enteritis, diarrhoea and death, and* C. galli *infects the surface, ductal, and glandular epithelium of the proventriculus of adult hens and some wild birds.*

Detection of the agent: *Laboratory identification is required for diagnosis. Microscopic observation of stained oocysts is used commonly. Enzyme immunoassays, immunochromatographic lateral flow assays, and molecular diagnostic tests are widely available. The infecting species cannot be identified by oocyst morphology or antibody-based assays, but downstream analysis of DNA amplified by the polymerase chain reaction can be used to determine species. Most cases of cryptosporidiosis in young mammalian livestock are likely to be caused by* C. parvum*, which is also the most important zoonotic species. There is no standardised subtyping scheme, but sequencing the gp60 gene may be informative in outbreak investigations. Multilocus subtyping schemes are in development but need to be standardised. Oocysts can survive in moist environments for many months, and foodborne and waterborne transmission occurs. However, application of genotyping to the small numbers of oocysts likely to be present in food, water and environmental samples is challenging.*

Requirements for vaccines: *There is no commercially available vaccine for cryptosporidiosis.*

A. INTRODUCTION

Cryptosporidiosis is the pathological condition caused by infection with the protozoan Cryptosporidium. Infection of the gastrointestinal tract is most common; the primary symptom is diarrhoea. Respiratory infection occurs in some hosts, especially birds. Other sites may also be infected, again most commonly in birds, and in immunocompromised patients.

1. Nature and classification of *Cryptosporidium*

Cryptosporidiosis is caused by protozoa of the genus *Cryptosporidium,* classified traditionally as phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, and family *Cryptosporidiidae*. A revised classification of the Eukaryotes placed *Cryptosporidium* in the following descending hierarchical groups:

Diaphoretickes; Sar (Stramenopiles, Alveolata and Radiolaria) supergroup; Alveolata; and ultimately Conoidasida wherein *Cryptosporidium* was classified separately from the *Coccidia* and *Gregarinasina* (Adl *et al.,* 2012). Single-cell genomics and transcriptomics analyses also separated *Cryptosporidium* from the gregarines (Mathur *et al.,* 2019). At the time of writing (January 2022), there are 46 *Cryptosporidium* species with formally and adequately described biological and genetic characteristics (Table 1).

Laboratory detection of the genus is required for a diagnosis of cryptosporidiosis, but only molecular methods will differentiate *Cryptosporidium* species and subtypes as many of the oocyst sizes are similar (Table 1) and there are few species-distinguishing antigens. In the past, many 'species' were described on a generally false premise of host specificity (Fayer, 2010). In livestock, poultry and game birds, *C. parvum*, *C. andersoni*, *C. baileyi* and *C. meleagridis* have been reported to cause morbidity and outbreaks of disease. In humans, *C. parvum, C. hominis, C. meleagridis* and *C. cuniculus* are considered the main pathogenic species, causing sporadic cases and outbreaks (Table 1). Most cases of cryptosporidiosis in young mammalian livestock are likely to be caused by *C. parvum*, which is also the most significant zoonotic threat for humans. In addition, more than 120 *Cryptosporidium* 'genotypes' have been identified in animals on the basis of DNA sequencing but lack sufficient biological data for species status (Ryan *et al.,* 2021). The *Cryptosporidium* horse genotype, skunk genotype, ferret genotype, chipmunk genotype I, deer mouse genotype III and the *C. hominis* monkey genotype have also been described in humans.

Table 1. Some differences among species within the genus Cryptosporidium

Cryptosporidium species	Mean oocyst dimensions (μm)[a]	Major host(s)	Usual site of infection	Infections reported in humans
C. abrahamseni (previously piscine gt 7)	3.8 × 3.2	Fish	Intestine	No
C. alticolis	5.4 × 4.9	Voles	Small intestine	No
C. andersoni	7.4 × 5.5	Cattle	Stomach	Yes, but only rarely
C. apodemi	4.2 × 4.0	Mice	Intestine	No
C. avium (syn. avian genotype 5)	6.3 × 4.9	Birds	Ileum, ceacum, kidney, ureter, and cloaca	No
C. baileyi	6.2 × 4.6	Poultry	Upper respiratory tract	No
C. bollandi (previously piscine gt 2)	3.1 × 2.8	Fish	Gastric mucosa	No
C. bovis (previously bovine B genotype)	4.9 × 4.6	Cattle	Small intestine	Yes, but only rarely
C. canis (previously dog genotype)	5.0 × 4.7	Dog	Small intestine	Yes, occasionally
C. cuniculus (previously rabbit genotype)	5.6 × 5.4	Rabbit, humans	Small intestine	Yes, occasionally. One waterborne outbreak
C. ditrichi (syn. UK E6)	4.7 × 4.2	Mice	Small Intestine	Yes, but only rarely
C. ducismarci	5.0 × 4.8	Tortoises	Intestine	No
C. erinacei	4.9 × 4.4	Hedgehog	Small intestine	Yes, but only rarely
C. fayeri (previously marsupial genotype I)	4.9 × 4.3	Marsupials	Intestine	Yes, but only rarely
C. felis	4.6 × 4.0	Cat	Small intestine	Yes, occasionally
C. fragile	6.2 × 5.5	Black spined toad	Stomach	No
C. galli	8.3 × 6.3	Chicken	Proventriculus	No

Cryptosporidium species	Mean oocyst dimensions (μm)[a]	Major host(s)	Usual site of infection	Infections reported in humans
C. homai	Not reported	Guinea-pig	Intestine	No
C. hominis (previously referred to as *C. parvum* human genotype, genotype 1, and genotype H)	4.9 × 5.2	Humans	Small intestine	Yes, commonly. Outbreaks are reported frequently
C. huwi	4.6 × 4.4	Guppy	Stomach	No
C. macropodum (previously marsupial genotype II)	5.4 × 4.9	Eastern grey kangaroo	Intestine	No
C. meleagridis	5.2 × 4.6	Birds, mammals	Intestine	Yes, frequency depends on setting. One farm-related and one school-related outbreak
C. microti	4.3 × 4.1	Voles	Large intestine	No
C. molnari	4.7 × 4.5	Sea bream	Intestine	No
C. muris	7.0 × 5.0	Rodents	Stomach	Yes, but only rarely
C. myocastoris	5.0 × 4.9	Coypu	Small intestine	No
C. occultus	5.2 × 4.9	Rodents	Not reported	Yes, but only rarely
C. ornithophilus (previously avian gt 2)	6.1 x 5.2	Birds	Caecum, colon and bursa of Farbricius	No
C. parvum (also sometimes previously called bovine genotype, genotype II, and genotype B)	5.0 × 4.5	Humans, pre-weaned mammalian livestock, wild mammals	Small intestine	Yes, commonly and outbreaks are reported frequently
C. proliferans	7.7 × 5.3	Rodents	Stomach	No
C. proventriculi	7.4 × 5.7	Birds	Ventriculus and proventriculus	No
C. ratti (previously Rat gt 1)	4.9 × 4.6	Rats	Jejunum and ileum	No
C. rubeyi	4.7 × 4.3	Squirrels	Not reported	No
C. ryanae (previously deer-like genotype)	3.7 × 3.2	Cattle	Not reported	No
C. sciurinum (syn. ferret genotype)	5.5 × 5.2	Squirrels, ferrets, chipmunk	Not reported	Yes, but only rarely
C. scophthalmi	4.4 × 3.9	Turbot	Intestine	No
C. scrofarum (previously pig genotype II)	5.2 × 4.8	Pig	Small intestine	Yes, but only rarely
C. serpentis	6.2 × 5.3	Reptiles	Stomach	No
C. suis (previously pig genotype I)	4.6 × 4.2	Pig	Small intestine	Yes, but only rarely
C. testudinis	6.4 × 5.9	Tortoise	Not reported	No
C. tyzzeri (previously mouse genotype I)	4.6 × 4.2	Mice	Small intestine	Yes, but only rarely
C. ubiquitum (previously cervine genotype)	5.0 × 4.7	Various mammals	Small intestine	Yes, occasionally
C. varanii (syn. *C. saurophilum*)	4.8 × 4.7	Reptiles	Intestine	No

Cryptosporidium species	Mean oocyst dimensions (μm)[a]	Major host(s)	Usual site of infection	Infections reported in humans
C. viatorum	5.4 × 4.7	Humans	Not reported	Yes, occasionally
C. wrairi	5.4 × 4.6	Guinea pig	Small intestine	No
C. xiaoi (previously *C. bovis*-like genotype or *C. bovis* from sheep or *C. agni*)	3.9 × 3.4	Sheep, goat	Not reported	No

[a]From the original papers describing the species.

2. Description and impact of the disease in animals

Clinical and subclinical infections in animals have been reviewed by Santin (2013).

Occurrence of *Cryptosporidium* spp. in ungulate livestock has been reviewed systematically and subjected to meta-analysis by Hatam-Nahavandi *et al.* (2019). Most of the 245 studies included cattle (*n* = 163) and sheep (*n* = 46), and the overall sample positivity was 19% but with considerable variation. Occurrence was especially high in ruminant livestock, particularly in intensive animal production systems.

Cryptosporidium parvum is an important cause of diarrhoea in young, unweaned farmed livestock including calves, lambs, goat kids, alpaca and foals. In addition to welfare issues, production losses include death, diagnostic and treatment costs, additional feed and husbandry costs to achieve market weight and condition. Healthy and adult animals can also shed oocysts, often in large numbers, providing additional potential reservoirs of infection, environmental contamination (including water supplies), and risk of zoonotic transmission.

Cryptosporidium parvum infections of cattle are considered endemic globally. Prevalence and severity of disease peak in the second week of life. Endogenous stages infect enterocytes of the distal small intestine, caecum and colon. Villous atrophy, shortening of microvilli and sloughing of enterocytes are the major pathological changes. Affected animals usually recover within 2 weeks of showing signs of illness. Clinical signs can range from a mild to inapparent infection in older animals to severe diarrhoea in young animals, and can cause varying degrees of dehydration, dullness, anorexia, fever and loss of condition. Mortality is generally low unless occurring as a mixed infection with other enteric pathogens such as *Escherichia coli* or rotavirus, although severe outbreaks of cryptosporidiosis are sometimes reported. Infection has been correlated with low live weight gain and poor production performance.

Cryptosporidium parvum infections of small ruminants (sheep, goats) commonly cause neonatal diarrhoea sometimes associated with high morbidity and mortality especially with concurrent infections or deficiencies in nutrition and husbandry. In ewes, a periparturient rise in oocyst shedding has been observed. Low carcase weights have been reported following acute cryptosporidiosis in lambs.

Other *Cryptosporidium* species also infect livestock and companion animals.

Cryptosporidium bovis and *C. ryanae* are generally more common than *C. parvum* in post-weaned calves. Infections with these host-adapted cattle species have not yet been widely confirmed as associated with illness although there is a report in diarrhoeic calves in Sweden. There are no histological or pathological reports.

Cryptosporidium andersoni colonises the digestive glands of the abomasum of older calves and adult cattle. Infected cattle do not develop diarrhoea, but can excrete oocysts for several months. Some infected beef cattle exhibit reduced weight gain compared with uninfected controls, and one study found that infection may interfere with milk production in dairy cows.

Cryptosporidium ubiquitum and *C. xiaoi* infect lambs and kids. *Cryptosporidium ubiquitum* is prevalent in post-weaned lambs, but is not commonly linked to diarrhoea. *Cryptosporidium xiaoi* infection has been associated with outbreaks of neonatal diarrhoea in goats.

Cryptosporidium canis is the most frequently reported species in dogs and although usually subclinical, infection has been linked to severe diarrhoea, malabsorption and weight loss in younger animals.

Cryptosporidium felis is the most frequently reported species in cats, often in the absence of clinical signs, although occasionally linked to persistent diarrhoea. Cats with other enteric parasites, or with feline leukaemia virus infection, are more likely to develop cryptosporidiosis, which should be included in the differential diagnosis of chronic feline diarrhoea.

Cryptosporidium is a major protozoan parasite of birds and a primary pathogen in poultry, causing respiratory and/or intestinal disease, leading to morbidity and mortality, reviewed by Nakamura & Meireles, 2015. Three species cause disease in birds: *C. baileyi, C. meleagridis* and *C. galli*.

Cryptosporidium baileyi is the most frequently detected species among Galliformes (e.g. chickens, turkeys, quail). It most commonly infects the upper respiratory tract, although other sites include the renal tract, bursa of Fabricius and cloaca, while the trachea and the conjunctiva are lesser sites of infection. Intestinal infection does not normally result in gross lesions or overt signs of disease, but respiratory cryptosporidiosis can result in severe morbidity and, on occasion, mortality. Initially, severe disease is accompanied by sneezing and coughing, followed by head extension to facilitate breathing. Epithelial cell deciliation and hyperplasia, mucosal thickening and discharge of mucocellular exudate into the airways are major pathological changes associated with disease in young broilers. Severe signs of respiratory disease can last up to 4 weeks post-infection. Possible immunosuppression has been reported due to infection with *C. baileyi*.

Cryptosporidium meleagridis is also mainly reported among Galliformes, especially turkeys. Infections in chickens are infrequent. It is the only bird-adapted species also reported in mammals, including humans. Clinical infection is mainly of the small intestine with villous atrophy, crypt hyperplasia and shortening of microvilli reported as major pathological changes causing diarrhoea and weight loss.

Cryptosporidium galli is most frequently reported among Passeriformes and Psittaciformes, with infection limited to the epithelial cells of the proventriculus. Clinical signs include puffed plumage with head held under the wing, unresponsiveness to external stimuli, and failure to thrive. Histopathology of haematoxylin and eosin stained sections from finches demonstrated necrosis and hyperplasia of proventricular glandular epithelial cells, and a mixed inflammatory cell infiltration into the lamina propria of the proventriculus associated with large numbers of oocysts attached to the surface of glandular epithelial cells.

Outbreaks of disease in game birds (e.g. pheasant, partridge and grouse) suggest cryptosporidiosis should be included among respiratory and enteric diseases routinely tested for in these birds. Both *C. meleagridis* and *C. baileyi* are involved.

Other *Cryptosporidium* species have been reported in bird faeces, including *C. parvum*, for which birds may be transport vectors rather than highly susceptible to infection.

Cryptosporidium in terrestrial wildlife has been reviewed by Zahedi *et al*. (2016), indicating the widespread occurrence of this parasite including *C. parvum* and other zoonotic species.

3. Human health risk and zoonotic potential

Human cryptosporidiosis has been reviewed by Chalmers & Davies (2010). Cryptosporidiosis is usually an acute, self-limiting gastrointestinal disease, characterised by watery diarrhoea, abdominal cramps, vomiting, low-grade fever, and loss of appetite. Symptoms can last for up to 1 month during which time apparent recovery and relapse occurs in about one third of cases. It can be a serious disease in the young, malnourished, and immunocompromised, and is a major cause of moderate-to-severe diarrhoea in young children in sub-Sarharan Africa and South-East Asia where it carries a significant risk of death (Kotloff *et al.,* 2013).

Long-term sequelae have been linked to infection with *Cryptosporidium* including myalgia, arthralgia, fatigue, continued gastrointestinal upset, irritable bowel syndrome, and an association with bowel cancer that requires further investigation.

Patients with severe immunodeficiency may suffer from chronic, severe and intractable cryptosporidiosis with significant mortality. In malnourished young children, infection causes substantial morbidity and mortality (Kotloff *et al.*, 2013), and longer term consequences including growth faltering and cognitive defects. There is evidence for respiratory involvement in some populations.

Cryptosporidium parvum is the main zoonotic species (Table 1), but there are also human-adapted subtypes that seem to be transmitted without animal involvement. These occur globally but are most prevalent in Africa where anthroponotic transmission predominates in a landscape of generally extensive and pastoral husbandry (Robertson *et al.,* 2020).

Cryptosporidium hominis is an important cause of gastrointestinal disease in humans. Humans are the major host, and although there are a small number of reports of *C. hominis* infections in livestock, there is no evidence for maintenance of infection in, or transmission between, herds or flocks, and few reports of clinical signs in animals.

4. Transmission, biosafety and biosecurity requirements

Transmission is by the faecal-oral route and may involve a vehicle such as contaminated food or drinking water. Food- and especially water-borne outbreaks carry significant economic, health and social impacts. *Cryptosporidium parvum* is highly infectious for young livestock and humans; older livestock can remain infected and excrete oocysts that can be transmitted to other susceptible hosts. The nature of animal production systems and husbandry (e.g. whether intensive or extensive, pastoral or otherwise) is an influence on transmission to humans. A review of One Health approaches to tackle zoonotic cryptosporidiosis has been produced by Innes *et al.* (2020).

Transmission of *C. hominis* is considered to be anthroponotic; relatively rare findings in animals may be influenced by human activity.

Isolates vary in infectivity, and susceptibility is influenced by host-related factors (Borad & Ward 2010; Flores & Okhuysen, 2009; Yang *et al.,* 2010). Dose–response models indicate that there is a high likelihood of human and pre-weaned livestock infection with single numbers of *C. parvum* oocysts. There is a positive relationship between pre-existing antibodies and protection from infection, whether with the same or a different *Cryptosporidium* species. However, neither the level of immunity nor the extent of cross-protection has been determined. Ingestion of a sufficient quantity of good quality colostrum soon after birth is important in controlling cryptosporidiosis in livestock.

Oocysts can survive for long periods (>6 months) in cool, moist environments, and on fomites such as farm gates, buildings and utensils. Oocysts can be transmitted following direct contact with faeces from an infected individual, or contact with contaminated fomites, or by ingestion of contaminated food or water. Agricultural practices likely to enhance the spread of cryptosporidiosis include indoor calving and lambing and the communal feeding and husbandry of neonates, where young susceptible animals are in close contact with infected animals. Transmission from clinically normal dams to suckling neonates may occur, and improved sample preparation from large faecal mass and sensitive detection methods such as polymerase chain reaction (PCR) need to be applied to detect carriage in adult, subclinically affected animals. The disposal of faeces, farmyard manure or other contaminated waste in land-based dumps, and the spreading of slurry, when followed by periods of heavy rainfall or melting snow can lead to oocyst contamination of water courses and transmission through drinking water supplies. Manure and slurry should be well composted or fermented before spreading.

Farm management practices directed at controlling environmental contamination include removal of dung and contaminated bedding from animal housing, steam-cleaning and disinfection, although *Cryptosporidium* is resistant to many commonly used disinfectants. Thorough cleaning of surfaces and utensils with hot, soapy water > 60°C followed by drying can be effective.

Wild mammals may act as hosts to *Cryptosporidium* spp. (Fayer, 2010; Xiao *et al*., 2004) and provide a reservoir, but little is known of the scale of importance of their involvement in transmitting infection to, or maintaining infection in, livestock in agricultural environments (Sturdee *et al*., 1999). Animals such as birds or fish normally infected with host-adapted *Cryptosporidium* species may act as transport vectors of other species including *C. parvum.* Contamination of drinking water supplies by wildlife is a possible transmission route.

5. Differential diagnosis

The differential diagnosis for *Cryptosporidium* includes other entero-pathogens involved in diarrhoea. Multiple pathogens can be present in faeces including other parasites, viruses such as rotavirus or coronavirus, pathogenic strains of *E. coli,* and *Salmonella* spp. *Cryptosporidium* is a significant pathogen and cryptosporidiosis in livestock

is confirmed by finding significant numbers of oocysts in diarrhoeic faeces in the absence of other pathogens. Although it has been widely speculated that co-infection may lead to more severe cryptosporidiosis (Lorenz *et al.,* 2011) experimental data to support this are lacking. Gastro-intestinal upset may also have non-infectious causes, for example inflammatory bowel disease in humans.

B. DIAGNOSTIC TECHNIQUES

Table 2. Test methods available for diagnosis of cryptosporidiosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Conventional, stained microscopy	–	–	–	+++	++	–
FAT	–	–	–	+++	++	–
Antigen detection by IC	–	–	–	+	+	–
Antigen detection by ELISA	–	–	–	+++	+++	–
PCR	–	–	–	+++	+++	–
Detection of immune response						
Antibody detection by ELISA	–	–	–	–	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
FAT = fluorescent antibody test; IC = immunochromatography;
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[(a)]Positive reactions in ELISA or IC tests should be confirmed.

1. Introduction to tests available

Confirmation of the infection is most commonly by microscopic detection of *Cryptosporidium* oocysts in faeces (Casemore, 1991). Organisms can also be detected in intestinal fluid, tissue samples, or biopsy specimens; antigens in faeces or intestinal fluid; or nucleic acid by PCR-based detection in faeces, intestinal fluid, tissue samples, or biopsy specimens. Haematoxylin and eosin stain can be used for histological diagnosis in biopsy material or confirmation of the diagnosis post-mortem.

Species identification is usually by a reference laboratory test, for which the benchmark is sequencing the small subunit (SSU) rRNA gene (Roellig & Xiao, 2020). Subtyping tools targeting the gp60 gene may be used in epidemiological investigations of *C. parvum, C. hominis, C. meleagridis, C. ubiquitum* and *C. felis* infections (Roellig & Xiao, 2020). There is no standardised multilocus subtyping scheme, and although sequencing or fragment size analysis of mini- and microsatellite markers has been described (Xiao, 2010), marker selection, analysis and relationship algorithms need to be harmonised (Widmer & Caccio, 2015). High throughput sequencing technologies

are not yet routinely applied to *Cryptosporidium* in diagnostic laboratories. There are no reproducible *in-vitro* culture techniques available routinely to amplify parasite numbers prior to identification.

Serological tests are not appropriate for diagnosis but can be used for seroepidemiological surveys of exposure.

2. Detection of *Cryptosporidium*

2.1. Safety and quality

Cryptosporidium is a risk to laboratory workers and all laboratory procedures that can give rise to infectious aerosols must be conducted in a biosafety cabinet. Specimens may contain other pathogenic organisms and should be processed accordingly. To safeguard the health of laboratory workers, all laboratory manipulations must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

2.2. Collection and submission of samples

Specimens for primary diagnosis should be collected during acute infection. If *Cryptosporidium* only is sought, short-term storage of faeces at 4°C is appropriate as oocyst morphology and antigen structure will be retained. For longer term storage, –20°C can be used. Alternatively, an equal volume of 5% potassium dichromate ($K_2Cr_2O_7$) can be added to facilitate storage at ambient temperature (as potassium dichromate is toxic, all procedures should be done in a fume hood or a ducted Class II biosafety cabinet [ducted = vents externally to the room]). However, if other parasites and especially trophozoites are sought in differential diagnosis, faeces need to be examined promptly. The deterioration of the morphology of other parasite stages, and overgrowth by other microorganisms particularly yeasts, can be reduced by the addition of preservatives including 10% (v/v) aqueous formalin, merthiolate–iodine–formaldehyde (MIF), sodium acetate–acetic acid–formalin (SAF) and polyvinyl alcohol (PVA). Downstream tests must be considered for preservative compatibility; for example formalin and SAF are generally compatible with enzyme-linked immunosorbent assay (ELISA) and immunochromatographic (IC) kits, but refer to manufacturers' instructions. Preservatives may interfere with PCR-based tests; faecal samples can be preserved in 90% ethyl alcohol for later PCR testing. Faeces in preservative may require concentration by a recognised method before microscopy, but this is not appropriate if ELISA and IC kits are used because soluble antigens may be lost in the process. Some faeces may need additional processing, for example very liquid faeces may be concentrated, high fat faeces may require defatting, mucoid samples may need treatment with KOH or dithiothreitol, high-fibre faeces may need sieving to remove fibres.

Procedures for packaging and shipping of specimens must be as outlined in the International Air Transport Association's Dangerous Goods Regulations (IATA, 2021). These regulations are summarised in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*.

2.3. Microscopy – sample preparation and staining

2.3.1. Preparation of faecal (or appropriate body fluid) smears

Most unpreserved samples can be smeared directly on to microscope slides prior to staining. Include a positive control slide each time this procedure is performed.

2.3.1.1. Test procedure

i) Wear protective clothing and disposable gloves. Score the reference number of the specimen on a microscope slide with a diamond marker[1], and use separate microscope slides for each specimen. For formed faeces, place 1 drop of saline (about 50 µl) in the centre of the slide.

1 Alternatively, a pencil can be used to mark the etched (frosted) portion of a frosted glass microscope slide.

ii) For liquid faeces (or other appropriate body fluid) dispense one drop (about 20 µl) directly on to the slide. For formed faeces, use the tip of a clean applicator stick to remove about 2 mg sample[2] and emulsify in the saline by thorough mixing.

iii) Prepare a medium to thick smear with areas of varying thickness. Ensure that the smear is of the correct transparency[3].

iv) Air dry the smear at room temperature.

v) Fix the smear[4] in methanol for 3 minutes.

vi) Stain using modified Ziehl–Neelsen or auramine phenol stains as described below.

2.3.2. Concentration of oocysts from preserved or liquid samples by flotation

2.3.2.1. Preparation of flotation solution

Sucrose, zinc sulphate or sodium chloride solutions may be used to separate oocysts from faecal debris. Selection may depend on downstream applications.

2.3.2.1.1. Preparation of sucrose or zinc sulphate solution

Either prepare sucrose solution (specific gravity 1.18) in a glass beaker by adding 256 g of sucrose to 300 ml of deionised water or prepare zinc sulphate solution (specific gravity 1.18) in a glass beaker by adding 100 g of zinc sulphate to 300 ml of deionised water. Gently heat the solution (<60°C) and stir continuously on a hot plate stirrer until the sucrose or zinc sulphate has dissolved completely. Place the solution on ice or in a refrigerator until it cools to 4°C. Pour the cold solution to a 500 ml measuring cylinder and adjust the specific gravity to 1.18 by adding sufficient cold, deionised water (4°C). Pour the solution into a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C for up to 12 months.

2.3.2.1.2. Preparation of saturated salt solution

Prepare saturated salt solution (specific gravity 1.2) by adding approximately 200 g of sodium chloride to 200 ml of deionised water. Gently heat the solution (<60°C) and stir continuously on a hot plate stirrer. Add further, small amounts of sodium chloride (approximately 10 g) at 10-minute intervals until the solution becomes saturated. Pour the saturated salt solution into a clean glass bottle and either place on ice or in a refrigerator until it has cooled to 4°C. Pour the solution into a 500 ml measuring cylinder and adjust its specific gravity to 1.2 by adding cold, deionised water (4°C). Pour the saturated salt solution into a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C for up to 12 months.

Alternatively, a cold method may be used; approximately 1.5 kg of sodium chloride is required to saturate 4 litres of deionised water by adding the sodium chloride in small quantities, taking care not to stop the magnetic stirrer and keeping the solution stirring briskly. Continue to add sodium chloride until specific gravity 1.2 is reached. Pour the saturated salt solution into screw-cap glass bottles, labelled, dated, initialled and stored at 4°C for up to 12 months.

Before use, ensure that the salt solution is mixed by inversion and allowed to settle for 5 minutes.

2.3.2.2. Recovery of *Cryptosporidium* oocysts by centrifugal flotation

2.3.2.2.1. Test procedure

i) Wear protective clothing and disposable gloves. Transfer approximately 1 to 2 g of faeces[5] with an applicator stick, or pipette 1 to 2 ml liquid faeces, into 10 ml of flotation solution in a 15 ml centrifuge tube and mix thoroughly.

2 For formed stools, the sample should include portions from the surface and from within the stool.
3 Moderately thick smears are recommended for this procedure. If the smear is too thin or thick, oocysts will be missed. An acceptable thickness can be achieved when either the hands of your watch or the print on this page can just be read when viewed through the preparation.
4 Air-dried, methanol-fixed smears can be kept at room temperature for >6 months before staining.
5 For formed stools, the sample should include portions from the surface and from within the stool.

ii) Place the centrifuge tube in a bench top centrifuge with swing out buckets, add a balance tube, if necessary, and centrifuge at 1100 ***g*** for 5 minutes[6].

iii) Remove the top 2 ml of fluid (containing the oocysts) from the meniscus, wash 3× in deionised water and finally resuspend in a minimum volume (up to 1 ml) of deionised water.

iv) Transfer the re-suspended contents on to a microscope slide with a disposable pipette, and air dry.

2.3.3. Concentration of oocysts in preserved or liquid samples by sedimentation

All steps that can generate aerosols (excluding centrifugation) should be performed in an operator protection safety cabinet.

2.3.3.1. Test procedure

i) Wear protective clothing and disposable gloves. Sample approximately 500 mg to 1 g faeces[7] with an applicator stick[8] and place in a clean 12–15 ml centrifuge tube containing 7 ml of 10% formalin. If the stool is liquid, dispense about 750 µl into the centrifuge tube.

ii) Break up the sample thoroughly and emulsify using the applicator stick.

iii) Filter the resulting suspension through a sieve[9] into a beaker, then pour the filtrate back into the same centrifuge tube.

iv) Add 3 ml of ethyl acetate[10] to the formalinised solution, seal the neck of the tube with a rubber bung and shake the mixture vigorously for 30 seconds. Invert the tube a few times during this procedure and release the pressure developed gently by removing the rubber bung slowly.

v) Centrifuge the tube at 1100 ***g*** for 2 minutes[11].

vi) Loosen the fatty plug with a wooden stick by passing the stick between the inner wall of the tube and the plug. Discard the plug and the fluid both above and below it by inverting the tube, allowing only the last one or two drops to fall back into the tube. Discard this fluid, containing ethyl acetate and formalin, into a marked re-sealable liquid waste container.

vii) Re-suspend the pellet[12] by agitation. Transfer the re-suspended contents on to a microscope slide with a disposable pipette, and air dry.

Commercial devices for concentrating helminth ova, larvae and protozoan cysts and oocysts using the formalin-ether method are available.

2.3.4. Staining methods

2.3.4.1. Modified Ziehl–Neelsen (mZN)

i) Strong carbol fuchsin

Dissolve 20 g basic fuchsin in 200 ml absolute methanol and mix on a magnetic stirrer until dissolved. Add 125 ml liquid phenol (general purpose reagent [GPR; 80% w/w in distilled water]) carefully until well mixed, and make up to the final volume with 1675 ml deionised water. Mix thoroughly. Filter before use through Whatman No.1 filter paper to remove debris

6 Centrifugation at speeds higher than 1100 ***g*** for longer (>5 minutes) periods of time is not advised as some parasites may deform or rupture and collapse.

7 This is the size of a pea.

8 The sample should include portions from the surface and from within a formed stool.

9 425 µm aperture, 38 mm diameter is equivalent to 36 mesh British Standard (BS 410-86) or 40 mesh American Standard (ASTM E11-81). The skirt of the sieve should fit neatly into the rim of the beaker. Both the sieve and the beaker should be washed thoroughly in running tap water between each sample.

10 Ethyl acetate, although less flammable than diethyl ether, which was used previously, is nevertheless flammable, therefore the procedure should be performed in well ventilated areas, ensuring that they contain no naked flames. Avoid prolonged breathing or skin contact.

11 Centrifugation at speeds higher than 1100 ***g*** for longer (>5 minutes) periods of time is not advised as some other parasites may deform or rupture and collapse.

12 Too large a pellet is indicative of one or more of the following: centrifuging above the recommended speed or time, insufficient shaking (step iv), taking too large a faecal sample.

and store in a stock reagent bottle. Label, date and initial. Store the stock reagent in a dark cupboard at room temperature. Commercial supplies are also available. The concentration of basic fuchsin can vary within the acceptable range of 1 to 3%.

ii) 1% acid methanol

Carefully add 20 ml concentrated hydrochloric acid to 1980 ml of absolute methanol and mix. Transfer to a stock reagent bottle, and label, date and initial. Commercial supplies are also available.

iii) 0.4% malachite green

Add 2 g malachite green to 480 ml deionised water and mix on a magnetic stirrer. Filter through Whatman No.1 filter paper into a stock reagent bottle, label, date and initial. Commercial supplies are also available.

2.3.4.1.1. Test procedure

Include a positive control slide each time this procedure is performed.

i) Wear protective clothing and disposable gloves. Fix the air-dried smear[13] in methanol for 3 minutes.

ii) Immerse or flood the slide in cold strong carbol-fuchsin and stain for 15 minutes.

iii) Rinse the slide thoroughly in tap water.

iv) Decolourise in 1% acid methanol for 10–15 seconds[14].

v) Rinse the slide in tap water.

vi) Counterstain with 0.4% malachite green for 30 seconds.

vii) Rinse the slide in tap water.

viii) Air-dry the slide.

ix) Examine for the presence of oocysts by scanning the slide systematically using the ×40 objective lens of a bright-field microscope. Confirm the presence of oocysts under the oil immersion objective lens[15].

x) Measure the size and shape of the red-stained bodies using a calibrated eyepiece graticule.

Cryptosporidium spp. oocysts stain red on a pale green background. The degree and proportion of staining varies with individual oocysts. In addition, the internal structures take up the stain to varying degrees. Some may appear amorphous while others may contain the characteristic crescentic forms of the sporozoites. *Cryptosporidium parvum* oocysts appear as discs, 4–6 μm in diameter. Yeasts and faecal debris stain a dull red. Some bacterial spores may also stain red, but these are too small to cause confusion.

2.3.4.2. Auramine-phenol

i) Auramine phenol (AP)

Dissolve 3 g phenol in 100 ml deionised water and slowly add 0.3 g Auramine O. Filter through Whatman No. 1 filter paper into a stock reagent bottle. Label, date and initial the stock reagent. Store at room temperature in a light-proof glass bottle with an airtight stopper. Commercially available stains, such as Lempert's reagent, are also acceptable. Commercial supplies are also available.

ii) 3% Acid methanol

Carefully add 60 ml concentrated hydrochloric acid to 1940 ml absolute methanol and mix. Transfer to a stock reagent bottle, and label, date and initial. Commercial supplies are also available.

13 Moderately thick smears are recommended for this procedure.
14 Over-destaining must be avoided.
15 The smear can be examined with or without a cover-slip.

iii) 0.1% potassium permanganate

Add 0.5 g potassium permanganate to the 499.5 ml deionised water and mix using a magnetic stirrer. Filter through Whatman No. 1 filter paper into a stock reagent bottle, and label, date and initial. Commercial supplies are also available.

2.3.4.2.1. Test procedure

Include a positive control slide each time you perform this procedure.

i) Wear protective clothing and disposable gloves. Fix air-dried smears or concentrates[16] in absolute methanol for 3 minutes.

ii) Immerse the slides in AP stain for 10 minutes.

iii) Rinse in tap water to remove excess stain.

iv) Decolourise with 3% acid alcohol for 5 minutes.

v) Counterstain in 0.1% potassium permanganate for 30 seconds.

vi) Air dry slide at room temperature[17].

vii) Examine for the presence of oocysts, using an epifluorescence microscope equipped with fluorescein isothiocyanate (FITC) or UV filters, by scanning the slide systematically under the ×20 objective lens. Confirm the presence of oocysts under the ×40 objective lens.

viii) Measure the size and shape of the fluorescent bodies using a calibrated eyepiece graticule (see below)[18].

Cryptosporidium spp. oocysts appear ring or ovoid shaped and exhibit a characteristically bright apple-green fluorescence against a dark background. *Cryptosporidium parvum* oocysts are ring or doughnut shaped, measuring 4–6 μm in diameter. If available, view the preparation under a UV filter (excitation 355 nm, emission 450 nm), as sporozoites are more readily seen under the UV rather than the FITC filter set. Under the UV filter, oocysts appear light green and sporozoites appear yellow green.

2.3.4.3. Reporting results of microscopic examination

Negative specimens should be reported as '*Cryptosporidium* oocysts NOT seen'.

Positive specimens should be reported as '*Cryptosporidium* oocysts seen'.

A scoring system for positive samples can be used, based on the number of oocysts observed under the ×40 objective lens. However, microscopic examination cannot be considered as a quantitative determination as oocyst numbers vary considerably during the course of infection.

+	=	less than 1 per field of view
++	=	1 to 10 oocysts per field of view
+++	=	11 or more oocysts per field of view

2.3.5. Immunological methods

Three approaches to the immunological detection of *Cryptosporidium* oocyst antigens have proven useful, immunofluorescence microscopy (IFM), ELISA and IC, and a variety of commercial kits are available. IFM kits are more specific for, and can be more sensitive at, detecting *Cryptosporidium* oocysts in faecal smears than conventional stains (Chalmers & Katzer, 2013). Detection limits of ELISA and IC have been reported in the region of 3×10^5 to 10^6 oocysts per ml (Anusz *et al.,* 1990; Smith, 2008), which is no more sensitive than conventional microscopical methods, and less sensitive than IFM. However, ELISA in 96-well plate format offers the

16 Moderately thick smears are recommended for this procedure.
17 Do not blot slides dry, as some blotting papers contain fluorescent fibres.
18 Putative oocysts are measured by slowly increasing the voltage (light intensity) of the bright-field light source so that both fluorescent and bright-field images can be seen concurrently. Objects can then be measured with the eye-piece graticule.

advantage of streamlined testing of large numbers of samples while IC can be applied outside of the laboratory with less qualified staff. Positive reactions should be confirmed by another method.

2.3.5.1. Direct immunofluorescence microscopy (dIFM)

In dIFM, a FITC-conjugated anti-*Cryptosporidium* MAb (FITC-C-MAbs) that recognises surface-exposed epitopes of oocysts is used. It does not distinguish different species of *Cryptosporidium*. Epifluorescence using a FITC filter system causes the labelled oocysts to exhibit a bright apple-green fluorescence. Materials provided with commercial kits vary but *C. parvum* oocyst positive and negative controls, FITC-labelled anti-*Cryptosporidium* MAb (provided at the working dilution; it is false economy to dilute), and glycerol-based mounting medium containing a photo-bleaching inhibitor may be included.

Include a positive and negative control slide (usually supplied with the kit) each time this procedure is performed. *Cryptosporidium parvum* oocysts can be purchased from commercial suppliers, diagnostic veterinary laboratories or research facilities.

After staining and mounting slides according to manufacturers' instructions, scan the preparation for oocysts under the ×20 and confirm under the ×40 objective of an epifluorescence microscope equipped with an FITC filter set (maximum excitation wavelength 490 nm, mean emission wavelength 530 nm). Measure oocysts under the ×100 objective[18]. If necessary, slides can be stored at room temperature, in the dark, until read.

Cryptosporidium spp. oocysts are round or slightly ovoid objects that exhibit a bright apple-green fluorescence under the FITC filter set. Their measurements (measured length × breadth) are presented in Table 1. Often the fluorescence has an increased intensity around the entire circumference of the oocyst, with no visible breaks in oocyst wall staining. If Evans' blue, which reduces nonspecific fluorescence, is included in the kit, the background fluorescence will be red. If DAPI is included as counterstain, the nuclei will show blue fluorescence. Nonspecific fluorescence is usually yellow. Always refer to the positive control to ensure that the size, shape and colour of the putative oocyst is consistent with those of the positive control.

Results should be reported as for conventional microscopical methods (above). Numbers can be recorded as identified previously.

2.3.5.2. Detection of *Cryptosporidium* antigens by enzyme linked immunosorbent assay (ELISA)

In the ELISA, the presence of soluble *Cryptosporidium* antigens in faeces (coproantigen) is sought. Depending on the commercial kit, *Cryptosporidium* coproantigens are captured and detected using a mixture of monoclonal and polyclonal antibodies.

Commercially available sandwich ELISA antigen detection kits contain anti-*Cryptosporidium*-coated well strips for capturing *Cryptosporidium* coproantigens, anti-*Cryptosporidium* antibodies for developing the reaction that is conjugated to an enzyme (frequently horseradish peroxidase), substrate, chromogen/substrate development system and stopping solution (which inhibits further enzyme catalysis when added to the reaction mixture). The test may also be provided as a rapid membrane enzyme immunoassay in a single-test cartridge system.

These tests have been developed to detect *C. parvum* antigens in stool samples, but they are also capable of detecting common epitopes from infections with other *Cryptosporidium* species. Known negative and positive samples are included in commercial kits. Commercial kits normally contain all the necessary reagents to perform the analysis and the manufacturers' instructions must be followed. It is false economy to dilute kit reagents to increase testing capacity. A comprehensive method and a formula for calculating the cut-off value and assigning positive or negative status to samples are usually included. Kit reagents are normally stored at 4°C when not in use. All reagents should reach room temperature before being used. The diagnostician should always determine whether any contraindications apply to the use of a commercial test and any stool/sample fixative used. Because of the variation in the methods described for different commercial kits, no method for ELISA or IC coproantigen detection is included in this chapter.

Negative reactions should be reported as "*Cryptosporidium* antigen NOT detected'.

Positive reactions should be reported as '*Cryptosporidium* antigen detected'.

It is good practice to confirm positive ELISA reactions using a test of equal or better sensitivity and specificity, such as dIFM or PCR with the result included in the report.

2.3.5.3. Detection of *Cryptosporidium* antigens by immunochromatography (IC)

Rather than relying on molecular diffusion to dictate the rate of antigen binding by the capture antibody as in the ELISA format, which normally takes about an hour per reaction, in lateral flow IC, the speed of antigen binding to the solid phase-bound capture antibody is increased by a wicking action. This draws all fluids rapidly through a membrane enclosed in the immunochromatography cassette and reduces the time required for analysis from hours to minutes or seconds. Soluble *Cryptosporidium* antigens in the test sample are drawn through the membrane and come into contact with, and bind to, immobilised antibodies raised against *Cryptosporidium* antigens, which dramatically increases the speed of antigen–antibody interaction. Positive reactions are qualitative and are seen as a band of colour at a specific location on the membrane, normally identified by a line on the cassette. The assay format can vary between commercial kits. The diagnostician should always determine whether any contraindications apply to the use of a commercial test and any fixative used.

IC is a convenient and rapid method for detecting *Cryptosporidium* antigen in stool samples, although false-positive reactions have been reported and positive reactions must be confirmed by a different test. Sensitivity is less than reported for ELISA, dIFM and PCR.

Negative reactions should be reported as '*Cryptosporidium* antigen NOT detected'

Positive reactions should be reported as '*Cryptosporidium* antigen detected'

It is good practice to confirm positive IC reactions using a test of equal or better sensitivity and specificity, such as dIFM or PCR, with the result included in the report.

2.3.6. Nucleic acid recognition methods

PCR offers improved diagnostic sensitivity compared with microscopy and immunological assays for detecting *Cryptosporidium* in faeces (de Waele *et al.,* 2011). The target is the sporozoite DNA within the oocysts. The reported sensitivity of published PCR methods can range between 1 and 10^6 oocysts, depending on the copy number of the gene target, the oocyst disruption, DNA extraction, amplification and detection reagents, procedures and platforms.

Faecal samples can contain many PCR inhibitors. In addition to bilirubin and bile salts, complex polysaccharides are also significant inhibitors. Boiling faecal samples in 10% polyvinylpolypyrrilidone (PVPP) before extraction can reduce inhibition, but may not be necessary if abrogation steps are taken during DNA extraction (for example, spin columns) and in the PCR (including bovine serum albumin or appropriate mastermix). Faeces or partially purified oocysts stored in an equal volume of 5% ($K_2Cr_2O_7$) and intended for PCR should be washed in deionised water to remove residual preservative prior to DNA extraction; (as potassium dichromate is toxic, all procedures should be done in a fume hood or a ducted Class II biosafety cabinet [ducted = vents externally to the room]). For oocysts in suspension, a series of three washes each followed by centrifugation (1100 ***g*** for 10 minutes), removal of the supernatant and resuspension of the pellet in deionised water should minimise PCR inhibition.

No standard method for disrupting oocysts and extracting *Cryptosporidium* sporozoite DNA exists. *Cryptosporidium* DNA can be extracted either following partial purification of oocysts using one of the flotation/sedimentation techniques described above, or directly from oocysts in faeces. If concentration by formol–ethyl acetate sedimentation is the routine laboratory test, oocyst concentrates suitable for lysis and amplification by PCR can be made by washing the pellets by centrifugation in deionised water. Options for oocyst disruption include bead-beating, freeze–thaw cycles, heating or chemical/enzymatic treatments (Elwin *et al*., 2012). Options for subsequent DNA extraction include commercial spin columns, glassmilk, and chelex resin.

Care is necessary when choosing PCR primers, as some are genus-specific whereas others are species-specific. Validated hydrolysis probe-based real-time PCR assays have been used for detection and identification of *Cryptosporidium parvum* and other selected species in livestock (De Waele *et al.,* 2011) and human (Robinson *et al.,* 2020) samples.

Commercial single and multiplex PCR-based assay kits for the detection of comprehensive panels of gastrointestinal pathogens are becoming available (including for veterinary applications), utilising robotic platforms for DNA extraction, assay set-up and amplicon detection. Pathogen panels should ideally be tailored for the population under investigation. Loci incorporated in such assays include SSU rRNA and *Cryptosporidium* oocyst wall protein (COWP) genes.

2.3.7 Detection of oocysts in drinking water and food

There are standard methods for the detection of *Cryptosporidium* oocysts in drinking water (e.g. International Organization for Standardization [ISO, 2006], The Environment Agency [2010], Environmental Protection Agency [2012]) and fresh leafy green vegetables and berry fruits (ISO, 2016), based on high volume filtration, elution, concentration, immunomagnetic separation and IFM.

2.3.8. Typing and subtyping for disease and source tracking

Molecular tools for inter- and intra-species discrimination are usually applied in specialist or reference laboratories for investigating transmission, identifying sources of infection and identifying specific risk factors.

DNA sequence analysis of the *Cryptosporidium* SSU rRNA gene using the "Xiao/Jiang primers" (Roellig & Xiao, 2020; Xiao & Ryan, 2008) is widely regarded as the benchmark for species identification, as not all *Cryptosporidium* species or genotypes can be identified by procedures such as restriction fragment length polymorphisms (RFLP); however, most of the species that are currently known to be commercially important for livestock can be identified by PCR-RFLP using *Vsp*I and *Ssp*I (Xiao & Ryan, 2008). For bovine samples, *Dde*I can be included for differentiation of *C. andersoni* and *C. muris*, and *Mbo*II for differentiation of *C. parvum* from *C. bovis* or *C. ryanae* (Feng *et al.,* 2007; Roellig & Xiao, 2020). Sequencing of the amplicon is needed for investigation of water or environmental samples where any species or genotype may be present.

Recent work has confirmed the utility of mini- and micro-satellite markers in the study of the population structure of *Cryptosporidium*, but there is a need to harmonise the entire method from marker selection to analytical algorithms (Widmer & Caccio, 2015).

3. Serological tests

Most assays for *Cryptosporidium* antibodies are ELISA based, using various aqueous extracts of native antigens (e.g. Hill *et al*., 1990) or recombinant proteins (e.g. Priest *et al.,* 2006) derived from *C. parvum* oocysts. They have limited application for epidemiological surveillance, and results should be interpreted with caution as the tests are not fully validated.

C. REQUIREMENTS FOR VACCINES

There is no commercial or rigorously tested vaccine available.

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NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.10.3.

CYSTICERCOSIS (INCLUDING INFECTION WITH *TAENIA SOLIUM*)[1]

SUMMARY

Description of the disease: *Cysticercosis of farmed and wild animals is caused by the larval stages (metacestodes) of cestodes of the family* Taeniidae *(tapeworms), the adult stages of which occur in the intestine of humans, dogs, cats or wild* Canidae *and* Mustellidae*. Bovine cysticercosis (primarily in muscle) and porcine cysticercosis (primarily in muscle and the central nervous system [CNS]) are caused by the metacestodes (cysticerci) of the human cestodes* Taenia saginata *and* T. solium*, respectively. Cysticerci of* T. solium *also develop in the CNS, musculature and subcutaneous tissue of humans.* Taenia asiatica *is a less widespread cause of cysticercosis in pigs, with the cysts locating in the liver and viscera and the adult tapeworm occurring in humans. Cysticercosis and coenurosis of sheep and goats, and occasionally cattle, with the cysts occurring in the muscles, brain, liver or peritoneal cavity, are caused by* T. ovis, T. multiceps *and* T. hydatigena*, with the adult tapeworms occurring in the intestines of dogs and wild canids.*

Most adult and larval tapeworm infections cause little or no disease. Exceptions are severe, potentially fatal human neurocysticercosis (NCC) caused by T. solium*, and occasionally neuro-coenurosis caused by* T. multiceps *in humans. These parasites are also occasional causes of muscle or ocular signs in humans. Rare but severe cysticercoses in humans can be caused by the fox tapeworm* Taenia crassiceps *and the mustelid tapeworms* Taenia martis *and* Versteria *spp. 'Gid' caused by* T. multiceps *in ruminants can require surgery or slaughter of the animal. Acute* T. multiceps *coenurosis and* T. hydatigena *cysticercosis in sheep and goats are rare but may be fatal. Cysticercosis causes economic loss through condemnation of infected meat and offal.*

Detection of the agent: *Adult* Taenia *tapeworms are dorsoventrally flattened, segmented and large, reaching from 20 to 50 cm (species in dogs) to several metres (species in humans). Anteriorly, the scolex (head) has four muscular suckers and may have a rostellum, often armed with two rows of hooks, the length and number of these being relatively characteristic of a species. A neck follows the scolex, and this is followed by immature and then by mature reproductive segments, and finally gravid segments filled with eggs. Segment structure, although unreliable, can aid in the identification of the species. Adult* Taenia *are recognised at post-mortem or by passage of segments or eggs in faeces.* Taenia *species cannot be differentiated by egg structure. Metacestodes consist of a fluid-filled bladder with one or more invaginated scoleces. These 'bladderworms' are each contained within a cyst wall at the parasite–host interface. This structure comprises the cysticercus or coenurus. Metacestodes are grossly visible at post mortem and meat inspection, but light infections are often missed. NCC can be diagnosed by imaging techniques.*

Immunological and molecular tests: *Adult human* Taenia *infections can be diagnosed by detection of* Taenia *coproantigen in faeces using an antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA). Several species-specific DNA-based techniques have been developed that can be applied on parasite material or on faecal extracts, but remain to be fully validated.*

Serological tests: *Commercial antibody-detecting tests (enzyme linked immunoelectrotransfer blot) are available for the diagnosis of* T. solium *cysticercosis in people and in pigs, though for the latter the diagnostic performance is limited due to a high false positive rate. A commercial test (ELISA format)*

1 Although certain diseases caused by *Taeniidae* are included in some individual species sections of the WOAH List, this chapter covers several species and thus gives a broader description.

is available for detection of circulating parasite-derived antigen in the serum of pigs or humans with T. solium *cysticercosis, but the use in pigs is limited due to a high false positive rate and low positive predictive value.*

Requirements for vaccines: *Excellent vaccines based on recombinant antigens derived from oncosphere proteins have been developed for immunising animals against cysticercosis caused by* T. ovis *in sheep,* T. saginata *in cattle and* T. solium *in pigs. Currently, no vaccines are available for the adult stages of* Taenia *spp. A* T. solium *vaccine for pigs is commercially available; it is approved in some countries and undergoing regulatory approval in other countries. A combination of vaccination and oxfendazole treatment was highly effective in experimental control of natural transmission to pigs. Immunisation of pigs for* T. solium *cysticercosis requires at least two vaccinations.*

A. INTRODUCTION

The metacestodes (or larval cestodes) of *Taenia* spp. tapeworms are the cause of cysticercosis in various farmed and wild animals and in humans. Adult tapeworms are found in the small intestine of carnivore definitive hosts: humans, dogs, cats and wild canids and mustelids. *Taenia saginata* of humans causes bovine cysticercosis, which occurs virtually world-wide, but particularly in Africa, Latin America, Caucasian and South/Central Asia and eastern Mediterranean countries. The infection occurs in many countries in Europe and sporadically in the United States of America (USA), Canada, Australia and New Zealand. *Taenia solium* of humans causes porcine cysticercosis and human (neuro)cysticercosis ((N)CC), the most important cause of acquired epilepsy in endemic areas. It is found principally in Mexico, Central and South America, sub-Saharan Africa, non-Islamic countries of Asia, including India and China (People's Rep. of) in regions with poor sanitation and free-ranging, scavenging pigs. In 2016, central nervous system syndromes, including seizures and depressed spirit, have been observed in pigs naturally infected with *T. solium* (Trevisan *et al.,* 2016). The cysticerci of *T. asiatica* in South-East Asia occur in the liver of pigs. Dogs and wild canids are the definitive hosts of *Taenia* spp. that have sheep, goats and other ruminants and pigs as intermediate hosts, which occur throughout most of the world, although *T. multiceps* has disappeared from the USA, Australia and New Zealand. *Taenia ovis* cysticerci occur in the muscles of sheep, *T. multiceps* in the brain (occasionally in the muscles) of sheep, goats, sometimes other ruminants and rarely humans, and *T. hydatigena* is found in the peritoneal cavity and on the liver of ruminants and pigs. *Taenia crassiceps*, a species mainly occurring in foxes, causes, though rarely, ocular and neural cysticercoses in immunocompetent human patients and severe subcutaneous disseminated and proliferative cysticercoses in immunodeficient patients; *T. martis*, a tapeworm of martens in Europe causes rare cysticercosis in immunocompetent humans. A *Versteria* sp. distinct from *V. mustelae* caused severe and lethal cysticercoses in heavily immunosuppressed patients in North America (Deplazes *et al.,* 2019). Diagnosis in animals usually is based on identification of the metacestode at meat inspection or necropsy. Adults in definitive hosts are acquired by the ingestion of viable metacestodes in meat and offal that has not been adequately cooked or frozen to kill the parasite.

Gravid segments are shed by the adult tapeworms. Tapeworm eggs, within proglottids or separate, are released into the environment with the faeces. For certain species (*T. saginata*, canid taeniids), active migration of the proglottids out of the anus occurs. Eggs may be disseminated from faeces by physical means or transport hosts. Flies particularly ingest eggs and transport these eggs, so eggs are deposited at high intensity within 150 m of the faeces and at low intensity for 10 km (Lawson & Gemmell, 1990). Eggs are immediately infective when passed. Animals acquire infection from ingestion of food or water contaminated with sticky eggs, ingestion of segments or faeces containing eggs. or by eating arthropods such as dung beetles that carry eggs. Humans may be infected with *T. solium* by eggs on vegetables, in water, etc., that have been contaminated by faeces, or food contaminated by dirty hands, by faeco–oral transmission or possibly through retro-peristalsis and hatching of eggs internally (auto-infection). Disease clusters where a human carrier exists. Routine diagnosis of taeniosis continues to be mainly based on the morphology of the adult tapeworm and the presence of eggs or segments in the faeces of infected definitive hosts.

Taenia spp. should be handled with appropriate biosafety and biocontainment measures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

1.1. *Taenia saginata* (the beef tapeworm)

The adult is large, 4–8 metres long and can survive (many) years, usually singly, in the small intestine of humans. The scolex (or head) has no rostellum or hooks. Useful morphological features are presented in Table 1 (Khalil *et al*., 1994; Loos-Frank, 2000; Soulsby, 1982; Verster, 1969). Gravid segments have >14 uterine branches. They usually leave the host singly and many migrate spontaneously from the anus. Tapeworm eggs are also released separately, outside the proglottid, in the faeces.

The eggs are typical 'taeniid' eggs that cannot be differentiated morphologically from other *Taenia* or *Echinococcus* spp. eggs. *Taeniid* eggs measure about 25–45 μm in diameter; contain an oncosphere (or hexacanth embryo) bearing three pairs of hooks; have a thick, brown, radially striated embryophore or 'shell' composed of blocks; and there is an outer, oval, membranous coat, the true egg shell, that is lost from faecal eggs.

Metacestodes of *T. saginata* usually occur in the striated muscles of cattle (beef measles), but also buffalo, and various *Cervidae*. Viable cysts are oval, fluid-filled, about 0.5–1 × 0.5 cm, translucent and contain a single white scolex that is morphologically similar to the scolex of the future adult tapeworm. They are contained in a thin, host-produced fibrous capsule. Cysts occasionally are found in the liver, lung, kidney, fat and elsewhere.

1.2. *Taenia solium* (the pork tapeworm)

Taenia solium is typically smaller than *T. saginata* being 1–5 metres and is generally assumed to survive for 2–3 years. The scolex has an armed rostellum bearing two rows of hooks. Gravid segments have <14 uterine branches and do not usually leave the host spontaneously, but passively (in small chains) with the faeces. Tapeworm eggs are also released separately, outside the proglottid, in the faeces.

Taenia solium cysticerci occur primarily in the muscles and central nervous system (CNS) of pigs (pork measles), and in the muscles, subcutaneous tissues, CNS and, rarely the eye, of humans. Cysts are grossly similar to those of *T. saginata*. They have a scolex bearing a rostellum and hooks similar to the adult. Occasionally, in the brain cisterns of humans, cysts can develop in the space available as racemose cysts up to 10 cm or more across that lack a scolex.

Table 1. Useful features for identification of scoleces and segments of Taenia *spp.*

Parasite species	Number of hooks	Length of hooks (μm)		No. testes	Layers of testes	Cirrus sac extends to longitudinal vessels	No. uterine branches	
		Large hooks	Small hooks					
T. hydatigena	28–36 (26–44)	191–218 (170–235)	118–143 (110–168)	600–700	1	Yes	6–10 that re-divide	Lobes of ovary unequal in size. No vaginal sphincter. Testes extend to vitellarium, but not confluent behind.
T. ovis	30–34 (24–38)	170–191 (131–202)	111–127 (89–157)	350–750	1	No	11–20 that re-divide	Lobes of ovary unequal in size. Well developed vaginal sphincter. Testes extend to posterior edge of ovary.

Parasite species	Number of hooks	Length of hooks (μm)		No. testes	Layers of testes	Cirrus sac extends to longitudinal vessels	No. uterine branches	
		Large hooks	Small hooks					
T. multiceps	22–30 (20–34)	157–177 (120–190)	98–136 (73–160)	284–388	2	Yes	14–20 that re-divide	Lobes of ovary equal in size. Pad of muscle on anterior wall of vagina. Testes extend to vitellarium, but not confluent behind.
T. saginata	– without rostellum	–	–	765–1200	1	No	14–32 that re-divide Ratio of uterine twigs to branches 2.3	Lobes of ovary unequal in size with small Well developed vaginal sphincter. Testes extend to vitellarium, but not confluent behind.
T. solium	22–36	139–200	93–159	375–575	1	Yes	7–14 that re-divide	Lobes of ovary unequal in size with small accessory lobe. No vaginal sphincter. Testes confluent behind vitellarium
T. asiatica	Vestigial hooks some with small rostellum	–	–	868–904		No	16–32 that re-divide Ratio of uterine twigs to branches 4.4	Ovary, vaginal sphincter and extent of testes as *T. saginata.* Posterior protuberances on some gravid segments

1.3. *Taenia asiatica* (Asian *Taenia*)

Closely related to but genetically distinguishable from *T. saginata*, the adult in humans has an ovary, vaginal sphincter muscle and cirrus sac like those of *T. saginata*, but *T. asiatica* has a small rostellum and posterior protuberances on some segments and 16–32 uterine buds with 57–99 uterine twigs on one side. Segments are passed singly and often spontaneously.

The metacestodes are small, about 2 mm, and have a rostellum and two rows of primitive hooks, those of the outer row being numerous and tiny. They occur mainly in the parenchyma and on the surface of the liver of domesticated and wild pigs; they may be found on the mesenteries and, rarely, are described in cattle, goats, and monkeys.

1.4. *Taenia ovis*

Adults in the intestine of dogs and wild canines reach 1–2 metres in length and have an armed rostellum; the number and size of hooks can aid differentiation of *Taenia* spp. (Table 1). Metacestodes that occur in the musculature (skeletal and cardiac) of sheep and less commonly goats reach 0.5–1.0 × 0.5 cm. A similar parasite (*T. ovis krabbei*) occurs in wild canines and dogs and the muscles of reindeer and deer in northern areas.

1.5. *Taenia hydatigena*

Adults are up to 1 metre or more long, are found in the intestine of dogs and wild canids, and have an armed rostellum (Table 1). Metacestodes can be large, from 1 cm up to 6–7 cm, and the scolex has a long neck. They are found attached to the omentum, mesentery and occasionally protruding from the liver surface, particularly of sheep, but also of other domesticated and wild ruminants and pigs. A wolf and reindeer/deer cycle exists in northern latitudes, in which the metacestodes are found in the liver of the intermediate host; canids are definitive hosts.

1.6. *Taenia multiceps*

Adults, up to a metre long in the intestine of canids, have an armed rostellum (Table 1). The metacestodes (*Coenurus cerebralis*) are large, white fluid-filled cysts that may have up to several hundred scoleces invaginated on the wall in clusters. Coenuri grow to 5 cm or more in size in the brain of sheep, the brain and intermuscular tissues of goats, and also the brain of cattle, wild ruminants and occasionally humans. The cysts induce neurological signs that in sheep are called 'gid', 'sturdy', etc.

1.7. Diagnosis of adult parasites in humans or canine carnivores

All parasite or faecal material from humans with possible *T. solium* infections must be handled with suitable safety precautions to prevent accidental infection with the eggs. *Taenia multiceps* and *Echinocccus* spp. also infect humans and, as taeniid eggs in dogs cannot be differentiated to species or genus level, in areas where these are endemic, the same safety precautions apply. In addition to *Taenia* spp., humans and canine carnivores may be infected by *Diphyllobothrium* and *Hymenolepis* spp., while six other cestode genera are recorded occasionally in humans. These are described by Lloyd (2011) and all can be differentiated from *Taenia* spp. by egg/proglottid morphology. In canids, *Echinococcus* spp. eggs cannot be distinguished from *Taenia* spp. eggs, but the presence of the former can be determined by tapeworm size and *Echinococcus* -specific antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA) (Allan *et al*., 1992). Other worms in canids, *Dipylidium, Diplopylidium, Mesocestoides* and *Diphyllobothrium* spp., have morphologically distinct eggs and proglottids (Lloyd, 2011; Soulsby, 1982).

Adult cestodes can be expelled from humans using an anthelmintic (praziquantel, niclosamide, albendazole) followed by a saline purgative and are identified on the basis of scolex and proglottid morphology, though scolices are often not recovered and comparison of proglottid morphology is not always a reliable method. A self-detection tool was used in Mexico (Flisser *et al*., 2011); medical staff in health centres are supplied with preserved tapeworm segments in a bottle and a manual of questions to ask patients to try to identify carriers (no species identification). In animals, an anthelmintic, such as praziquantel, can be used as well; again, the recovered tapeworms are identified morphologically. Arecoline can no longer be recommended because of its side-effects. Tapeworms can be recovered after anthelmintic treatment, and require appropriate disposal.

Verster (1969) and Loos-Frank (2000) have given descriptions of parasitic diagnosis of all the *Taenia* spp. of humans and animals, their hosts and geographical distributions. Keys for identification are given by Khalil *et al.* (1994). Loos-Frank (2000) gives methods for mounting, embedding, sectioning and staining the proglottids. Worms, after relaxation in water, can be stained directly, although small worms should be fixed in ethanol for a few minutes. Alternatively, worms can be fixed and stored in 70% ethanol containing 10% lactic acid, the scolex and worm being stored separately. The rostellum, hooks and suckers of scoleces or protoscoleces should be cut off and mounted *en face* in Berlese's fluid (made by dissolving 15 g gum arabic in 20 ml distilled water and adding 10 ml glucose syrup and 5 ml acetic acid, the whole then being saturated with chloral hydrate, up to 100 g). The stain is lactic acid carmine: 0.3 g carmine is dissolved at boiling point in 42 ml lactic acid and 58 ml distilled water, 5 ml of 5% iron chloride solution ($FeCl_2.4H_2O$) is added after cooling and can be used again to refresh older solutions. Specimens are allowed to sink in the stain in a vial and are left in the stain for some more minutes to allow the stain to penetrate. Specimens are then washed in 1-day-old tap water until blue in colour. They are then fixed in 50–70% ethanol and dehydrated under the slight pressure of plastic foil keeping the segments flat. Salicylic acid methyl ester is used as clearant.

When segments break from the end of the worm, eggs are expelled in the intestine and can be found in the faeces. Segments of *T. saginata, T. asiatica* and the dog *Taenia* spp. may migrate spontaneously from the anus and this is likely to be noticed (>95% in the case of *T. saginata*). When the segments migrate, the

sticky eggs are deposited in the perianal area and might be detected by application and examination of sticky tape. These signs are far less likely for *T. solium*. Segments of all three may be found on the faeces, but are passed intermittently. Even if a segment has shed all its eggs, it can be identified as a cestode by the many concentric calcareous corpuscles contained within its tissues. Faeces, after mixing to reduce aggregation, can be examined for eggs. Various techniques are used throughout the world and include ethyl acetate extraction and flotation. For the latter, $NaNO_3$ or Sheather's sugar solution (500 g sugar, 6.6 ml phenol, 360 ml water), with their higher specific gravities, are superior to saturated NaCl as flotation media for taeniid eggs. Flotation can be carried out in commercially marketed qualitative or quantitative flotation chambers (Mc Master technique) or by centrifugal flotation that includes a modified Wisconsin technique (faeces, diluted in water, are sieved and centrifuged, the pellet is resuspended in sugar or Sheather's solution and centrifuged at 300 ***g*** for 4 minutes). Eggs adhering to the cover-slip can then be detected. Faecal egg examination will be less sensitive for *T. solium* than the other species. Species cannot be determined by egg morphology. Cheesbrough (2005; 2006) reports that *T. saginata* eggs can be differentiated from *T solium* on staining with Ziehl–Neelsen as used for acid-fast bacilli: the striated embryophore of *T saginata* is acid fast (stains red), that of *T. solium* is not acid fast. DNA probes, the polymerase chain reaction (PCR), PCR restriction fragment length polymorphism (RFLP) or multiplex PCR, have proved useful for differentiation though largely used experimentally to differentiate faecal eggs of *T. solium*, *T. saginata* and *T. asiatica* (Gasser & Chilton, 1995; Geysen *et al.,* 2007; Gonzalez *et al*., 2004; Yamasaki *et al.,* 2004). While equally applicable to differentiation in dogs, the same examinations have not been done for *Taenia* spp.

Ag-ELISAs to detect *Taenia* coproantigen have been developed and can be established in-house if laboratory facilities are available (Allan *et al*., 1992; Deplazes *et al.,* 1991), with prior evaluation of appropriate controls. One Ag-ELISA was developed experimentally by Allan *et al.* (1992) to detect coproantigen in dogs, and so, with appropriate controls, could be used to detect *Taenia* infection in this species. The technique, however, is only *Taenia*-genus specific. A modification of the test, including polyclonal antibodies directed against excretory secretory antigens has been developed, rendering the test species specific (Guezala *et al.,* 2009). The test is an in-house, solid-phase, microwell assay with wells coated with polyclonal, rabbit anti-*Taenia*-specific antibody (TSA).

1.8. Diagnosis of metacestodes

Taenia solium metacestodes might be palpable in the tongue but, both in the living animal and on post-mortem examination or meat inspection, tongue palpation is of diagnostic value only in pigs heavily infected with metacestodes; these will also be difficult to differentiate from large sarcocysts, or other (mechanical) lesions. Nevertheless, tongue palpation is a common diagnostic method used by pig traders in endemic, resource poor areas. A sensitivity of 21% was determined by Dorny *et al.* (2004).

1.8.1. Meat inspection – the main diagnostic procedure

Metacestodes are visible first as very small, about 1 mm, cysts, but detection of these requires thin slicing of tissues in the laboratory. Many young cysts are surrounded by a layer or capsule of inflammatory cells (mononuclear cells and eosinophils being prominent histologically). The parasites' abilities to evade the immune response mean that later in infection, as the cyst matures, few inflammatory cells are present in its vicinity and the cysticercus in its intermuscular location is surrounded by a delicate fibrous tissue capsule.

In theory, cysts can be visualised or felt in tissues such as the tongue of heavily infected animals as early as 2 weeks after infection. Cysts are readily visible by 6 weeks and, when mature and viable, are usually oval, about 10 × 5 mm or larger (depending on the species), with a delicate, fairly translucent, white parasite membrane and host capsule. Pale fluid within the cyst and the scolex, visible as a white dot within the cyst, usually invaginates midway along the long axis of the cyst.

At meat inspection many of the *T. saginata* cysts detected, often as many as 85–100%, are dead. The rate at which cysts age and die and so degenerate varies with the parasite species and the tissue within which the cyst is embedded, the host's immunological status and possibly with host age at infection. In general, cysts tend to die more rapidly in the muscular predilection sites, such as the heart. The preferential distribution of parasites in these areas may be because of greater blood circulation to these muscles. Conversely, the higher rate of activity in these muscles may damage the parasites, allowing leakage of fluid and perhaps disrupting the parasites' abilities to evade the immune response. Cysts at different stages of viability and degeneration can be found

in the same host. Death in skeletal muscles may occur within 2 months of infection of adult cattle with *T. saginata,* but cysts may remain viable for several years. Cysts of *T. hydatigena* in the peritoneal cavity of sheep and those of *T. solium* in pigs also have been described as surviving for long periods.

Degenerating cysts vary in appearance. Marked infiltration of eosinophils, macrophages, lymphocytes and collagen deposition thickens the capsule, which becomes opaque, but initially the cyst within remains apparently normal. The fluid gradually becomes colloid and inflammatory cells infiltrate. The cyst cavity becomes filled with greenish (eosinophilic) and then yellow, caseous material, usually being larger in size and certainly more obvious in meat than the original viable cyst. Later the cyst may calcify. Where very young (without a scolex) or degenerate cysts need to be differentiated from other lesions, compression of the cyst, smears of the caseous contents, histological examination of haematoxylin and eosin (H&E) stained sections are used. Microscopic examination may reveal the calcareous corpuscles (concentric concretions of salts that are around 5–10 μm in size). These indicate a cestode origin of the tissue. The presence of hooks and their length together with knowledge of the host and tissue may aid in identification of cestode species. Experimentally, immunohistochemical staining has differentiated *T. saginata* cysts from non-*Taenia* structures. Molecular tools can be used as well, especially for species identification (see above), also in the finding of a new cestode in a host species or geographical area from which, historically, the parasite was absent. In one study PCR identified only 50% of degenerate presumed *T. saginata* cysts (Abuseir *et al*., 2006), whereas Eichenberger *et al.* (2011) using different primers identified 80% of calcified lesions.

After treatment of *T. solium* in pigs with drugs such as oxfendazole (single dose, 30 mg/kg), the cysts may lose their fluid and collapse. The resultant lesion is smaller than lesions observed following natural death but can take 3–6 months to resolve.

Meat inspection procedures are defined, amongst others, by the regulations within a country or region (e.g. EU) and the animal species. Additional inspections can be performed on doubt or identification of a lesion, or specific origin of an animal. Examinations tend to be more extensive with the zoonotic infections *T. saginata* and *T. solium*.

In general, meat inspection procedures related to *Taenia* spp. consist of:

i) Visual inspection of the carcass, its cut surfaces and the organs within it. This may reveal *T. saginata, T. solium* and *T. ovis* in the muscles, *T. hydatigena* on the liver (and *T. asiatica*), mesenteries or omentum, or *T. multiceps* in the brain in the specific animal species.

ii) The external and internal masseters and the pterygoid muscles are each examined and one or two incisions made into each, the cuts being parallel to the bone and right through the muscle.

iii) The freed tongue is examined visually and palpated, particularly for *T. solium*.

iv) The pericardium and heart are examined visually. The heart usually is incised once lengthwise through the left ventricle and interventricular septum so exposing the interior and cut surfaces for examination. Incisions may go from the base to the apex and regulations also may require additional, perhaps four, deep incisions into the left ventricle. Alternately, the heart may be examined externally and then internally after cutting through the interventricular septum and eversion.

v) The muscles of the diaphragm, after removal of the peritoneum, are examined visually and may be incised.

vi) The oesophagus is examined visually.

vii) In some countries, the triceps brachii muscle of cattle is incised deeply some 5 cm above the elbow. Additional cuts into it may be made. The gracilis muscle also may be incised parallel to the pubic symphisis. These cuts are usually undertaken for *T. solium* in pigs. Such incisions into the legs are made, particularly in African countries as it is suspected that more parasites lodge in these muscles in working or range animals walking long distances because of the exercise and consequent increased blood flow to these muscles. Other countries may also require such incisions into the legs. However, as this devalues the meat,

such incisions are made most commonly once one or more cysts have been found at the predilection sites so as to determine the extent of the infection.

Additional incisions may be required either by the regulations or if cysts are found on the initial incision(s). Eichenberger *et al.* (2013) reported an increase in sensitivity by multiple incisions. Details on meat inspection are supplied by Herenda *et al.* (2000).

Additional or fewer procedures may be required for specific parasites and the judgements on the carcass, viscera, offal and blood will vary dependent on *Taenia* spp. and regulations within a country.

1.8.2. Meat inspection – species differentiation and decision making

i) *Taenia saginata:* predilection site

Calves under 6 weeks are not examined in certain countries (e.g. EU regulation). Predilection sites are the heart, tongue, masseters and diaphragm, presumably because they receive the greatest blood circulation. Nonetheless, cysts may be found in any muscle (or less frequently organs) of the body. Lesions of *T. saginata* may need to be differentiated from *Sarcocystis* sarcocysts and other lesions. In PCR studies in Germany, Switzerland and New Zealand up to 20% of viable, presumed *T. saginata* cysts could not be positively identified (Abuseir *et al*., 2006). Meat inspection has a very low sensitivity (<16%) for the detection of *T. saginata*, especially with low infection levels (Dorny *et al.,* 2000; Eichenberger *et al.,* 2013; Kyvsgaard *et al.,* 1990). In general, meat inspection procedures detect only about 15–50% of the animals that are actually infected. Light infections are easily missed on palpation and meat inspection – in one study involving *T. saginata*, 78% of carcasses infected with >20 cysts were detected compared with those detected following dissection and slicing, while only 31% of those with fewer cysts were detected (Walther & Koske, 1980). Meat inspection efficacy will vary with the number and location of incisions (and the skill and experience of the inspector). For example, in Zimbabwe, 58% of cattle were positive in the head only, 20% in the shoulder only and 8% in the heart only, although overall 81% were found to be infected if all three organs were included. In Kenya Walther & Koske (1980) also found that the predilection sites were not necessarily infected in 57% of the cattle found positive on dissection. They also confirmed the importance of the shoulder incisions in detection of infection in African countries as 20% of the cattle found to be infected were positive in the shoulder only. Animals infected as very young calves may have few or no cysts in tongue and masseters. Wanzala *et al*. (2003), also in Kenya, described the insensitivity of meat inspection in detecting cysticerci: only 50% of naturally or artificially infected cattle were identified. Their observations indicated that a number of viable cysticerci may be missed. A recent large-scale study on Belgian cattle, characterised by mostly light infections, even estimated the sensitivity as low as 0.54% (Jansen *et al.,* 2018a). While Eichenberger *et al.* (2011) detected substantially more cases when implementing additional incisions in the heart muscle (estimated sensitivity of 24.2%), this could not be confirmed by Jansen *et al.* (2018b) who defined a sensitivity of 2.87%.

ii) *Taenia solium:* predilection site

The predilection sites are as for *T. saginata* although there are reports of higher prevalence in shoulder and thigh. Commonly one or more cuts are required 2.5 cm above the elbow joint. This is said to detect some 13% of infected carcasses that would otherwise have been missed. Incisions to be made are country regulation dependent. As for *T. saginata*, meat inspection has a low sensitivity (22.1%), especially for light infections (Dorny *et al.,* 2004). A recent study in South Africa found that the level of agreement (Kappa statistic) between carcass dissection (gold standard) and meat inspection was negative, which is an indication of disagreement between the two methods and confirms that the current meat inspection procedures alone are not sufficiently sensitive to detect all cases of porcine cysticercosis (Sithole *et al.,* 2019).

iii) *Taenia asiatica:* predilection site

The small size means detection of cysts in the liver is difficult except in heavy infection.

iv) *Taenia hydatigena:* predilection site

The parasite migrating in the liver leaves haemorrhagic tracks that then become green/brown with inflammation and later white due to fibrosis. For the records, these must be differentiated from those of liver flukes, if possible, by identification of the cysticerci or adult flukes. White spot from *Ascaris* infection is differentiated as the lesions appear as pale to white, small, isolated foci. Some cysts remain trapped below the liver capsule. These usually are small and degenerate early and then calcify into cauliflower-like lesions. Those that are retained at the liver surface are usually superficial and subserosal, while most of an *Echinococcus granulosus* hydatid cyst is deeper in the parenchyma. *Taenia hydatigena* cysts usually mature in the omental or mesenteric fat. If viable, the *T. hydatigena* cyst has a long-necked single scolex in virtually translucent cyst fluid. Fertile *Echinococcus* hydatid cysts have thicker walls and may contain many brood capsules containing protoscoleces; these appear as a sandy, whitish deposit within the cysts. Differentiation can be important in the implementation and monitoring of hydatid disease control measures so histology may be required. H&E-stained sections will reveal the laminated membrane of very young hydatid cysts as indicated by Lloyd *et al.* (1991). Its presence or absence can be confirmed by periodic acid–Schiff staining when the highly glycosylated proteins in the laminated membrane stain red. *Taenia hydatigena* lesions in cattle and pigs can be similar to tuberculosis. However, the portal and mesenteric lymph nodes are not involved, the contents of parasite cysts are more easily shelled-out and remainders of hooks and calcareous corpuscles may be seen or Ziehl–Neelsen staining may reveal bacteria.

v) *Taenia multiceps:* predilection site

The parasites have a predilection site for the brain and spinal cord. Early migrating parasites can cause reddish haemorrhagic and later grey purulent tracks in the brain, and in heavy infections, the sheep may have a meningoencephalitis. Clinical signs caused by the mature cyst relate to pressure atrophy of adjacent nervous tissue and vary according to location in the brain. There may be impaired vision or locomotion if cysts are in the cerebral hemispheres and the sheep gradually may be unable to feed and will become emaciated. Cerebellar cysts may precipitate more acute and severe signs of ataxia or opisthotonus. In heavy infections, parasites migrate and begin development in other tissues, but they die early. These produce small lesions, 1 mm or so in size, that first contain an encapsulated cyst, then eosinophilic, caseous material that later may calcify. The site of *T. multiceps* cysts in sheep brain might be identified by clinical signs presented and, possibly, softening of the skull overlying the coenurus. Sometimes, the coenurus can be observed at the superficial surfaces of affected muscles, especially in the neck region.

vi) *Taenia ovis:* predilection site

The predilection sites are as for *T. saginata.* Cysts may be confused with large *Sarcocystis gigantea* sarcocysts.

1.9. Detection of circulating antigens

The development of an automated sensitive and specific diagnostic test would greatly reduce the costs of damage to the carcass and also the costs of labour at meat inspection. Sensitivity of serological tests for animals has not reached the stage where commercialisation for individual diagnosis or large-scale detection of infected carcasses in slaughter houses is possible. All assays tested – Ag-ELISA, antibody ELISA, enzyme-linked immunoelectro transfer blot (EITB) and tongue inspection – show low sensitivity in rural pigs infected naturally with low levels of *T. solium* (Dorny *et al*., 2005; Sciutto *et al*., 1998). This finding is also true for *T. saginata* infections in cattle (Jansen *et al.,* 2018b; Van Kerckhoven *et al*., 1998). For example, only a small percentage (13–22%) of cattle carrying fewer than 30–50 viable cysticerci is detected by Ag-ELISA. Recent results estimate a sensitivity and specificity of 26.9 (increasing to 40% if only viable cysts are considered) and 99.4%, respectively for Ag ELISA in Belgian cattle (Jansen *et al.,* 2018b). Results of test performances can vary substantially between studies, representing different populations and study designs/analyses. In a Swiss study, a sensitivity and specificity of 14.3% (increasing to 40% if only viable cysts are considered) and 93.7%, were determined, respectively for the same Ag ELISA (Eichenberger *et al.,* 2013).

For the diagnosis of *T. solium* in pigs based on the B158/B60 Ag ELISA, for which a commercial kit is now available, latest results indicate a rather poor sensitivity and specificity (Chembensofu *et al.,* 2017; Chilundo *et al.,* 2018; Kabululu *et al*., 2020; Sithole *et al*., 2019). The Ag ELISA was recently shown to have a low positive predictive value of 35.2 % (Kabululu *et al.,* 2020).

Nonetheless, Ag-ELISAs do have a use in field-based epidemiological studies for indicating transmission. The detection of viable infections in cattle or pigs could indicate point sources of infection, season of transmission and age of animals at risk. The development of more sensitive and specific assays with recombinant antigens for diagnosis of NCC should improve immunodiagnosis of *T. solium* in pigs.

2. Serological tests for antibodies

Tests for circulating antibodies have little role in animals, except for epidemiological studies. A number of EITB and ELISAs for antibodies to *T. solium* in humans are now widely available. These were reviewed by Rodriguez *et al.* (2012) with comparisons of sensitivity and specificity. Recent results indicate a sensitivity and specificity of 13.8% and 92.9%, respectively for antibody detection in Belgian cattle (Jansen *et al.,* 2018b), while the same test used in Swiss cattle in a different study, performed better with sensitivity and specificity of 81.6% and 96.3%, respectively (Eichenberger *et al.,* 2013).

In pigs, specific antibody detection, based on EITB and assuming the presence of one reactive band as a positive test result leads to a fairly good sensitivity of 89%, but a poor specificity of 48%. The latter can be improved by setting the cut-off at three bands needed for test positivity, leading to an increase in specificity reaching 76%, but with sensitivity decreasing to 78% (Jayashi *et al.*, 2013). Recent results suggest a cross-reactivity to GP50 in pigs infected with *Taenia hydatigena* (Muro *et al.,* 2017).

C. REQUIREMENTS FOR VACCINES

Effective vaccines have been developed against infection with the larval stages of several *Taenia* spp., but internationally accepted standards for the production of vaccines are not available. Considerable information is available in the scientific literature on immunogenic molecules, their recombinant technology and extraction, and their efficacy experimentally and in a number of field trials.

A recombinant vaccine based on the 45W antigen was developed in Australia and New Zealand against infection with *Taenia ovis* (Rickard *et al*., 1995). The vaccine was approved for use in sheep in New Zealand but is not available commercially.

An effective recombinant vaccine has been developed against bovine cysticercosis based on the TSA9/18 antigens of *T. saginata* (Lightowlers *et al*., 1996), but it has never been developed as a commercial product.

For *Taenia solium*, a vaccine based on the recombinant antigen TSOL18 (Flisser *et al*., 2004) has been evaluated in independent experimental studies conducted in Mexico, Peru, Honduras and Cameroon in which the vaccine achieved 99–100% protection (Lightowlers, 2013). Subsequently, the vaccine has been used in the field in Nepal, Tanzania, Zambia, Uganda, as well as in Peru where it was used in a project involving >55,000 pigs (Gabriel *et al*., 2020; Garcia *et al*., 2016; Kabululu *et al*., 2020; Poudel *et al*., 2019). The TSOL18 vaccine has been found to be both safe and efficacious. There is only one commercially available approved TSOL18 vaccine for pigs, produced in India. The TSOL18 vaccine targets the parasite during its early development in the pig, preventing infection. It does not affect cysticerci if they were already established in the tissues prior to vaccination. For that reason, use of the vaccine is recommended in combination with treatment using oxfendazole (single dose, 30 mg/kg), where the drug clears any pre-existing infection, while the vaccine prevents infection from any subsequent exposure to the parasite.

The commercial TSOL18 vaccine incorporates 150 μg of the TSOL18 antigen expressed in yeast, together with an oil adjuvant. Pigs of 2 months of age or older are immunised by intramuscular injection with 1 ml of the vaccine. A booster vaccination is required after 3–4 weeks. Booster vaccination may be applied after 6 months, however recent evidence suggests that pigs become naturally resistant to new *T. solium* infections as they age (Poudel *et al*., 2019), suggesting that booster vaccination of animals fully vaccinated as young animals may not be required. Generally, no significant side effects are noticed, however in some animals, temporary pyrexia, lethargy for 1–2 days and local injection site reactions for up to 7 days may be observed after vaccination. Following administration of a 5-fold overdose of vaccine, no adverse reactions were observed under farm conditions other than those described above.

The vaccine should be stored and transported between 2°C and 8°C; it should not be frozen. Although the manufacturer recommends the vaccine be maintained refrigerated, being a defined antigen, non-living vaccine, the vaccine is known to be relatively insensitive to exposure to room temperatures.

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NB: At the time of publication (2021) there were no WOAH Reference Laboratories for cysticercosis (including infection with *Taenia solium*) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.10.4.

INFECTION WITH *CAMPYLOBACTER JEJUNI* AND *C. COLI*

SUMMARY

Description of the disease: Campylobacter jejuni (C. jejuni) *and* Campylobacter coli (C. coli) *can colonise the intestinal tract of most mammals and birds and are the most frequently isolated* Campylobacter *species in humans with gastroenteritis. Although poultry is the main reservoir of* Campylobacter, *transmission to humans is only partly through handling and consumption of poultry meat; other transmission routes are also considered to be important. This chapter focuses on* C. jejuni *and* C. coli *in primary livestock production with regard to food safety.*

Campylobacter jejuni *and* C. coli *do not normally cause clinical disease in adult animals except for sporadic cases of abortion in ruminants and very rare cases of hepatitis in ostriches. The faecal contamination of meat* (*especially poultry meat*) *during processing is considered to be an important source of human food-borne disease. In humans, extraintestinal infections, including bacteraemia, can occur and some sequelae of infection, such as polyneuropathies, though rare, can be serious.*

Identification of the agent: *In mammals and birds, detection of intestinal colonisation is based on the isolation of the organism from faeces, rectal swabs or caecal contents, or the use of polymerase chain reaction* (*PCR*). Campylobacter jejuni *and* C. coli *are thermophilic, Gram-negative, highly motile bacteria that, for optimal growth, require microaerobic environment and incubation temperatures of 37–42°C. Agar media containing selective antibiotics are required to isolate these bacteria from faecal/intestinal samples. Alternatively, their high motility can be exploited using filtration techniques for isolation. Enrichment techniques to detect intestinal colonisation are not routinely used. Preliminary confirmation of isolates can be made by examining the morphology and motility using a light microscope. The organisms in the log growth phase are short and S-shaped in appearance, while coccoid forms predominate in older cultures. Under phase-contrast microscopy the organisms have a characteristic rapid corkscrew-like motility. Phenotypic identification is based on reactions under different growth conditions. Biochemical and molecular tests, including PCR and MALDI-TOF* (*matrix assisted laser desorption ionisation–time of flight*) *mass spectrometry can be used to identify* Campylobacter *strains at species level. PCR assays can also be used for the direct detection of* C. jejuni *and* C. coli.

Serological tests: *serological assays are not routinely in use for the detection of colonisation by* C. jejuni and C. coli.

Requirements for vaccines: *There are no effective vaccines available for the prevention of enteric* Campylobacter *infections in birds or mammals.*

A. INTRODUCTION

1. Disease

Campylobacter jejuni and *C. coli* are generally considered commensals of livestock, domestic pet animals and birds. Large numbers of *Campylobacter* have been isolated from young livestock with enteritis, including piglets, lambs and calves, but the organisms are also found in healthy animals. Outbreaks of avian hepatitis have been reported, but although *C. jejuni* is associated with the disease, it is not the causative agent (Jennings *et al.,* 2011). Recently, a new *Campylobacter* was isolated as the causative agent of spotty liver disease in layers (Crawshaw *et al.,* 2015). *Campylobacter* is the main cause of human bacterial intestinal disease identified in many industrialised

countries (Havelaar *et al.,* 2013; Scallan *et al.,* 2011). Over 80% of cases are caused by *C. jejuni* and about 10% of cases are caused by *C. coli*. In humans, *C. jejuni/C. coli* infection is associated with acute enteritis and abdominal pain lasting for 7 days or more. Although such infections are generally self-limiting, complications can arise and may include bacteraemia, Guillain–Barré syndrome, reactive arthritis, and abortion (WHO, 2013). Attribution studies show that poultry is the main reservoir of *Campylobacter* and responsible for between 50 and 80% of the human infections. In the European Union (EU), an estimated 30% of the human infections are associated with handling and consumption of poultry meat; but a considerable proportion of the poultry-derived strains has a non-poultry meat transmission route, e.g. via environmental contamination (EFSA, 2010b). Contact with pets and livestock, the consumption of contaminated water or raw milk and travelling in high prevalence areas are also considered risks factors in human disease (Domingues *et al.,* 2012). The control of *Campylobacter* in the food chain has now become a major target of agencies responsible for food safety world-wide.

Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: standard for managing biological risk in the veterinary diagnostic laboratory and animal facilities*).

2. Taxonomy

There are currently 34 *Campylobacter* species recognised, but with the improved diagnostic techniques and genomic analysis, this number is expected to increase over time (*cf* List of prokaryotic names with standing in nomenclature: http://www.bacterio.net/index.html). Members of the genus *Campylobacter* are typically Gram-negative, non-spore-forming, S-shaped or spiral shaped bacteria (0.2–0.8 μm wide and 0.5–5 μm long), with single polar flagella at one or both ends, conferring a characteristic corkscrew-like motility. These bacteria require microaerobic conditions, but some strains also grow aerobically or anaerobically. They neither ferment nor oxidise carbohydrates. Some species, particularly *C. jejuni*, *C. coli* and *C. lari*, are thermophilic, growing optimally at 42°C. They can colonise mucosal surfaces, usually the intestinal tract, of most mammalian and avian species tested. The species *C. jejuni* includes two subspecies (*C. jejuni* subsp. *jejuni* and C. *jejuni* subsp. *doylei*) that can be discriminated on the basis of several phenotypic tests (nitrate reduction, selenite reduction, sodium fluoride, and safranine) and growth at 42°C (subsp. *doylei* does not grow at 42°C) (Garrity, 2005). Subspecies *jejuni* is much more frequently isolated then subspecies *doylei*.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of* Campylobacter jejuni *and* C. coli *and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[(a)]						
Isolation	+++	–	+++	+++	+++	–
MALDI-TOF	+++	–	+++	+++	+++	–
Antigen detection	++	–	++	–	++	–
16S rRNA sequencing	++	–	++	++	++	–
Real-time PCR	++	–	++	++	++	–
Detection of immune response: not applicable for *Campylobacter jejuni* and *C. coli*						

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
MALDI-TOF = matrix assisted laser desorption ionisation–time of flight; PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Isolation and identification of the agent

Two ISO (International Organization for Standardization) procedures for detection of *Campylobacter* exist, a horizontal method for detection and enumeration of thermotolerant *Campylobacter* spp. (ISO 10272) in food and animal feeding stuffs with 2 parts: (part 1 detection method and part 2 colony count technique. Both parts of the ISO are under revision and will be published in 2017. The revised standard will include methods for the isolation of *Campylobacter* from live animals, and a procedure for ISO 17995 concerns water quality, with detection and enumeration of thermotolerant *Campylobacter* spp. from water (ISO, 2005 – last reviewed in 2014).

1.1. Collection of specimens

1.1.1. Poultry at the farm

Poultry is frequently colonised with *C. jejuni* (65–95%), less often with *C. coli* and rarely with other *Campylobacter* species (Newell & Wagenaar, 2000). Colonisation rates in broiler chickens are age-related. Most flocks are negative until 2 weeks of age. Once *Campylobacter* colonisation occurs in a broiler flock, transmission, via exposure to faecal contamination, is extremely rapid and up to 100% of birds within a flock can become colonised within a few days. Samples from live birds, destined for the food chain, should therefore be taken as close to slaughter as possible (Newell & Wagenaar, 2000). The majority of birds shed large numbers of organisms (>10^6 colony-forming units/g faeces). Campylobacters can be isolated from fresh faeces/caecal droppings or cloacal swabs. For reliable detection of *Campylobacter* by culture, freshly voided faeces (preferably without traces of urine) should be collected. Such samples must be prevented from drying out before culture. When swabs are used, a transport medium such as Cary Blair, Amies, or Stuart must be used. Sampling strategy in primary poultry has been reviewed (Vidal *et al.,* 2013) and is normally based on boot-swab samples, faecal/caecal droppings or cloacal swabs.

1.1.2. Cattle, sheep and pigs at the farm

Campylobacters are frequent colonisers of the intestine of livestock such as cattle, sheep and pigs; data have been reviewed by Newell *et al.,* in press. Cattle and sheep are found to be colonised mainly with *C. jejuni*, *C. coli*, *C. hyointestinalis*, and *C. fetus*, whereas pigs are predominantly colonised by *C. coli*. In young animals, the numbers are higher than in older animals. In older animals, the organisms can be intermittently detected in faeces, probably due to low numbers or due to intermittent shedding. Fresh samples have to be taken (rectal samples if possible) and they should be prevented from drying out. When swabs are used, a transport medium (like Cary Blair, Amies, or Stuart) must be used.

1.1.3. At slaughter

In poultry, the caeca are usually used for the detection of *Campylobacter*. They can be cut with sterile scissors from the remaining part of the intestines and submitted intact to the laboratory in a suitable container.

Samples from cattle, sheep and pigs are collected from the intestines by aseptically opening the gut wall or by taking guarded rectal swabs.

1.2. Transportation and treatment of specimens

1.2.1. Transport

Campylobacters are sensitive to environmental conditions, including dehydration, atmospheric oxygen, sunlight and elevated temperature. Transport to the laboratory and subsequent processing should therefore be as rapid as possible, preferably the same day, but within at least 3 days. The samples must be protected from light, extreme temperatures and desiccation.

No recommendation on the ideal temperature for transportation can be made, but it is clear that freezing or high temperatures can reduce viability. High temperatures (>20°C), low temperatures (<0°C) and fluctuations in temperature must be avoided. When the time between sampling and processing is longer than 48 hours, storage at 4°C (±2°C) is advised.

1.2.2. Transport media

Swabs: When samples are collected on boot-swabs or rectal swabs, the use of commercially available transport tubes, containing a medium, such as Cary Blair or Amies, is recommended. This medium may be plain agar or charcoal-based. The function of the medium is not for growth of *Campylobacter* spp., but to protect the swab contents from drying and the toxic effects of oxygen.

When only small amounts of faecal/caecal samples can be collected and transport tubes are not available, shipment of the specimen in transport medium is recommended. Several transport media have been described: Cary-Blair, modified Cary-Blair, modified Stuart medium, Campythioglycolate medium, alkaline peptone water and semisolid motility test medium. Good recovery results have been reported using Cary-Blair (Luechtefeld *et al*., 1981; Sjogren *et al*., 1987).

1.2.3. Maintenance of samples

On arrival at the laboratory, samples should be processed as soon as possible, preferably on the day of arrival but no longer than 3 days after collecting the samples. To avoid temperature variation, samples should only be refrigerated when they cannot be processed on the same day, otherwise they should be kept at room temperature. When samples are submitted or kept in the laboratory at 4°C, they should be allowed to equilibrate to room temperature before processing to avoid temperature shock.

1.3. Isolation of *Campylobacter*

For the isolation of *Campylobacter* from faecal/caecal or intestinal samples, no pre-treatment is needed; samples can be plated on selective medium or the filtration method on non-selective agar can be used. In the case of caecal samples, caeca are aseptically opened by cutting the end with a sterile scissors and squeezing out the material to be processed. Enrichment is recommended to enhance the culture sensitivity of potentially environmentally stressed organisms or in the case of low levels of organisms in faeces, for example from cattle, sheep or pigs. However, enrichment of faecal samples is usually subject to overgrowth by competing bacteria and is not carried out routinely.

1.3.1. Selective media for isolation

Many media can be used in the recovery of *Campylobacter* spp. Modified charcoal, cefoperazone, desoxycholate agar (mCCDA), is the most commonly recommended medium, although alternative media may be used. A detailed description on *Campylobacter* detection by culture and the variety of existing media is given by Corry *et al.* (Corry *et al*., 1995; 2003). The selective media can be divided into two main groups: blood-based media and charcoal-based media. Blood components and charcoal serve to remove toxic oxygen derivatives. Most media are commercially available. The selectivity of the media is determined by the antibiotics used. Cefalosporins (generally cefoperazone) are used, sometimes in combination with other antibiotics (e.g. vancomycin, trimethoprim). Cycloheximide (actidione) and more recently amphotericin B are used to inhibit yeasts and molds (Martin *et al*., 2002). The main difference between the media is the degree of inhibition of contaminating flora. All the selective agents allow the growth of both *C. jejuni* and *C. coli*. There is no medium available that allows growth of *C. jejuni* and inhibits *C. coli* or vice versa. To some extent, other *Campylobacter* species (e.g. *C. lari*, *C. upsaliensis*, *C. helveticus, C. fetus* and *C. hyointestinalis*) will grow on most media, especially at the less selective temperature of 37°C.

Examples of selective blood-containing solid media:

i) Preston agar
ii) Skirrow agar
iii) Butzler agar
iv) Campy-cefex

Examples of charcoal-based solid media:

i) mCCDA (modified charcoal cefoperazone deoxycholate agar), slightly modified version of the originally described CCDA) (Bolton *et al*., 1984; 1988)
ii) Karmali agar or CSM (charcoal-selective medium) (Karmali *et al*., 1986)
iii) CAT agar (cefoperazone, amphotericin and teicoplanin), facilitating growth of *C. upsaliensis* (Aspinall *et al*., 1993).

1.3.2. Passive filtration

Passive filtration, a method developed by Steele & McDermott (1984) obviates the need for selective media; thus it is very useful for the isolation of antimicrobial-sensitive *Campylobacter* species. As the method does not use expensive selective media, it may be used in laboratories with fewer resources. For passive filtration, faeces are mixed with PBS (approximately 1/10 dilution) to produce a suspension. Approximately 100 µl of this suspension are then carefully layered on to a 0.45 or 0.65 µm filter, which has been previously placed on top of a non-selective blood agar plate. Care must be taken not to allow the inoculum to spill over the edge of the filter. The bacteria are allowed to migrate through the filter for 30–45 minutes at 37°C or room temperature and the filter is then removed. The plate is incubated microaerobically at 42°C.

1.3.3. Incubation

i) Atmosphere

Microaerobic atmospheres of 5–10% oxygen, 5–10% carbon dioxide are required for optimal growth (Corry *et al*., 2003; Vandamme, 2000). Appropriate atmospheric conditions may be produced by a variety of methods. In some laboratories, (repeated) gas jar evacuations followed by atmosphere replacement with bottled gasses are used. Gas generator kits are available from commercial sources. Variable atmosphere incubators are more suitable if large numbers of cultures are undertaken.

ii) Temperature

Media may be incubated at 37°C or 42°C, but it is common practice to incubate at 42°C to minimise growth of contaminants and to select for optimal growth of *C. jejuni* and *C. coli*. The fungistatic agents cycloheximide or amphothericin are added in order to prevent growth of yeasts and mould at 37°C (Bolton *et al*., 1988). In some laboratories, incubation takes place at 41.5°C to harmonise with *Salmonella* and *Escherichia coli* O157 isolation protocols (ISO, 2006).

iii) Time

Campylobacter jejuni and *C. coli* usually show growth on solid media within 24–48 hours at 42°C. As the additional number of positive samples obtained by prolonged incubation is very low, 48 hours of incubation is recommended for routine diagnosis (Bolton *et al*., 1988).

1.4. Confirmation

A pure culture is required for confirmatory tests, but a preliminary confirmation can be obtained by direct microscopic examination of suspect colony material.

1.4.1. Identification on solid medium

On Skirrow or other blood-containing agars, characteristic *Campylobacter* colonies are slightly pink, round, convex, smooth and shiny, with a regular edge. On charcoal-based media such as mCCDA, the characteristic colonies are greyish, flat and moistened, with a tendency to spread, and may have a metal sheen.

1.4.2. Microscopic examination of morphology and motility

Material from a suspect colony is suspended in saline and evaluated, preferably by a phase-contrast microscope, for characteristic, spiral or curved slender rods with a corkscrew-like motility. Older cultures show less motile coccoïd forms.

1.4.3. Detection of oxidase

Take material from a suspect colony and place it on to a filter paper moistened with oxidase reagent. The appearance of a violet or deep blue colour within 10 seconds is a positive reaction. If a commercially available oxidase test kit is used, follow the manufacturer's instructions.

1.4.4. Aerobic growth at 25°C

Inoculate the pure culture on to a non-selective blood agar plate and incubate at 25°C in an aerobic atmosphere for 48 hours.

1.4.5. Latex agglutination tests

Latex agglutination tests for confirmation of pure cultures of *C. jejuni* and *C. coli* (often also including *C. lari*) are commercially available.

1.5. Identification of *Campylobacter* to the species level

Among the *Campylobacter* spp. growing at 42°C, the most frequently encountered species from samples of animal origin are *C. jejuni* and *C. coli*. However, low frequencies of other species, including *Helicobacter* species, have been described. Generally, *C. jejuni* can be differentiated from other *Campylobacter* species on the basis of the hydrolysis of hippurate as this is the only hippurate-positive species isolated from veterinary or food samples. The presence of hippurate-negative *C. jejuni* strains has been reported (Steinhauserova *et al*., 2001). Table 2 gives some basic classical phenotypic characteristics of the most important thermophilic *Campylobacter* species (ISO, 2006). More extensive speciation schemes have been described in the literature (On, 1996; Vandamme, 2000). Speciation results should be confirmed using defined positive and negative controls.

Table 2. Basic phenotypic characteristics of selected thermophilic Campylobacter *species*

Characteristics	*C. jejuni*	*C. coli*	*C. lari*
Hydrolysis of hippurate	+*	–	–
Hydrolysis of indoxyl acetate	+	+	–

Key: + = positive; – = negative; *not all strains.

The confirmatory tests for the presence of thermophilic campylobacters and the interpretation (ISO, 2006) are given in Table 3. Confirm results of confirmation tests using positive and negative controls.

Table 3. Confirmatory tests for thermophilic Campylobacter

Confirmatory test	Result for thermophilic *Campylobacter*
Morphology	Small curved bacilli
Motility	Characteristic (highly motile and cork-screw like)
Oxidase	+
Aerobic growth at 25°C	–

1.5.1. Detection of hippurate hydrolysis

Suspend a loopful of growth from a suspect colony in 400 µl of a 1% sodium hippurate solution (care should be taken not to incorporate agar). Incubate at 37°C for 2 hours, then slowly add 200 µl 3.5% ninhydrin solution to the side of the tube to form an overlay. Re-incubate at 37°C for 10 minutes, and read the reaction. Positive reaction: dark purple/blue. Negative reaction: clear or grey. If commercially available hippurate hydrolysis test disks are used, follow the manufacturer's instructions.

1.5.2. Detection of indoxyl acetate hydrolysis

Place a suspect colony on an indoxyl acetate disk and add a drop of sterile distilled water. If indoxyl acetate is hydrolysed a colour change to dark blue occurs within 5–10 minutes. No colour change indicates hydrolysis has not taken place. If commercially available indoxyl acetate hydrolysis test disks are used, follow the manufacturer's instructions.

Biochemical speciation may be supplemented or replaced with molecular methods or MALDI-TOF mass spectrometry. MALDI-TOF can be used to identify *Campylobacter* isolates rapidly and efficiently at the genus and species level (Bessede *et al.*, 2011). A variety of DNA probes and polymerase chain reaction (PCR)-based identification assays has been described for the identification of *Campylobacter* species (On, 1996; Vandamme, 2000). On & Jordan (2003) evaluated the specificity of 11 PCR-based assays for *C. jejuni* and *C. coli* identification. A fast method to differentiate *C. jejuni* and *C. coli* strains is a duplex real-time PCR, targeting gene *mapA* for *C. jejuni* identification and gene *CeuE* for *C. coli* identification (Best *et al*., 2003). Another real-time PCR method commonly used to identify and differentiate between *C. jejuni, coli* and *lari* is described by Mayr *et al.* (2010). A gel-based method that is commonly used differentiates between *C. jejuni, C. coli, C. lari and C. upsaliensis* (Wang *et al.,* 2002). *Campylobacter* isolates can also be molecular identified at species level with 16S rRNA sequencing (Gorkiewicz *et al.,* 2003).

1.6. Molecular detection of *Campylobacter*

PCR-based methods for the detection of *Campylobacter* in animal faecal samples and enriched meat samples have been extensively described in the literature (Bang *et al*., 2001; Lund *et al*., 2003; Olsen *et al*., 1995). Many molecular tests are available to identify *Campylobacter* species, but there is not a specific recommended one. *Campylobacter* isolates can be identified at species level with 16S rRNA sequencing (Gorkiewicz *et al.,* 2003). Inclusion of positive and negative reference strains and process controls to detect inhibition of the PCR reaction by the sample matrix are required for all molecular *Campylobacter* detection methods.

1.7. Antigen-capture-based tests

Enzyme immunoassays are available for the detection of *Campylobacter* in human and animal stool samples. Some are of the lateral flow format. The sensitivity and specificity should be critically evaluated through an in-house validation.

2. Serological tests

There are no serological assays in routine use for the detection of colonisation of *C. jejuni/C. coli* in livestock.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines specifically developed for *C. jejuni* or *C. coli* in animals or birds.

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NB: There is a WOAH Reference Laboratory for campylobacteriosis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for campylobacteriosis

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 3.10.5.

LISTERIA MONOCYTOGENES

SUMMARY

Listeria monocytogenes *is a Gram-positive facultatively anaerobic rod-shaped bacterial species. A wide variety of animal species can be infected, but clinical listeriosis in animals is mainly a ruminant disease, with occasional sporadic cases in other species. The main clinical manifestations of animal listeriosis are encephalitis, septicaemia and abortion, and the disease is often associated with stored forages, usually silage, and contaminated farm environments. Post-mortem findings and histopathology depend on the clinical presentation.*

Listeriosis is one of the most severe food-borne diseases of humans. The disease manifestations include septicaemia, meningitis (or meningoencephalitis) and encephalitis. In pregnant women, intrauterine or cervical infections may result in spontaneous abortion or stillbirths (maternal neonatal listeriosis), and may be preceded by influenza-like signs, including fever. Listeria monocytogenes *has also been associated with gastroenteric manifestations with fever, and, rarely, with cutaneous or eye infections reported by veterinarians and farmers. Although the morbidity of listeriosis is relatively low, the mortality of the systemic/encephalitic disease can be very high, with values in the vicinity of 20–30%. In Europe, the hospitalisation rate is estimated at more than 95%. The elderly, pregnant women, newborns and the immunocompromised are considered to be at high risk of contracting the disease.*

A number of molecular and cellular determinants of virulence have been identified for this facultative intracellular pathogen, and there is evidence of polymorphism among different strains of L. monocytogenes *for some of these virulence determinants. This heterogeneity is correlated with the ability of the organism to cause forms of the disease. The definition of hypervirulent and hypovirulent clones for* L. monocytogenes *was established from clones that were epidemiologically associated either with food or with the human central nervous system or maternal neonatal listeriosis. For ruminants, a strong association of sequence type ST1 (obtained by multi-locus sequence typing) with rhombencephalitis has been observed, suggesting its neurotropism. Whole genome sequencing will provide more precise insights in the coming years. Therefore, all* L. monocytogenes *strains are considered to be potentially pathogenic.*

Detection of the agent: *A variety of conventional and rapid methods are available for the detection and identification of* L. monocytogenes *in primary production, feed and food samples, food processing environment samples and specimens from animal listeriosis. Conventional bacteriological methods remain the 'gold standard' against which other methods are validated. These methods use selective agents and enrichment procedures to reduce the number of competitive microflora and allow multiplication of* L. monocytogenes*. Development of chromogenic media and polymerase chain reaction methods has allowed more reliable detection of this microorganism. Recently, rapid identification of strains was improved with matrix-assisted laser desorption ionisation – time of flight (MALDI-TOF) mass spectrometry. Immunohistochemical detection of* L. monocytogenes *antigens is a useful tool for the diagnosis of the encephalitic form of the disease.*

Although not required for regulatory purposes, different levels of subtyping of L. monocytogenes *strains are available, including serotyping by classical agglutination or molecular genotyping using polymerase chain reaction grouping, and whole-genome sequencing, which replaced pulse-field gel electrophoresis at the international level as the reference method for subtyping* L. monocytogenes*. The structure of population and phylogeny may be studied by multi-locus sequence typing. Subtyping tests have been standardised and validated at the international level by the PulseNet International Network.*

Serological tests: *Serological tests for the detection of antibodies have not been traditionally used for the diagnosis of listeriosis. A number of formats have been tried and they have all been found to be largely unreliable, lacking sensitivity and specificity.*

Requirements for vaccines: *Although experimental vaccines in laboratory animals are being explored, it has proven very difficult to develop effective vaccines against* L. monocytogenes, *which, as an intracellular microorganism, requires effector T cells for an effective immune response.*

A. INTRODUCTION

A wide variety of animal species can be infected by *Listeria monocytogenes,* including mammals, birds, fish and crustaceans (Table 1), although most clinical listeriosis cases occur in ruminants; pigs rarely develop disease and birds are generally subclinical carriers of the organism (Dhama *et al.,* 2015). Greatest attention is placed on infection of dairy and beef cattle, or sheep and goats, according to the country or region. Most infections in animals are subclinical, but invasive disease can occur either sporadically or as an outbreak. In addition to the economic impact of listeriosis in ruminants and other animal species, ruminants may play a role as a source of infection for humans, primarily from consumption of contaminated animal products. Individual risk factors for ruminants are still poorly understood (Walland *et al*., 2015). It is still poorly understood how *L. monocytogenes* circulates among animals, humans and various environments such understanding being restricted to specific *L. monocytogenes* subtypes (Walland *et al.,* 2015). Direct transmission from infected animals, especially during calving or lambing can occur but these infections are very rare (Wesley *et al.,* 2007). Animals kept in zoological or other wildlife parks have occasionally been reported with listeriosis, such as Celebese ape, bushy-tailed jirds, adult cougar and wild-caught monkeys (Czuprynski *et al.,* 2010). The relative importance of the direct zoonotic transmission of the disease is not clear, and contamination from the food-processing environment is of greater public health importance (Roberts & Wiedmann, 2003).

Table 1. Species with reported isolation of **Listeria monocytogenes**

		Mammals		
Cattle	Cats	Rabbits	Sheep	Deer
Guinea-pigs	Goats	Raccoons	Chinchillas	Pigs
Rats	Skunks	Horses	Mice	Mink
Dogs	Lemmings	Ferrets	Foxes	Voles
Moose	Humans	Monkeys	Otter	Gerbils
Cougar	Buffalos	Camel	Hedgehogs	
		Birds		
Canaries	Ducks	Owls	Chaffinches	Eagles
Parrots	Chickens	Geese	Partridges	Cranes
Hawks	Pheasants	Doves	Lorikeets	Pigeons
Seagulls	Turkeys	Whitegrouse	Whitethroat	Woodgrouse
Cockateil	Poults	Turkeys	Snowy owl	
		Others		
Frogs	Crustaceans	Ticks	Fish	Ants
Flies	Snails			

The clinical manifestations of listeriosis in animals include rhombencephalitis (or in some cases more disseminated encephalitic changes), septicaemia and abortion, especially in sheep, goats and cattle. During an outbreak within a flock or herd, usually only one clinical form of listeriosis is encountered. The rhombencephalitic form is referred to as 'circling disease' because of the affected animal's tendency to circle in one direction, and it is the most common manifestation of the disease in ruminants. It is also amongst the most common causes of neurological disease in ruminants. Clinical signs include depression, anorexia, head pressing or turning of the head to one side and

unilateral cranial nerve paralysis. The latter is due to involvement of cranial nerves and their nuclei within the brainstem. Abortion is usually late term (after 7 months in cattle and 12 weeks in sheep) (Hird & Genigeorgis, 1990; Walker, 1999). The septicaemic form is relatively uncommon and generally, but not invariably, occurs in the neonate. It is marked by depression, inappetence, fever and death. Bovine and ovine ophthalmitis have also been described. Rarely, mastitis of ruminants has been associated with *L. monocytogenes* infection. Gastrointestinal infections can occasionally occur in sheep and goats (Clark *et al.*, 2004). When listeriosis occurs in pigs, the primary manifestation is septicaemia, with encephalitis reported less frequently, and abortions rarely. Although birds are usually subclinical carriers, sporadic cases of listeriosis have been reported, most frequently septicaemia and far less commonly meningoencephalitis. Avian listeriosis may be the result of a secondary infection in viral disease conditions and salmonellosis (Wesley, 2007). *Listeria monocytogenes* occasionally infects fish (Czuprynski *et al.,* 2010; Jami *et al.,* 2014). The bacterium is also present in many environmental niches, including soil, water and plants.

The post-mortem findings and histopathology in animal listeriosis depend on the clinical presentation. In the encephalitic form, the cerebrospinal fluid may be cloudy and the meningeal vessels congested. Gross lesions are generally subtle and characterised by vascular congestion and mild tan discoloration of the brainstem. On occasion, the medulla shows areas of softening (malacia) and abscessation. Characteristic histopathological changes consist of foci of intraparenchymal neutrophils and macrophages (microabscesses) in the brainstem with adjacent perivascular mononuclear cuffing. The microabscesses often affect one side more severely. More extensive malacic pathology may occur. The medulla and pons are most severely involved. In the septicaemic form, multiple foci of necrosis in the liver and, less frequently the spleen, may be noted. Aborted fetuses of ruminants show very few gross lesions, but autolysis may be present if the fetus was retained before being expelled (Low & Donachie, 1997; Walker, 1999).

The evidence indicates that animal listeriosis is frequently associated with stored forage and with the environment as the main source of contamination. In the environment, this saprophytic microorganism can live in soil, water, and decaying vegetables from which it could contaminate animal feed (Whitman *et al.,* 2020). Silage (in silos and bunkers) is the most frequent source (Fenlon *et al.,* 1996). Emphasis should therefore be placed on reducing the likelihood of the multiplication of the organism, which occurs more frequently at pH values of silage greater than 5.0, particularly where ineffective fermentation has occurred and where there is concomitant growth of moulds. Every effort should be made to produce silage of good quality, with early cutting of grass, minimal contamination with soil or faeces and ensuring optimal anaerobic fermentation, which will insure that the pH falls below 5.0; at that level, growth of *Listeria* spp. is inhibited. The best silage for feeding should be selected, especially in the case of sheep, discarding material that has obvious signs of contamination with mould. Material a few centimetres from the top, front and sides of an opened bale or bag, should also be discarded. Leftover silage should be removed (Low & Donachie, 1997). Barn equipment like bedding, water and feeding troughs can be contaminated at a higher rate than silage (Walland *et al.,* 2015).

In septicaemic/abortive listeriosis, the intestinal mucosa is the main route of entry after oral ingestion. The incubation period can be as short as 1 day. In rhombencephalitis, *L. monocytogenes* likely invades the brainstem via cranial nerves after breaching of the oral mucosa (Walland *et al.,* 2015). The pathogenesis of neurolisteriosis in ruminants is not at present entirely understood and the infectious dose has not been established (Walland *et al.,* 2015). The incubation period is significantly longer than in the septicaemic form, usually 2–3 weeks. The course of the disease is usually acute in sheep and goats, 1–4 days (Roberts & Wiedmann, 2003), although it can be more protracted in cattle. Control measures in animals were described by Dhama *et al.* (2015).

Although *L. monocytogenes* has been recognised as an animal pathogen for many years, its significant role as a food-borne human pathogen became evident only in the 1980s, when a documented report of a Canadian listeriosis outbreak, traced to contaminated coleslaw, was published (Schlech *et al.*, 1983). Data from this outbreak and the level of the contaminated coleslaw were used several years later to establish the microbiological criteria at 100 colony-forming units/g as the *Codex Alimentarius* level. Today, *L. monocytogenes* is considered to be one of the most important agents of food-borne disease (de Noordhout *et al.,* 2014). More than 110 outbreaks worldwide have been reported in the literature, including the largest one in South Africa in 2018. Although outbreaks have been reported from several countries, the majority of human cases are sporadic and represent a real challenge to controlling them definitively. Possible explanations for the emergence of human food-borne listeriosis as a major public health concern include major changes in agricultural methods and animal husbandry, food production, processing and distribution, increased use of refrigeration as a primary preservation means for foods, changes in human eating habits, particularly towards convenience and ready-to-eat foods, and an increase in the number of people considered to be at high risk for the disease (elderly, pregnant women, newborns, immunocompromised) (Buchanan *et al.*, 2016). If *L. monocytogenes* has been reported in several countries, its incidence depends on eating habits, cooking practices, use of refrigeration and food importation.

The invasive forms of listeriosis in humans include septicaemia, meningitis (or meningoencephalitis), and encephalitis (rhombencephalitis) (Charlier *et al.,* 2017). Gastroenteric manifestations with fever also occur. Although the morbidity of listeriosis is relatively low, the mortality can reach values between 20 and 30%. In Europe, the hospitalisation rate is estimated at more than 95%. In pregnant women, infection may result in abortion, stillbirth or premature birth and may be preceded by influenza-like signs including fever (Charlier *et al.,* 2017).

Listeria monocytogenes is a Gram-positive facultatively anaerobic rod and is responsible for almost all *Listeria* infections in humans; although rare cases of infection caused by *L. ivanovii* have been reported (Charlier *et al.*, 2017). In animals, *L. monocytogenes* is responsible for the majority of infections, but *L. ivanovii* (abortion in ungulates such as cattle and sheep), *L. innocua* (encephalitis in sheep) and *L. seeligeri* infections have also been recorded. *Listeria ivanovii* has been associated with abortions and has been reported very occasionally to cause meningoencephalitis in sheep (Table 2). Although *L. monocytogenes* has definite zoonotic potential, it is also one of the main environmental contaminants of public health significance. The most feasible and practical means to reduce the risk of listeriosis in humans is through dietary and food preparation measures, including hazard analysis critical control points (HACCP).

Table 2. Virulence of* Listeria *species

***Listeria* species**	**Virulence in humans**	**Virulence in animals**
Listeria sensu stricto:		
L. monocytogenes	+	+
L. ivanovii subsp. *ivanovii*	–[(a)]	+
L. ivanovii subsp. *londoniensis*	–	+
L. innocua	–[(b)]	–
L. welshimeri	–[(b)]	–
L. seeligeri	–[(b)]	+
L. grayi subsp. *grayi*	–[(b)]	–
L. grayi subsp. *murrayi*		
Listeria sensu lato:		
L. aquatica	–	–
L. booriae	–	–
L. cornellensis	–	–
L. costaricensis		
L. fleischmannii subsp. *coloradensis*	–	–
L. fleischmannii subsp. *fleischmanii*		
L. floridensis	–	–
L. goaensis	–	–
L. grandensis	–	–
L. marthii	–	–
L. newyorkensis	–	–
L. riparia	–	–
L. rocourtiae	–	–
L. thailandensis	–	–
L. weihenstephanensis	–	–

[(a)]only 11 human cases of infection reported; [(b)]only 1 human cases of infection reported.

Listeria monocytogenes can infect humans. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

B. DIAGNOSTIC TECHNIQUES

Table 3. Test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Bacterial isolation and identification	++	++	++	++	++	–
PCR methods	+++	+++	+++	++	+++	–
Chromogenic isolation and identification media	+++	+++	+++	+++	+++	–
Detection of immune response						
Serology	–	+	–	+	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.

1. Detection and characterisation of the agent

There is a variety of conventional and rapid methods currently available for the detection and identification of *L. monocytogenes* in samples from the food chain (primary production samples, feed, food samples, and environmental samples) and specimens from animal listeriosis. As low levels of *L. monocytogenes* could be difficult to detect, methods could also target *Listeria* spp. that have been used as bioindicators of a higher risk of the presence of *L. monocytogenes* in food and plant environmental samples. For animals and humans, conventional bacteriological methods are important for various reasons: their use results in a pure culture of the organism, which is useful for regulatory, epidemiological surveillance and outbreak management purposes. They remain the 'gold standards' against which other methods are compared and validated. These methods are usually very sensitive and they do not require sophisticated and expensive equipment, allowing widespread use. Some of the disadvantages of this group of methods include the relatively long period of time that the protocols require for completion, several 'hands-on' manipulations, the requirement for many different chemicals, reagents and media, the possibility of contaminating microorganisms in the sample masking the presence of the target ones, including overgrowth, the potential overlook of atypical variants of the target organism and the relative subjectivity involved when interpreting typicality of colony on selective and differential agar plates (Jadhav *et al.,* 2012).

The isolation and identification of *L. monocytogenes* from samples from the food chain and specimens from animal listeriosis require the use of selective agents and enrichment procedures that keep the levels of competing microorganisms to reasonable numbers and allow for the multiplication of *L. monocytogenes* to levels that are enough for detection of the organism. In the early days of listerial clinical bacteriology, cold enrichment (Dhama *et al.,* 2015) was regularly used to this end, exploiting the ability of the organism to multiply at refrigeration temperatures (around 4°C), whereas contaminating bacteria would not multiply under these conditions. This cold enrichment or a period of freezing the sample (≤–15°C, 15 days), can always be used for detection of

L. monocytogenes in faeces of human or zoo animals. A number of selective compounds that allow growth of *L. monocytogenes* at classical incubation temperatures have been incorporated into culture media, shortening the time required for selective growth of the organism. Examples of these selective compounds include cycloheximide, colistin, cefotetan, fosfomycin, lithium chloride, nalidixic acid, acriflavine, phenylethanol, ceftazidime, polymixin B and moxalactam. Development of chromogenic media has allowed better isolation of this microorganism in samples from the food chain. When testing the presence of *L. monocytogenes* from primary production samples (faeces, environment, etc.) that contain huge amounts of competitor micro-organisms (including non-targeted species of *Listeria* genus, not differentiated on non-chromogenic isolation agar), the use of these chromogenic isolation media is crucial to avoid a strong underestimation of the prevalence of *L. monocytogenes*.

Bacteriological diagnosis of animal listeriosis has traditionally involved direct plating of specimens on blood agar or other enriched media and concomitant use of the 'cold enrichment' technique, with weekly subculturing for up to 12 weeks (Dhama *et al.,* 2015; Walker, 1999). Immunohistochemical detection of *L. monocyte-genes* antigens in formalin-fixed tissue has proven to be more sensitive than direct plating and cold enrichment bacterial culture for the diagnosis of the encephalitic form of the disease in ruminants (Campero *et al.*, 2002; Johnson *et al.*, 1995). This is also the case for diagnosis of rhomboencephalitis in humans. Nevertheless, in contrast to human medicine, in animals it is very difficult or not possible to isolate the microorganism from the cerebrospinal fluid or to identify the microorganism by polymerase chain reaction (PCR) in the cerebrospinal fluid. At present, therefore, confirmative diagnosis of listeric rhomboencephalitis in the living animal is not possible and is only achieved post-mortem by finding characteristic histopathological lesions or immunohistochemistry, bacterial isolation from the brainstem, or PCR on the brainstem.

In spite of advances made in the selective isolation of *L. monocytogenes* from samples from the food chain, there is still room for improvement in a number of areas. No single procedure can be credited with being sensitive enough to detect *L. monocytogenes* from all types of food (Jadhav *et al.,* 2012). In addition, sublethally injured *L. monocytogenes* cells can be found in processed food resulting from freezing, heating, acidification and other types of chemical or physical treatment. These sublethally injured and viable but not cultivable bacteria require special culture conditions for damage repair, before being able to be detected in culture.

The introduction of alternative enrichment procedures and selective agents for the isolation of *L. monocytogenes* from food and environmental samples has opened up the possibility of using some of these techniques for the bacteriological analysis of samples from animal listeriosis. Nevertheless, it should be stressed that performance characteristics cannot be ensured when these last methods are used outside the scope of their validation.

1.1. Bacterial isolation methods

Conventional methods for the isolation of *L. monocytogenes* from samples from the food chain that have gained acceptance for international regulatory purposes include the European Committee for standardization (CEN, EN) and the International Organization for Standardization (ISO) (ISO, 2017a; 2017b); the Nordic Committee on Food Analysis (NMKL) method; the United States Food and Drug Administration (FDA) method; and the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) method.

The EN ISO, FDA and USDA methods should be used according to their respective scope and cover a large variety of food and environmental matrices. Food samples intended for analysis must be representative from the food, including the outer surface and the internal part. The conventional culture methods include an enrichment procedure based on the use of liquid culture media containing selective agents. The various Association of Official Analytical Chemists (AOAC)[1]-certified methods call for different selective enrichment schemes containing different selective agents, and optimised for different enrichment duration and temperatures.

The ISO Technical Committee ISO/TC 34, Agricultural Food Products, Subcommittee SC 9, Microbiology, in agreement with the CEN Technical committee CEN/TC 463, Microbiology of the food chain, state that the (EN) ISO Standard 11290, parts 1 and 2, internationally validated by interlaboratory studies, can be used for the detection of *L. monocytogenes* or *Listeria* spp. in a large variety of samples from the food chain: food and feed products but also samples from primary production (breeding) and food processing

1 AOAC International (2019). Official methods of analysis. Chapter 17: *Listeria*. AOAC, Gaithersburg, MD, USA. http://www.eoma.aoac.org/

environment. Although they recognise that this standard might not be appropriate in every detail in certain very specific instances, they recommend that every effort should be made to apply this horizontal method as far as possible.

The principle of the ISO 11290 Part 1 method version 2017[2] for the detection of *Listeria monocytogenes* or *Listeria* spp., covering all food chain and primary production samples, is outlined below. Briefly, the first step is a selective primary enrichment in half-Fraser broth, which is incubated at 30±1°C for 25±1 hours. The second step is an enrichment in Fraser broth with a culture suspension obtained in the first step, and this enrichment is incubated at 37±1°C for 24±2 hours, possibly for 24 hours more, to detect *Listeria* spp. other than *L. monocytogenes*. After incubation, samples from the cultures obtained in the first (half-Fraser broth) and second (Fraser broth) steps are streaked on selective solid *Listeria* agar according to Ottaviani and Agosti, which contains lithium chloride, nalidixic acid, ceftazidime, polymyxin B and amphotericin B (or cycloheximide), and also any other solid selective medium at the choice of the laboratory, such as Oxford or PALCAM (polymixin-acriflavine-lithium chloride-ceftazidime-esculin-mannitol agar). Inoculated selective solid *Listeria* agar according to Ottaviani and Agosti is incubated at 37±1°C and examined after 24±2 hours to check for the presence of presumptive colonies of *L. monocytogenes* (incubate an additional 24±2 hours at 37±1°C in the absence of typical colonies). Presumptive colonies of *L. monocytogenes* on *Listeria* agar according to Ottaviani and Agosti are green-blue surrounded by an opaque halo. Oxford agar contains lithium chloride, cycloheximide, colistin, acriflavine, cefotetan and fosfomycin as selective agents, and presumptive colonies of *Listeria* spp. are small, black and surrounded by a black halo. Incubate the second selective medium at the appropriate temperature and examine after the appropriate time according to the manufacturer's instructions. Subculture the presumptive *L. monocytogenes* or *Listeria* spp. on a non-selective medium and confirm by means of appropriate morphological, physiological and biochemical tests described in the standard. For the enumeration method described in ISO 11290 Part 2 version 2017[3], only *Listeria* agar according to Ottaviani and Agosti shall be used.

There are two general groups of chromogenic media for *Listeria*. The first group of media employs a chromogen that detects β-D-glucosidase activity, which is indicative of *Listeria* species, and the formation of a distinct halo, indicative of the organism's lecithin use, surrounding the colony is used to identify *L. monocytogenes* and *L. ivanovii*. Media in this group include *Listeria* agar according to Ottaviani and Agosti. In the second group, a chromogenic substrate is used to detect phospatidylinositol-specific phospholipase C (PI-PLC) activity (Jinneman *et al.*, 2003). With this group of agars, *L. monocytogenes* and some *L. ivanovii* cleave the chromogen and the remaining *Listeria* species remain white. In some media of this last group, sugar as xylose has been added to the media to distinguish between *L. monocytogenes* and *L. ivanovii* by the presence of a yellow halo surrounding the *L. ivanovii* colonies. *Listeria monocytogenes* develops blue colonies (PI-PLC positive) without a yellow halo (xylose negative) and *L. ivanovii* produces greenish-blue colonies (PI-PLC positive) with a yellow halo (xylose positive). Other *Listeria* spp. colonies are white (PI-PLC negative). No xylose and PI-PLC negative *L. monocytogenes* has been reported. Some *L. ivanovii* strains from sheep milk with a slow xylose activity could be difficult to differentiate from *L. monocytogenes* on some chromogenic media for *Listeria*.

For the FDA method described in chapter 10 (version 2017)[4] of Bacteriological Analytical Manual (BAM), which can be accessed online, the buffered *Listeria* enrichment broth (BLEB) is the base enrichment. The Tryptone soya broth with yeast extract base has been supplemented with monopotassium phosphate to improve the buffering capacity, and pyruvic acid is added to aid in the recovery of stressed or injured cells. Analytical portions are pre-enriched in BLEB for 4 hours at 30°C, selective agents, acriflavin HCl (10 mg/litre), nalidixic acid (40 mg/litre) and cyclohexamide (50 mg/litre) are added and the enrichment is continued at 30°C for 48 hours. Enriched samples are streaked at 24 and 48 hours to one esculin-based selective/differential agar plate and one chromogenic selective agar plate. The esculin-based agar plates contain esculin and ferric iron such as Oxford or a modification, MOX agar (MOX), or lithium

2 ISO (2017). Microbiology of the food chain – Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. – Part 1: Detection method. International Standard ISO 11290–1, Geneva, Switzerland.

3 ISO (2017). Microbiology of the food chain – Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. – Part 2: Enumeration method. International Standard ISO 11290–2, Geneva, Switzerland.

4 Food and Drug Administration (2017). Chapter 10: Detection of *Listeria monocytogenes* in foods and environmental samples, and Enumeration of *Listeria monocytogenes* in foods. *In:* Bacteriological Analytical Manual (BAM). Hitchins A.D., Jinneman K. & Chen Y. Available online: https://www.fda.gov/food/laboratory-methods-food/bam-detection-and-enumeration-listeria-monocytogenes

chloride/phenylethanol/moxalactam (LPM) supplemented with Fe^{3+}. Presumptive *L. monocytogenes* are subcultured and confirmed by means of appropriate morphological, physiological, biochemical tests, and real-time PCR described in the method[5].

The USDA-FSIS method (version 2019)[6] uses two enrichment steps: the 'primary' enrichment is done in University of Vermont medium (UVM) containing nalidixic acid and acriflavine, and the 'secondary' enrichment is carried out in Fraser broth, containing nalidixic acid, lithium chloride and acriflavine or morpholine-propanesulfonic acid-buffered *Listeria* enrichment broth (MOPS-BLEB). Incubation conditions are described in this method and distinct depending on the matrix chosen for the enrichment step. After selective enrichment, cultures are then plated on MOX agar that contains lithium chloride, colistin and moxalactam. Presumptive *L. monocytogenes* are subcultured and confirmed by means of appropriate morphological, physiological and biochemical tests described in the method.

For the NMKL 136 method (version 2007)[7]), primary enrichment in half-Fraser broth at 30°C for 24 hours, is followed by a secondary enrichment in Fraser broth at 37°C for 48 hours. The cultures obtained from both the enrichment steps are plated out on a *L. monocytogenes*-specific isolation medium, agar *Listeria* according to Ottaviani and Agosti or *Listeria monocytogenes* blood agar medium (LMBA) or chromogenic *Listeria* agar medium, which is basically like agar *Listeria* according to Ottaviani and Agosti, and on another solid selective isolation medium; the latter is optional. Subculture the presumptive *L. monocytogenes* and confirm by means of appropriate morphological, physiological and biochemical tests described in the standard.

All culture media prepared should be subjected to quality control, such as according to ISO 11133 standards for the preparation, production, storage and performance testing of culture media.

The original and traditional procedure for the isolation of *L. monocytogenes* from animal tissues has been direct plating of specimens on sheep blood agar or other rich culture media and concomitant use of the 'cold enrichment' technique, with weekly subculturing for up to 12 weeks (Dhama *et al.,* 2015; Walker, 1999). The cold enrichment technique is not currently performed. Isolation of the organism by direct plating is relatively easy when numbers are large in a normally sterile site, such as in the case of the septicaemic form of the disease, but isolation is difficult when the organism is present in low numbers, as in the case of the encephalitic form or when samples are heavily contaminated.

For sampling and preparation of samples taken at the primary production stage in the aim of detection of *L. monocytogenes* or *Listeria* spp., ISO standards 13307 (Primary production Stage – Sampling techniques) and 6687-6 (Specific rules for the preparation of samples taken at the primary production stage) should be used.

In the case of animal listeriosis, the samples should be chosen according to the clinical presentation of the disease: material from lesions in the liver, kidneys or spleen, in the case of the septicaemic form; spinal fluid, pons and medulla in the case of the rhombencephalitic form; and placenta (cotyledons), fetal abomasal contents or uterine discharges in the case of abortion. Refrigeration temperatures (4°C) must be used for handling, storing and shipping specimens. If the sample is already frozen, it should be kept frozen until analysis.

The protocol recommended for isolation of *L. monocytogenes* from animal necropsy material is described below as originally published (Eld *et al.*, 1993).

5 FOOD AND DRUG ADMINISTRATION (2018). BAM Protocol: Simultaneous confirmation of *Listeria* species and *L. monocytogenes* isolates by real-time PCR. *In:* Bacteriological Analytical Manual (BAM), Available online: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm279532.htm

6 USDA-FSIS (2019). Isolation and Identification of *Listeria monocytogenes* from Red Meat, Poultry, Egg and Environmental Samples. *In:* Microbiology Laboratory Guidebook, MBLG 8.11 pp 1–18, Available online: https://www.fsis.usda.gov/wps/wcm/connect/1710bee8-76b9-4e6c-92fc-fdc290dbfa92/MLG-8.pdf?MOD=AJPERES

7 NMKL (2007). Method no. 136, Fourth Edition, *Listeria monocytogenes.* Detection in foods and feeding stuffs and enumeration in foods. NMKL, Secretary General, c/o Norwegian Veterinary Institute, Oslo, Norway

1.1.1. Isolation procedure from animal necropsy material

i) Inoculate 10–25 g or ml of sample (depending on the amount of sample available) into 225 ml Listeria enrichment broth. When dealing with samples from animal listeriosis, the size of the sample for inoculation may be limited and less than that recommended for food samples (25 g or ml). If that is the case, as much sample material as possible (aim at 10–25 g or ml) should be inoculated (Eld et al., 1993). (Listeria enrichment broth base: 30 g Oxoid tryptone soya broth; 6 g Difco yeast extract; 1 litre water; selective agents: 2.3 mg Acriflavine; 9.2 mg nalidixic acid; 11.5 mg cycloheximide; add selective agents to 225 ml of the broth base.)

ii) Incubate broth at 30°C for 48 hours.

iii) Spread 0.1 ml of the enrichment broth culture onto Oxford agar plates.

iv) Incubate plates at 37°C. Examine bacterial growth after 24 and 48 hours.

v) Test five colonies (or all when fewer available) with typical appearance of L. monocytogenes for cell shape, Gram reaction, haemolytic activity on blood agar (defibrinated horse blood), tumbling motility at 20°C, fermentation of glucose (+), rhamnose (+) and xylose (–), hydrolysis of esculin and production of catalase.

1.1.2. Alternative protocol

Alternative protocols exist at the national level for veterinary laboratories; here is one example:

i) Check that the sample has not been contaminated by the environment. If there is a doubt, sterilise with a Bunsen burner or cauterise with a brand, for example in the case of brain sample contaminated during extraction from skull. The test portion is homogenised in buffered-peptone water with a crusher to give a consistent initial suspension. Any sample that has not yet been crushed is stored at 2–8°C.

ii) The initial suspension is inoculated in enrichment broth such as brain–heart broth or Rosenow broth. In parallel, it is spread, for direct observation, on modified Palcam and a Columbia sheep blood agar with nalidixic acid (15 mg/litre) and colistine sulphate (10 mg/litre), if it is presumed that the sample is not contaminated. The Palcam base is modified as follows: a supplement (containing 100,000 International Units of Polymyxin B sulphate, 20 mg ceftazidin, 5 mg acriflavin chlorhydrate, 200 mg of cycloheximide, and 10 ml of sterile water) is prepared, sterilised by filtration and 10 ml is added to 1000 ml of Palcam base medium.

iii) Incubate at 37±1°C for 24 hours for liquid culture and 24–48 hours for solid media.

iv) After 24 hours, if colonies presumed to be *Listeria* appear on the Petri plates, select them for further confirmation tests. If none is present, incubate the plates again in the same conditions for 24 hours. Enrichment broth is streaked on Palcam and Columbia sheep blood agar with nalidixic acid (15 mg/litre) and colistine sulphate (10 mg/litre), and incubated at 37±1°C for 24 hours. On Palcam and modified Palcam, expose the plates in the air for 1 hour to allow the medium to regain its pink to purple colour. After 24 hours, *Listeria* spp. grow on these last media as small or very small greyish green or olive green colonies, 1.5–2 mm in diameter, sometimes with black centres, but always with black halos. After 48 hours, *Listeria* spp. appear in the form of green colonies about 1.5–2 mm in diameter, with a central depression and surrounded by a black halo. On Columbia sheep blood agar with nalidixic acid and colistine sulphate, *Listeria* spp. grow as grey and flat colonies and *L. monocytogenes* presents a small haemolysis zone that could be observed after removing the colony. *Listeria ivanovii* presents a weak haemolytic activity around the colony.

v) At 48 hours and 72 hours, if colonies presumed to be *Listeria* appear on Petri plates, select them for further confirmation tests. If there are five presumed *Listeria* colonies on the plate, select them all. If more than five presumed *Listeria* colonies are on the plate, pick five colonies only.

For faeces and silage, and placental envelop, there are two modifications to this last protocol.

For faeces and silage, a 1/10 suspension (25 g in 225 ml) is performed in half-Fraser broth and incubated at 30±1°C for 24 hours. At 24 hours, this suspension is streaked on modified Palcam and a subculture in Fraser broth at 0.1 ml in 10 ml is performed. Media are incubated at 37±1°C for 24 hours. At 48 hours, this incubated Fraser broth is streaked on modified Palcam and Petri plates are incubated at 37±1°C for 24–48 hours. Fraser broth is re-incubated at 37±1°C for 24 hours before to be streaked on modified Palcam.

For placental envelop, the test portion is diluted at 1/2 and 1/5 in buffered-peptone water and directly isolated on selective media. The Palcam is replaced, in this case, by modified Palcam.

Agar *Listeria* according to Ottaviani and Agosti, and other chromogenic media for *Listeria* allow the growth of most *Listeria* spp. and are to be used in clinical microbiology to screen human or animal faeces (Dhama *et al.,* 2015; Jadhav *et al.,* 2012).

1.2. Culture-based identification methods

Typical *Listeria* spp. colonies, on the above selective/differential agar plates or preferably after subculture to a non-selective medium, are then selected for further identification to the species level, using a battery of tests. The tests include the Gram-staining reaction, catalase, motility (both in a wet mount observed under phase-contrast microscopy and by inoculation into semi-solid motility agar [0.2–0.4% agar] or U/Graigie's tube), haemolysis and carbohydrate use (Tables 3 and 4).

To observe of tumbling motility, a hanging drop preparation is made from a young broth culture, such as tryptone soya yeast extract broth, and incubated at room temperature for 8–24 hours. When semi-solid motility agar is used after stab inoculation (about 1 cm) and incubation at 20–28°C, listeriae swarm through the medium, which becomes cloudy. At about 0.5 cm below the surface of the agar, a characteristic layer of increased growth is observed, like an umbrella. This occurs because of the better development of *Listeria* under aerobic conditions as opposed to strictly anaerobic conditions.

For haemolysing activity, horse and sheep blood-containing agar plates shall be used. After incubation at 37°C for 24 hours and inoculation by stabbing the medium, *L. ivanovii* exhibits a wide zone of haemolysis. The haemolysis zone of *L. monocytogenes* is narrow, frequently not extending much beyond the edge of colonies. In this case, removal of the colonies could help interpretation. Rare strains of *L. monocytogenes* are not haemolytic and rare strains of *L. innocua* are haemolytic. The hybrid sub-lineage of the major lineage II (HSL-II) of *L. monocytogenes* exhibit a wide zone of haemolysis.

The Christie–Atkins–Munch–Peterson (CAMP) test is a very useful tool to help identify the species of a *Listeria* spp. isolate. It is required in the ISO standards and some AOAC protocols and it is considered to be optional in the FDA and USDA-FSIS methods. The test is simple to perform and easy to read. It consists of streaking a ß-haemolytic *Staphylococcus aureus* (ATCC™ strain 49444® or 25923®, NCTC™ strain 7428® or 1803®) and *Rhodococcus equi* (ATCC™ strain 6939®, NCTC™ strain 1621®) in single straight lines in parallel, on a sheep blood agar plate or a double-layered agar plate with a very thin blood agar overlay. The streaks should have enough separation to allow test and control *Listeria* strains to be streaked perpendicularly, in between the two indicator organisms, without quite touching them (separated by 1–2 mm). After incubation for 24–48 hours at 35–37°C (12–18 hours if using the thin blood agar overlay), a positive reaction consists of an enhanced zone of ß-haemolysis, at the intersection of the test/control and indicator strains. *Listeria monocytogenes* is positive with the *S. aureus* streak and negative with *R. equi*, whereas the test with *L. ivanovii* gives the reverse reactions (Jadhav *et al.,* 2012). HSL-II *L. monocytogenes* is positive with both *S. aureus* and *R. equi* streak.

Within the genus *Listeria*, twenty species have been taxonomically described: *Listeria sensu stricto*, comprising the species *Listeria monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii* and *L. marthii*, and (ii) *Listeria sensu lato, comprising the species L. grayi, L. rocourtiae, L. fleischmannii, L. weihenstephanensis, L. floridensis, L. aquatica, L. cornellensis, L. riparia, L. grandensis, L. booriae, L. newyorkensis, L. costaricensis, L. goaensis* and *L. thailandensis* (Orsi & Wiedmann, 2016). New species (*L. rocourtiae, L. marthii, L. weihenstephanensis, L. fleischmannii* subsp. *fleischmannii* and susbp. coloradensis, *L. newyorkensis*) are mostly isolated from environmental samples and are rare. *Listeria fleischmannii* could be isolated in primary production samples from farms and soil from plants or cellars.

***Table 4. Principal characteristics of the* Listeria *species* sensu stricto**

Test	*Listeria* spp. reaction
Gram stain	Positive
Cell morphology	Short (0.4-0.5 μm × 0.5-2.0 μm) nonspore forming rod with or without a few peritrichous flagella
Growth conditions	Aerobic and facultative anaerobic
Motility	Positive tumbling motility or in umbrella in motility agar at 20–28°C, negative at 37°C
Catalase	Positive
Oxidase	Negative
Aesculin hydrolysis	Positive
Indole	Negative
Urease	Negative

***Table 5. Differentiation of* Listeria *species* sensu stricto**

Species	β-haemolysis	Production of acid from			Christie, Atkins, Munch-Petersen (CAMP) reaction on sheep blood with	
		L-Rhamnose	D-Xylose	D-Mannitol	*S. aureus*	*R. equi*
L. monocytogenes	+[(a)]	+[(b)]	–	–	+	–[(c)]
L. innocua	–[(d)]	V	–	–	–	–
L. ivanovii subsp. *ivanovii*	+	–	+	–	–	+
L. ivanovii subsp. *londoniensis*	+	–	+	–	–	+
L seeligeri	(+)	–	+	–	(+)	–
L. welshimeri	–	V	+	–	–	–
L. grayi subsp. *grayi*	–	–	–	+	–	–
L. grayi subsp. *murrayi*	–	+	–	+	–	–

V: variable; (+): weak reaction; +: >90% positive reactions; –: no reaction.
[(a)]Rare strains of *L. monocytogenes* are not haemolytic;
[(b)]HSL-II *L. monocytogenes* strains associated with ovine listeriosis are rhamnose negative; some lineage III strains of *L. monocytogenes*, which are primarily associated with animal listeriosis, are rhamnose negative;
[(c)]Rare strains are S+ and R+. The R+ reaction is less pronounced than that of *L. ivanovii;*
[(d)]rare strains of *L. innocua* are haemolytic.

1.3. Rapid identification methods

The following protocols include conventional and nonconventional commercially available tests, and nucleic acid assay kits, to help in the identification of *L. monocytogenes* (Valimaa *et al.,* 2015). PCR, targeting the *hly* gene, has been found to be a sensitive and rapid technique for confirmation of the identification of suspect *L. monocytogenes* isolated on selective/differential agar plates (Dhama *et al.,* 2015; Jadhav *et al.,* 2012).

Alternative commercially available methods for identification have been validated by one or more recognised formal validation systems, such as AOAC, MicroVal, Nordval International and Afnor Certification. A new standard ISO 16140-6 was published in 2019 for the validation of alternative methods for microbiological confirmation and typing procedures in the Microbiology of the food chain. The list is growing steadily as new technologies are exploited for application to the needs of laboratories. Regular updates of these alternative methods are published online on the websites of validation/certification bodies, together with key references and scope, validation status and certification of the method. For the validation of these methods, sets of strains of *L. monocytogenes* that reflect the diversity of this bacteria

(https://foodsafety.foodscience.cornell.edu/research-and-publications/ilsi-collection/) has to be used but an additional set to reflect the diversity for animal origin needs to be established and also used.

In addition to the chromogenic isolation media, chromogenic confirmation media or broth for identification of *L. monocytogenes* have been developed. They are mostly based on detection of PI-PLC activity and fermentation of L-rhamnose. A presumed *L. monocytogenes* colony is selected and spread on a form of band (2 cm). *Listeria monocytogenes* shows a PI-PLC activity and a yellow zone of L-rhamnose fermentation. Rare strains of *L. monocytogenes* are rhamnose negative.

A system is commercially available for the presumptive identification of *Listeria* species isolated from samples from the food chain. It provides an alternative to conventional biochemical testing of *Listeria* spp. isolates by the reference methods. It is based on testing miniaturised microtubes on a strip or a card that give reactions by fermentation, utilisation or enzymatic activity, which can be detected after 24 hours at 37°C. For biochemical identification, differentiation of *Listeria* species is based on a code derived after adding the numerical values for each group of several tests and, an additional test such as the reactions obtained from the CAMP test and haemolysis characteristics, which are assayed separately. A commercial method based on the presence or absence of arylamidase, distinguishes between *L. monocytogenes* and *L. innocua* without the need for further tests for haemolytic activity.

Identification can be done by sequencing the 16S rDNA or *iap* genes (Dhama *et al.,* 2015; Jadhav *et al.,* 2012). After extraction of DNA with commercial kits, an end point PCR for 16S rDNA or *iap* genes is performed. PCR products are purified and sequenced with a sequencer in the laboratory. The sequence is compared with DNA database accessible via the internet using blast. Recently, a real-time PCR assay for the identification of isolates has been added to the online BAM manual of FDA methods http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm279532.htm

Identification can be also done by whole genome sequencing of the strain and determination of the species of this strain by comparison of its genomic sequence with other reference genomics sequences of each Type strains of *Listeria* species by the use of the average nucleotide identity based on BLAST (Basic Local Assignment Search Tool), called ANIb. Several tools are freely available on the internet. This identification at genus and/or species level is highly accurate at a taxonomical level.

An alternative method for the rapid identification of *Listeria* species is the matrix-assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF MS), which is increasingly being used worldwide in microbiology laboratories. MALDI-TOF MS identification systems are based on the comparison of the tested isolate mass spectrum for proteins, and also for lipids, with reference databases. Several databases and identification strategies have been developed. For *Listeria* isolates, the genus and species could be accurately and rapidly identified with a validation of one MALDI-TOF MS for *Listeria* system by the WHO[8] Collaborating Centre (Thouvenot *et al.,* 2018) and AOAC: First Action 2017.10.

1.4. PCR methods for detection of *Listeria*

A number of methods based on nucleic acid recognition have been developed to detect *L. monocytogenes* in samples from the food chain (Jadhav *et al.,* 2012). Target DNA sequences for diagnostic purposes include the *hly* gene, the *iap* gene, the *prfA* gene and 16S rDNA gene in a PCR or real-time PCR. Target ribosomal RNA sequences, in higher copies per cell than DNA, and the use of isothermal PCR format is a new promising development for diagnostic purposes. PCR-based methods for the detection of *Listeria* should be validated and used in accordance with Chapter 2.2.3. *Development and optimisation of nucleic acid detection assays.*

1.5. Antimicrobial susceptibility testing

Listeria monocytogenes is intrinsically resistant to cephalosporins (cefazolin, ceftiofur, cefpirome), quinolones (nalidixic acid and early fluoroquinolone such as ofloxacine), fosfomycine and clindamycine. Acquired resistance has been rarely identified. Most of the isolates are susceptible to Penicillin G, amoxicilline, aminoglycosides (gentamicin), tetracyclines, phenicols, trimethoprim and sulfonamides, rifampin, glycopeptides (vancomycin) (Granier *et al.*, 2011; Luque-Sastre *et al.,* 2018). In Europe, Eucast[9]

8 WHO: World Health Organization
9 Eurocast: www.eucast.org

proposed in 2011 a methodology for *L. monocytogenes* susceptibility testing by disc diffusion. In the USA, two documents from the Clinical and Laboratory Standards Institute (www.clsi.org), M31-A3 regarding susceptibility tests of bacteria from animals and M45-A2 regarding susceptibility tests of fastidious bacteria, provide guidelines and interpretation criteria to assess susceptibility of *L. monocytogenes* by the broth microdilution method.

1.6. Subtyping methods

Most regulatory controls of *L. monocytogenes* do not require any specific subtyping of the isolates. However, subtyping schemes can be useful in outbreak investigations, environmental tracking, control of recurrent or persistent clone(s) in a plant, and public health investigations.

Listeria monocytogenes has traditionally been subtyped by a number of different approaches including serotyping, phage typing, DNA restriction enzyme analysis (either using high-frequency cutting enzymes and conventional gel electrophoresis to separate fragments, or using rare-cutting enzymes and pulse-field gel electrophoresis [PFGE] to separate fragments), and nucleic acid sequencing-based typing, microarray analysis. Whole genome sequencing (WGS) is now used routinely to subtype *L. monocytogenes*.

Because of the requirement for specific reagents, stringent quality assurance procedures and some sophisticated equipment, it is recommended that subtyping of *L. monocytogenes* isolates be referred to appropriate reference laboratories. These reference laboratories could be set at the national, regional or international levels. At the international level, there is only one WHO Collaborating Centre for *Listeria*[10].

1.6.1. Serotyping and genoserotyping (PCR group)

Strains of *L. monocytogenes* can be assigned to 14 different serovars (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, 4h and 7), based on their combination of somatic (O) and flagellar (H) antigens, according to the Seeliger & Höhne protocol (1979). Serotyping antigens are shared among *L. monocytogenes, L. innocua, L. seeligeri* and *L. welshimeri*. There is only one commercial kit with these antifactor sera (Denka Seiken, Tokyo, Japan). Although all of them are considered to be potentially pathogenic, most (>95%) human clinical isolates belong to three serovars 1/2a, 1/2b, and 4b. Compared with other subtyping methods, serotyping has poor discriminatory power, but can provide valuable information to facilitate the ruling out of isolates that are not part of an outbreak or an investigation on a human sporadic case. Isolates from foods and from environmental sources are frequently nontypable using commercial standard antifactor sera and require additional sera. In this case, typing could be performed at the WHO Collaborating Centre for *Listeria*.

Because serotyping is not cost-effective, necessitates technical expertise and antisera, it is now often substituted by a quick and reproducible PCR-based method, developed by Doumith *et al.* (2004), which targets the five DNA fragments *prs, ORF2110, ORF2819, lmo1118, lmo0737*. This last genoserotyping method is now internationally recognised and validated. All *Listeria* species but *L. rocourtiae* possess an amplifiable *prs* gene fragment. PCR serogroup IIa comprises strains of serovars 1/2a and 3a (amplification of *prs* and *lmo0737* DNA fragments); PCR serogroup IIb comprises strains of serovars 1/2b, 3b, and 7 (amplification of the *prs* and *ORF2819* DNA fragments); PCR serogroup IIc comprises strains of serovars 1/2c and 3c (amplification of *prs, lmo0737* and *lmo1118* DNA fragments); PCR serogroup IVb comprises strains of serovars 4b, 4d and 4e (amplification of *prs, ORF2819* and *ORF2110* DNA fragments). Finally, PCR serogroup L comprises strains of other serovars of *L. monocytogenes* and other species, except *L. rocourtiae*. This PCR genoserotyping is now performed *in silico* directly from the genomic sequence of the strain (Moura *et al.*, 2016). The PCR method of Doumith *et al.* (2004) failed to distinguish newly named serovar 4h and Feng *et al.* (2020) proposed a multiplex PCR specific to serovar 4h (amplification of LMxysn 1095 and smcL).

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1.6.2. Lineage

After serotyping, *L. monocytogenes* can be classified into three lineages, of which lineage I encompasses serovars 1/2b, 3b, 4b, 4d and 4e; lineage II includes serovars 1/2a, 1/2c, 3a, 3c and 4h; and lineage III comprises serovars 4a, 4c and atypical 4b, according to Wiedmann *et al.* (1997). The lineage status of serovars 4ab and 7 remains unclear due to limited availability of such strains. Within the lineage III, three genetically distinct subgroups (IIIA, IIIB, and IIIC) have been identified after comparative analysis of *actA* and *sigB* gene sequences. Phenotypically, lineage IIIa strains behave like typical *L. monocytogenes* in their ability to ferment rhamnose, whereas lineages IIIB and IIIc strains are notably deficient in rhamnose utilisation. Lineages I and II are involved in the documented human listeriosis cases and lineage III are rarely associated with outbreaks despite their frequent isolation from food and environmental specimens. Lineage I and II isolates seem to be similarly prevalent in animals. Lineage I is more virulent than lineage II, whereas serovar 4h belonging to a hybrid sublineage of the major lineage II (HSL-II) is highly hypervirulent. Specific genes potentially associated with central nervous systems infections in ruminants between lineages I and II were described (Aguilar-Bultet *et al.,* 2018). Recently, lineage IIIB has been reclassified as lineage IV because it is significantly different from lineage IIIA and IIIC to warrant being its own lineage.

1.6.3. Chromosomal DNA restriction endonuclease analysis

Restriction endonuclease analysis (REA) of chromosomal DNA is a useful subtyping method for *L. monocytogenes*. As these enzymes are highly specific in recognising nucleotide sequences, the resulting DNA digestion fragments, of different size and electrophoretic mobility, reflect genomic differences, resulting in specific 'fingerprints' among otherwise related strains. Because of the restriction endonuclease specificity, the method is highly reproducible. Of the restriction endonucleases tested on *L. monocytogenes* in a WHO Multicentre study, *Hae*III, *Hh*aI and *Cf*oI were the most useful (Graves *et al.*, 2007). However, because of a potentially large number of enzyme recognition sites in the bacterial genome, sometimes complex fingerprints evolve, with overlapping or poorly resolved bands that are difficult to interpret. The technique is therefore not adequate for comparing a large number of strain patterns or for building dynamic databases (Graves *et al.*, 2007).One of these REA method, ribotyping, has been widely used for subtyping *L. monocytogenes*, mainly through the use of the restriction endonuclease *Eco*RI, but have a poor discriminatory power.

When restriction endonuclease enzymes that cut infrequently are used to digest unsheared chromosomal DNA, such as *Apa*I, *Sma*I, *Not*I and *Asc*I, very large fragments are obtained. Because of their size, these large fragments do not separate when run under conventional agarose gel electrophoresis. However, by periodically changing the orientation of the electric field across the gel, through pulses, the large fragments can 'crawl' through the agarose matrix and are separated according to size differences. This technique is known as pulsed-field gel electrophoresis (PFGE) and has revolutionised the precise separation of DNA fragments larger than 40 kilobases. PFGE has been applied to the subtyping of *L. monocytogenes* and has been found to be a highly discriminating and reproducible method. PFGE was particularly useful for subtyping serotype 4b isolates, which are not satisfactorily subtyped by most other subtyping methods before genomic area. The main disadvantages of PFGE are the time required to complete the procedure (2–3 days), the large quantities of expensive restriction enzymes required, and the need for specialised, expensive equipment (Graves *et al.*, 2007). The Centers for Disease Control and Prevention (CDC) in the USA has established PulseNet, a network of public health and food regulatory laboratories at the national or international levels that routinely subtype food-borne pathogenic bacteria by PFGE. PulseNet laboratories use highly standardised protocols for PFGE of *Listeria* with endonuclease enzymes *Apa*I and *Asc*I, and can quickly compare PFGE patterns from different locations via restriction profile picture exchange using internet. *Listeria monocytogenes* was added to PulseNet in 1999 and the last protocol published in 2009. In Europe, the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) are building databases with PGFE profiles of *L. monocytogenes* isolated from human cases and food and veterinary sources, respectively, with the aim of investigating transnational or cross-border outbreaks. Since 2017, pulsed field gel electrophoresis with endonuclease enzymes AscI and ApaI for *L. monocytogenes* has been progressively replaced by methods of subtyping using WGS (Moura *et al.,* 2016).

1.6.4. Nucleic acid sequence-based and whole genome sequencing typing

Although there have been some reports on the sequence analysis of single genes as a means to type *L. monocytogenes* strains, determination of allelic variation of multiple genes, has been introduced as a very promising subtyping methodology for this microorganism. This approach has been reported for a handful of other microorganisms and it is known as multi-locus sequence typing (MLST) (Ragon *et al.*, 2008). Direct amplification and nucleotide sequencing has been used with good discrimination between the strains analysed. Because MLST is based on nucleotide sequence, it is more discriminatory and provides unambiguous results. MLST allowed definition of a sequence type (ST) or clonal complex (CC), which gives a view of the phylogenetic structure of a population of isolates. Some of this clonal complex has been implicated in outbreaks or could be linked to clinical forms that give the risk manager additional information on isolates. Recent advancements have enhanced understanding of the virulence potential associated with different ST/CC of *L. monocytogenes*, and identified hypervirulent (such as CC1, CC2, CC4, CC6) and hypovirulent (such as CC9, CC121) clones (Maury *et al.,* 2016). *Listeria monocytogenes* ST1 of CC1 has been found to be strongly associated with rhombencephalitis, which could indicate an increased neurotropism of ST1 in ruminants (Dreyer *et al.,* 2016). Finally, hypervirulent *L. monocytogenes* clones have adapted to mammalian gut, which accounts for their association with dairy products (Maury *et al.,* 2019).

Subtyping by WGS analysis of *L. monocytogenes* strains by a variety of methods such as core genome MLST or single-nucleotide polymorphisms, is available for epidemiological investigations (Chen *et al.,* 2017; Moura *et al.,* 2016; Ruppitsch *et al.,* 2015). Definitions of *L. monocytogenes* clones and clusters using whole genome diversity have been proposed (Moura *et al.,* 2016). Continuing efforts in WGS and functional analysis of human, food and environment isolates are needed to gain more insights into the important subject of the virulence of *L. monocytogenes* isolates or clonal complexes.

Compared with gel-based methods, WGS analysis is more phylogenetically relevant. Some WGS analytical approaches have targeted the entire genome of *L. monocytogenes* (Moura *et al.,* 2016), while others have targeted the core genome (Moura *et al.,* 2016). Different genomic variations have been targeted: single nucleotide polymorphisms, allelic profiles, and k-mers. Moura *et al.* (2016) defines, with unprecedented precision, the population structure of *L. monocytogenes*, demonstrates the occurrence of international circulation of strains and reveals the extent of heterogeneity in virulence and stress resistant genomic features among clinical and food isolates. The implementation of WGS for global epidemiological surveillance has assisted investigations of numerous listeriosis outbreaks.

The development, management and curation of international databases of standardised and reliable WGS subtyping results and their metadata of isolates from different origins (clinical isolates of human origin, food and feed isolates, isolates from animals/veterinary surveillance, isolates from the farm and factory environment) contributes to the understanding of the transmission pathways, rapidly identifying the source of an outbreak and managing the pathogen (Moura *et al.,* 2016; Whitman *et al.,* 2020). The next step in the surveillance for the identification of animal sources of an outbreak and the investigation of listeriosis outbreaks is the use of artificial intelligence or machine learning such as for *Salmonella*.

2. Serological tests

Serological tests for the detection of antibodies have not been traditionally used for the diagnosis of listeriosis. They have been largely unreliable, lacking sensitivity and specificity. A number of formats, including enzyme-linked immunosorbent assay (ELISA), dot-blot and microagglutination (Gruber-Widal reaction) have been largely unsuccessful in the diagnosis of culture-proven human listeriosis, even in the absence of immunosuppression. Considerable cross-reactivity with antigenic determinants of other Gram-positive organisms has been observed. On the other hand, *L. monocytogenes* is a ubiquitous organism, and regular exposure of animals and humans to this microorganism is very common. Many healthy individuals are intestinal carriers (2–6%) and anti-*L. monocytogenes* serum antibody prevalence as high as 53% have been reported in humans. Carriage rate for animals is similar to that of humans, with some differences depending on the species and a slightly higher rate during the indoor season, as compared to animals on pasture (Dhama *et al.,* 2015).

The discovery that the *L. monocytogenes* haemolysin, listeriolysin O (LLO), is a major virulence factor and that it can stimulate an antibody response, has recently renewed interest in the possibility of using serological tests for the diagnosis of listeriosis, particularly in central nervous system patients, with sterile blood and cerebrospinal fluid, and in perinatal listeriosis. An indirect ELISA based on the detection of anti-LLO was used for the diagnosis of experimental listeriosis in sheep (Low *et al.*, 1992). However, LLO is antigenically related to a number of cytolysins, including streptolysin O (SLO) from *Streptococcus pyogenes*, pneumolysin from *S. pneumoniae* and perfringolysin from *Clostridium perfringens*. Problems of cross-reactivity of anti-LLO antibodies with these cytolysins, particularly SLO and pneumolysin, have hampered the development of specific reliable serological tests based on the detection of anti-LLO antibodies. In addition, anti-LLO antibodies have been found in a proportion of healthy individuals and patients with other bacterial, fungal or viral infections (27%, all combined), although at lower titres than in patients with listeriosis. Absorption of diagnostic antisera with SLO is only partially effective in eliminating all cross-reactivity. These experimental assays have been used in some epidemiological investigations and as support for the diagnosis of culture-negative central nervous system infections. Recombinant forms of LLO have been explored as alternatives to wild LLO as a diagnostic antigen in dot-blot assays. Full validation of these serological tests for the diagnosis of listeriosis is needed but a sera biobank needs to be developed.

C. REQUIREMENTS FOR VACCINES

It has proven very difficult to develop effective vaccines against *L. monocytogenes* which, as a facultatively anaerobic intracellular microorganism, requires effector T cells for an effective immune response. Experimental vaccines in laboratory animals are being explored to confer protection to *L. monocytogenes* infection, but these are still far from becoming available for human or farm animal use. These experimental approaches include immunisation with plasmid DNA, CD40 signalling along with heat-killed *L. monocytogenes*, LLO-deficient mutants inoculated along with liposome-encapsulated LLO, RNAi technology and immunisation with listerial antigens and IL-12 (Dhama *et al.,* 2015).

Genetically modified *L. monocytogenes* is also being considered as an effective vaccine vector for the expression, secretion and intracellular delivery of foreign antigens for the induction of potent immune responses against viral antigens and tumour cells. Safety concerns remain for the use of this approach in dogs.

The lack of well designed and tested vaccines for animal use, means that control of listeriosis in animals is most feasible by preventing the environmental conditions that favour its presentation.

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NB: At the time of publication (2021) there were no WOAH Reference Laboratories for *Listeria monocytogenes* (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 2004; MOST RECENT UPDATES ADOPTED IN 2021.

CHAPTER 3.10.6.

MANGE

SUMMARY

Mange is a contagious skin disease, characterised by crusty, pruritic dermatitis and hair/feather loss, and caused by a variety of parasitic mites burrowing in or living on the skin. Some alternative historical names for mange are 'la gale' (*in French*)*, 'itch', 'scab' a term that should be used in relation to* Psoroptes ovis*), and 'scabies'* (*a term that should be reserved only for mange caused by* Sarcoptes scabiei*). Specifically, on domestic hosts* (*i.e. livestock, poultry, companion and laboratory animals*)*, about 50 mite species in 16 families and 26 genera may cause mange. A number of other skin conditions* (*e.g. dermatitis, wheals, blisters, nodules*) *may be confused with mange and must be considered in differential diagnoses, including those resulting from allergic reactions to other kinds of mites, various arthropod bites, fungal diseases, or reactions to physical or chemical aspects of plants. Mange diagnosis in domestic animals is based on clinical manifestations and the demonstration of mites or their developmental stages in host skin scrapings.*

Detection of the agent: *Mange mites are mostly weakly sclerotised, slow-moving, very small* (*100–900 μm*)*, and live permanently on their hosts. Although the Acari is an extremely diverse and ubiquitous group of arachnid arthropods, all of the major mange mite species fall within only two acariform lineages, the Astigmata: Psoroptidia and the Prostigmata: Rhaphignathina. Some economically important mange mite genera are* Cheyletiella, Chorioptes, Demodex, Knemidokoptes, Notoedres, Otodectes, Psorobia, Psoroptes, *and* Sarcoptes*. Specialised illustrated diagnostic keys, taxonomic descriptions, and reference specimens should be consulted to properly identify the causative agents of mange. Special collecting techniques and compound microscopy usually are necessary for diagnosis. Certain identifying characteristics of each of the mange mite groups are highlighted in the following discussion. Although availability is limited, serodiagnostic tests have been developed for certain mange mites and are useful in some circumstances.*

Requirements for vaccines: *Currently, no commercial vaccines against mange are available.*

A. INTRODUCTION

Mange is a contagious skin disease, characterised by crusty, pruritic dermatitis and hair/feather loss, and caused by a variety of parasitic mites burrowing in or living on the skin. The French term for mange is 'la gale' (Pangui, 1994), 'sarna' in Spanish, and in English it has been called 'itch', 'scab' a term that should be used in relation to *Psoroptes ovis*), or 'scabies' (a term that should be reserved specifically for mange caused by *Sarcoptes scabiei*).

Numerous species of mites cause mange in literally hundreds of species of wild and domestic birds and mammals. In fact, approximately 60 mite families have members that live in or on the skin, hair, or feathers of homoeothermic vertebrates and are potential mange mites. Specifically, on domestic hosts (i.e. livestock, poultry, companion and laboratory animals), about 50 mite species in 16 families and 26 genera may cause mange. Humans are host to the readily transmitted *S. scabiei*. Some other mange mites may cause transient disease in humans, but infestations seldom persist.

Mites (Acari) are an extremely diverse, abundant, and ubiquitous group of arachnid arthropods with about 55,000 described species. Higher-level acarine classification is still an unsettled construct, but the following is a consensus system (Bochkov & Mironov, 2011; Krantz & Walter, 2009) encompassing the mange mites. Acari comprises two major evolutionary lineages, Parasitiformes and Acariformes, but only certain acariform mites cause mange in domestic animals. Moreover, both lineages – Trombidiformes and Sarcoptiformes – within the Acariformes contain mange mites. Trombidiformes includes the major suborder Prostigmata, with multiple

superfamilies and many families, five of which contain mange mites. Sarcoptiformes contains the major suborder Oribatida, with many cohorts, superfamilies, and families included, but only 11 constituent families in Astigmata: Psoroptidia contain mange mites.

Some other mites may cause less serious dermatitis in animals or humans (Yunker, 1964). Certain Parasitiformes (order Mesostigmata: e.g. *Ornithonyssus*, *Dermanyssus*) and other Prostigmata (e.g. *Trombicula* [and other chiggers], *Pymotes*) transiently bite a host while feeding, leaving itchy welts and wheals behind. Stored-products, animal-nest, and house-dust mites (e.g. *Acarus*, *Glycyphagus*, *Dermatophagoides*) may cause contact dermatitis (e.g. baker's itch, grocer's itch) but no persistent infestation. Certain free-living bird-nest mites (Hypoderatidae) have a parasitic nymphal stage (hypopus) that characteristically lives subcutaneously in the bird host (e.g. *Hypodectes propus* in domestic pigeons), causing skin irregularities. Pediculosis or certain fungal diseases, such as ringworm, can cause alopecia and crusty dermatitis, and even the physical (e.g. awns, urticarial hairs) or chemical (e.g. urushiol) aspects of some plants may cause host skin reactions that could be confused with mange.

B. DIAGNOSTIC TECHNIQUES

Mange diagnosis in domestic animals is based almost exclusively on clinical manifestations and the demonstration of mites or their developmental stages in host skin scrapings (Kettle, 1995). It is typified by hair/feather loss, crusty or scaly skin lesions, dermatitis, thickened skin, scurf, and pruritus. A possible alternative serological test is now available commercially for only one type of mange, i.e. sheep scab in domestic sheep (Burgess *et al.,* 2020).

1. Detecting the agent

1.1. Direct visualisation

Hair/feather loss and crusty or scaly skin are the most apparent clinical signs of mange. A number of other diseases must be considered when one is confronted with a possible case of mange, including fungi, bacteria, insect bites, irritating plants, mechanical abrasion, etc. In most cases, scrapings should be taken from the edge of the lesion, from obviously pruritic locations, and from where there are thick, crusty flakes. Take a skin scraping by holding a scalpel blade or other sharp instrument at a right angle to the skin and scraping off the outer surface of the skin. For those mite species that burrow into the skin, the scraping must be deep enough to cause a small amount of blood to ooze from the scraping site. A drop of mineral oil or glycerol may be placed on the blade to help hold the skin scrapings during the procedure. Skin scrapings should be placed in sealed containers (e.g. clean, empty salve tins; stoppered glass/plastic test tubes; small, sealable plastic bags) and promptly taken or sent to a laboratory for more thorough examination. An even more effective method of collecting mites from the skin surface and hair is by using a vacuum cleaner fitted with an in-line filter (Klayman & Schillhorn van Veen, 1981). The material collected, along with the filter, is then examined as a skin scraping would be. An otoscope can be valuable in revealing the presence of ear mites. A cotton-tipped applicator can be used to swab the ear canal if ear mites are observed or suspected; examine it in the same way as a skin scraping.

Perform an initial examination of the skin scraping under a dissecting microscope. Obviously visible mites, especially those that are alive and moving, may be picked up with a dissecting needle dipped in glycerine or a mounting medium and transferred to a drop of mounting medium on a glass slide. When the desired number of mites has been collected, gently place a cover-slip on the drop of mounting medium, taking care to avoid air bubbles. Hoyer's medium, Berlese's fluid, Vitzhum's fluid, and Heinze's modified PVA medium are all acceptable mounting media. If permanent mounts are desired, allow the slides to dry for at least 1 week at room temperature, then ring the cover-slips with nail polish or other sealant to keep them from drying out.

Mites that are embedded in oil and exudate, *Demodex* for example, may be demonstrated by placing a small amount of skin scraping directly on the slide with some glycerine or immersion oil and pressing a cover-slip on top of it. The slide then can be examined directly with a compound microscope.

Skin scrapings that contain dead mites, large amounts of skin flakes or scabs, or large amounts of hair should be processed further. Place the skin scraping (up to several grams of skin and hair) in a suitably sized beaker, then add sufficient 10% potassium hydroxide to immerse the sample. Cautiously bring the solution to a gentle boil, stirring frequently (a laboratory hot-plate with a magnetic stirrer works well for

this), for 5–10 minutes or long enough to digest most of the hair and skin. This step should be performed under a chemical fume hood to limit exposure to caustic fumes. Do not boil for an extended period of time, or the mites may disintegrate. Transfer the digested material to suitable test tubes, and centrifuge at 600 ***g*** for 10 minutes. Decant the supernatant. Resuspend the pellet in a small amount of flotation medium (e.g. Sheather's solution or a mixture of 50% corn syrup and 50% water); then, fill the tube completely with flotation medium, and place a cover-slip on top of the tube, assuring that it makes contact with the flotation medium. Let stand for 1 hour, or centrifuge for 10 minutes. Carefully remove the cover-slip by lifting straight up, so that a drop of fluid remains on the underside of the cover-slip, and place on a glass slide. Any mites in the sample will have floated to the top and will be found in the drop of fluid attached to the cover-slip. Another simpler but satisfactory technique, that is used in many laboratories, is to re-suspend the pellet in a small amount of distilled water, drop onto a large (76 × 51 × 1 mm) glass slide and cover with a 40 × 50 mm cover-slip. This is examined under a dissecting microscope (×40 or ×100) with understage lighting. The slide then may be examined under a compound microscope for the presence of mites.

DNA of *Sarcoptes scabiei* has been successfully amplified and detected by polymerase chain reaction (PCR) from human cutaneous scales (Bezold *et al*., 2001). This technique holds promise as an additional procedure for detecting specific, hard-to-find mange mites in skin scrapings.

In cases where mites are difficult to find in skin scrapings from small domestic pet animals, they sometimes may be demonstrated by faecal flotation.

1.2. Molecular methods

Little of practical value is available for mange mite detection, although DNA of *Sarcoptes scabiei* has been successfully amplified and detected by PCR from human cutaneous scales (Bezold *et al*., 2001). Angelone-Alasaad *et al.* (2015) successfully amplified and detected DNA of *Sarcoptes scabiei* mites from multiple host species by both conventional and real-time PCR. This technique might be useful for detecting specific, hard-to-find mange mites in animals, although its use still requires acquisition of mite materials from skin scrapings.

1.3. Serological tests

Researchers have shown that *Sarcoptes scabiei* and *Psoroptes ovis* infestations cause measurable specific antibody responses in hosts, namely pigs, sheep, dogs, and camels (Falconi *et al*., 2002; Lowenstein *et al*., 2004; Lower *et al*., 2001); this makes possible serological detection of sarcoptic and psoroptic manges. Enzyme-linked immunosorbent assays (ELISAs) that detect antibodies to *Sarcoptes* in pigs and dogs are commercially available in some countries and have been used for serodiagnosis of scabies (Lowenstein *et al*., 2004) in Sweden and Switzerland to support scabies eradication programmes in swine. Work is in progress on development of serodiagnostic methods based on recombinant proteins, such as dot-ELISA (Zhang *et al.,* 2012) and indirect ELISA (reviewed by Arlian & Morgan, 2017). Burgess *et al.,* (2020) reported a highly effective ELISA capable of detecting the presence of *Psoroptes ovis* mites in both affected and subclinically affected sheep hosts. However, at present and in most situations, the only unequivocal proof of mange is finding and identifying the offending mites, but this traditional (direct) method is being augmented by continually improving biochemical (indirect) methods.

2. Identifying the agent

Mange mites are traditionally, and most often, identified by mounting them on slides and examining their morphology under a microscope. They are mostly weakly sclerotised, slow-moving, very small (100–900 μm), and live permanently on their hosts. The general life cycle of mange mites is brief (1–5 weeks) and includes four stages: egg, six-legged larva, eight-legged nymph (one or more instars), and eight-legged adult (male and female.) Specialised illustrated diagnostic keys (e.g. Baker *et al*., 1956; Bochkov, 2010; Gaud & Atyeo, 1996; Giesen, 1990; Kettle, 1995; Klompen, 1992; Krantz & Walter, 1999; Yunker, 1973), taxonomic descriptions, and reference specimens should be consulted to properly identify the causative agents of mange. However, certain morphologically diagnostic characteristics of each of the mange mite groups are highlighted in the following discussion.

Genetic sequencing, typically of specific subunits of mitochondrial DNA, is becoming a more widely used tool to help identify organisms of all kinds and can be useful and reliable when identifying some well characterised species

of mange mites. However, a great many species of mites do not yet have many, or any, published mitochondrial DNA sequences on which to base an identification, so one should use caution when basing an identification solely on a genetic sequence. However, when combined with an examination of morphological features, genetic sequences can supply valuable confirmatory information when identifying mange mites. Mange in domestic animals results from the host's physiological, immunological, and behavioural responses to infestation by certain mites in any of eleven families of Astigmata or five families of Prostigmata.

2.1. Astigmata

Astigmatan mange mites are generally small, globose or oval in outline, and thin-skinned. The somatic cuticle often shows a pattern of fine, parallel striations (finger print patterns), with distinctively shaped and placed setae, spines, pegs, or scales, and sometimes, lightly sclerotised plates or shields. Adults usually have eight legs and anterior mouthparts that include paired palps and chelicerae used for cutting and feeding. The legs attach proximally to the body through distinct cuticular epimeres (coxal apodemes) and terminate distally in a variety of setal forms or in a pretarsal empodium that may be shaped like either a claw or a bell-like sucker (caruncle or ambulacrum.) Astigmatan mites do not have true, paired pretarsal claws. Males sometimes bear somatic suckers or other secondary sexual characteristics used in mating, but the form and placement of setae and empodia on the legs is usually sufficient to separate the sexes as well as identify the various mange mite species. Fertilised eggs are simple, soft, and translucent ovoids that are produced by mated females through a usually midventral ovipore.

2.1.1. *Sarcoptida*e

Sarcoptid mites are all obligate, burrowing skin parasites of mammals, with over 100 described species (Bochkov, 2010; Klompen, 1992). Survival time under moderate conditions for mites off the host is limited to about 10 days or less. Because of their activities in the epidermal layers of the skin, mange caused by these mites is generally more severe than that caused by mites dwelling above the surface of the skin. The body outline of sarcoptids is generally rounded, dorsoventrally flattened, and the cuticle is striated. The palps are one-segmented, and the legs are usually short. Three genera contain domestic animal parasites of interest.

2.1.1.1. *Sarcoptes scabiei*

This mite causes sarcoptic mange (scabies) in humans and other mammals. It is among the most common, widespread, and serious types of mange extant. More than 100 known species of infested hosts occur worldwide in at least 10 mammalian orders and 26 families (Bornstein *et al*., 2001). Domestic hosts include camels, cattle, dogs, cats, sheep, goats, horses, swine, llamas, and alpacas. Sarcoptic mange in dromedaries is a particularly debilitating chronic condition with high morbidity, and it may predispose afflicted hosts to other infections. Fain (1968) suggests that humans were the original host of *Sarcoptes*, and all other hosts were secondarily infested. Despite some dissention, current scientific consensus generally views all *Sarcoptes* mites on all hosts as no more than host-adapted variants of a single, variable species. Transmission between individuals within a host species or genus may occur easily by close contact, but taxonomically unrelated hosts are not readily infested or infestations are self-limiting. For example, *S. scabiei* var. *canis* easily transfers among dogs and can move to foxes, coyotes, and other canids, but humans serve as no more than transient hosts for this variant. Recent molecular analyses support the conspecificity of all *Sarcoptes* variants (Zahler *et al*., 1999), and an immune response has been demonstrated in Sarcoptes infested hosts (Arlian *et al*., 1994).

Mature female *S. scabiei* are approximately 500 μm long, with fingerprint-like striations on the cuticle, short and stubby legs, various characteristic setae and pegs, and with a dorsal patch of tooth-like spines. Males are similar but smaller (about 275 μm), and the tooth-like spines are reduced in size and number. The anus is posterior in both sexes, and the first pair of epimeres is fused in a midventral Y-shape. Long-stalked, unjointed pretarsal suckers occur on legs I and II in both sexes and on legs IV in males. The remaining legs all terminate in long, hair-like setae. In addition, each tarsus bears at its tip one or two highly modified setae in the form of short spurs. Nymphs resemble females but are smaller and lack an ovipore. Larvae are similar but smaller still and have only six legs.

2.1.1.2. *Trixacarus caviae*

This mite is a specific parasite of captive and laboratory guinea-pigs, *Cavia porcellus*, but it never has been found on wild-caught animals (Klompen, 1992). Although these mites are a little smaller, the morphology and life cycle are similar to *S. scabiei*. However, all dorsal setae in *T. caviae* are long and hair-like, unlike some of those in *Sarcoptes*, which are short and broad or peg-like; males of *Trixacarus* also lack pretarsal suckers on the fourth pair of legs, and the pedicels (stalks) of all suckers are a bit shorter than those typical of *Sarcoptes* mites. This mite may cause a severe mange in host animals, especially in the laboratory setting. A similar mite, *T. diversus*, rarely occurs on laboratory rats.

2.1.1.3. *Notoedres* spp.

Notoedres is a large genus comprising some 45 species, most of which are associated with bats (Chiroptera) (Klompen, 1992). Four species are of some concern with respect to notoedric mange in domestic animals. The cat mange mite, *N. cati*, is a cosmopolitan parasite of domestic cats, but it also infests several wild cats (e.g. bobcat, cheetah, serval, snow leopard), palm civets, coatimundis, mongooses, and domestic rabbits. These are highly contagious mites, and they cause intense mange, especially about the host's head and sometimes spreading to the legs, genital area, or even the tail. Laboratory rats are hosts to *N. muris*, which burrows into the stratum corneum and causes thickening and cornification of the skin on the pinnae, eyelids, nose, and tail. Additional hosts include other *Rattus* spp., several other rodents, two marsupials, and a hedgehog (Klompen, 1992). The laboratory mouse may be infested by two *Notoedres*, *N. musculi* and *N. pseudomuris*, but the latter primarily occurs in wild populations of this host. Each mite also infests a few other murid rodent species. The mange caused is similar to that caused by *N. muris* in rats. *Notoedres* mites are generally similar to *Sarcoptes* but about half the size, and they lack the mid-dorsal field of tooth-like cuticular spines and peg-like setae, which may be replaced by a slight scale-like pattern in the cuticular striations and short, stout setae. The anus is posterodorsal, the first pair of epimeres is not fused medially, and the tarsi of legs I and II each end in three or four short, spur-like setae, not just two.

2.1.2. *Psoroptidae*

Psoroptid mites are obligate parasites of mammals. They dwell and feed on the surface of the host's skin. Survival time for some of these mites off the host may be two weeks or more. The generally oval-shaped body is dorsoventrally flattened, has a striate cuticle with scattered setae but no spines, and bears longer legs and more prominent mouthparts than those of sarcoptid mites. The anus is posteroventral. Males usually each have a pair of terminal posterior lobes bearing diagnostic setae and a pair of ventral adanal suckers used in mating. The first pair of epimeres is not fused medially. Fifty species in about 30 genera of psoroptid mites are known from at least 11 mammalian orders, with the greatest number on primates (Bochkov, 2010). Three genera have veterinary importance for domestic animals.

2.1.2.1. *Psoroptes ovis*

For decades, conventional practice among acarologists has been to distinguish several species of *Psoroptes* among the mites that cause psoroptic mange worldwide in wild and domestic ungulates and rabbits, e.g. *P. cuniculi* in the ears of rabbits and various ungulates, *P. equi* on the bodies of English equids, *P. ovis* on the bodies of sheep and other ungulates (Bochkov, 2010). Distinctions between the species were based primarily on host and anatomical site infested and on morphology of the males. Recently, several workers have invalidated these criteria and used genetic analysis to show conspecificity of the traditionally different species. The earliest published description for *Psoroptes* mites is that for *P. ovis*, making this the proper designation for all such mange mites on all domestic hosts. Thus, the nomenclatural situation in *Psoroptes* becomes similar to that in *Sarcoptes*, with one morphologically and genotypically variable species occurring worldwide, albeit on a smaller spectrum of hosts and with a bit less stringent host specificity among the variants. Two other named *Psoroptes* spp. remain as tentatively valid taxa occurring only on wild mammal hosts (Bochkov, 2010). Psoroptic mange in both sheep and cattle seems to vary in its severity according to the variant of *P. ovis* present, with the most severe form being a reportable condition caused by an especially virulent genotype and known as 'sheep scab'.

Thus, particularly for further eradication efforts against psoroptic sheep mange, genotypic analysis of the involved mites may be an especially valuable tool (Falconi *et al*., 2002).

Mature female *Psoroptes* are 550–750 μm long, with a striate cuticle and four long and 16 short dorsal somatic setae. A noticeable anterodorsal cuticular plate is present behind the mouthparts, and the midventral ovipore is an inverted U-shape. Males are about one-fourth smaller, and they have an additional, larger posterodorsal cuticular plate, a pair of posteroventral adanal suckers, and two terminal posterior lobes, each equipped with four setae of varying lengths and structures. Nymphs and larvae are somewhat similar to adults but progressively smaller, and all *Psoroptes* are pearly white in colour. In all stages, the anterior two pairs of legs are thicker and more robust than the posterior pairs, which are thinner, and in the male, shortened in the fourth pair. Legs I and II terminate in pretarsal empodial suckers on long, segmented pedicels in both sexes, with similar structures on legs IV of the female and legs III of the males. The female's third tarsus ends in two long, whip-like setae, and the male has a single short seta on tarsus IV, plus a long, thin seta accompanying the empodial sucker on tarsus III.

2.1.2.2. *Chorioptes* spp.

This genus currently comprises six recognised species of obligate ectoparasitic mites that may cause chorioptic mange in domestic and wild mammals. Two of the species, collected rarely from wild animals, are poorly known and may not be valid entities, but *C. bovis* and *C. texanus*, primarily from domestic animals, have withstood modern biogenetic scrutiny and are accepted species (Bochkov, 2010; 2014). A number of allegedly host-specific varieties within these species are not separable from one another (Sweatman, 1957). The two species are morphologically distinguishable only by differences in the terminal posterior lobes and setae of males (Sweatman, 1957). Chorioptic mange, also called 'barn itch,' may be the most common form of mange in cattle and horses. It is a relatively mild condition that usually is more localised and less intensely pruritic than psoroptic or sarcoptic manges. This is probably because *Chorioptes* mites are able to feed and survive on host-produced epidermal debris at the skin surface, without necessarily attacking the living parts of the host's skin. Infestations tend to concentrate on the lower portions of the host, especially the feet and legs, but may include the udder/scrotum, tailhead, and perineum. In some cases, *C. texanus* infests the host's ears (Sweatman, 1957). *Chorioptes bovis* has been known for more than 165 years and occurs widely on cattle, goats, sheep, horses, camelids (mainly llamas), and possibly domestic rabbits. *Chorioptes texanus* was not discovered until 1924, and for 50 years, it was recognised only from goats and reindeer in the USA and Canada (Sweatman, 1957). Since 1975, it has been found on Taiwan serow, *Capricornis swinhoei*; fallow deer, *Dama dama*; European elk, *Alces alces*; and multiple times on cattle in several countries (Bochkov, 2014). Based on unpublished observations by the USDA, *C. texanus* may now be the prevalent *Chorioptes* species on cattle in the USA.

Both *Chorioptes* species on domestic animals are nearly identical morphologically in all stages. The circular body is dorsoventrally flattened, with a striate cuticle, and about 400 μm long in the female; males are about one-fourth smaller, and the somewhat similar nymphs and larvae are progressively smaller yet. Dorsally, adults of both sexes have both anterior and posterior cuticular shields and a variety of mostly short, hair-like setae. Ventrally, the female ovipore is a transverse slit with a pair of trailing apodemes. The mouthparts are unremarkable, and the legs are moderately long and robust, except the fourth pair in the male are very short, and the third and fourth pairs in the female are more slender. All legs in both sexes terminate distally in empodial suckers with short, unjointed stalks, except for the female's third pair, which end in two long, whip-like setae each. The male also has a long, whip-like seta on each third leg and a pair of adanal suckers. The terminal posterior lobes of males bear five setae each. The lobes of *C. bovis* each have a nearly rectangular margin, the seta at the external angle is long and whip-like, and the two spatulate setae are moderately shorter (ca. 115 μm) and broad. The lobes of *C. texanus* are each more angulate, almost bilobed, with a very short hair-like seta at the external angle and two much longer (ca. 215 μm) spatulate setae that seem narrowed basally.

2.1.2.3. *Otodectes cynotis*

Carnivores are the primary hosts for these highly contagious mites, which mainly infest the host's ear canals but sometimes spread to the pinnae and even beyond. Clinical signs of otodectic mange (otacariasis, 'ear canker') may include rubbing and scratching the ears, vigorous head-shaking, depression, excessive drainage, and haematoma of the ear. Worldwide, *Otodectes* is probably the most frequent mange mite infesting carnivores, both wild and domestic. In addition to companion animals (e.g. dogs, cats, ferrets), these mites also affect various farm-raised furbearers (e.g. foxes, mink) and occasionally may stray to humans. As with other mange mites, past workers often have treated *Otodectes* mites from different localities or different hosts as separate varieties, or even different species, but recent molecular and phenotypic studies conclude that the genus is monobasic.

Otodectes mites have a typical psoroptid morphology and life history mirroring those of *P. ovis*. The female body is about 435 μm long and oval-shaped; the male length is about 325 μm. The female ovipore is a transverse slit with trailing genital apodemes, and bilaterally, the epimeres of the first pair of legs are joined to those of legs II. The terminal posterior somatic lobes of the male are only weakly produced, but adanal suckers are present. Each lobe bears five hair-like setae of varying lengths. All of the legs are moderately long and robust, except for the fourth pair, which is much reduced, especially in the female. Empodial suckers with very short, simple pedicels occur distally on all legs except for the posterior two pairs in females, which each end in a pair of long setae. The third tarsus of the male also bears a pair of long, whip-like setae in addition to its ambulacrum.

2.1.4. *Epidermoptidae*

This acarine family comprises 23 genera and about 100 species of mites, particularly species in the subfamily Knemidokoptidae (six genera, 17 species), that inhabit the same microhabitats in birds that Sarcoptidae occupy in mammals (Krantz & Walter, 1999). As a result, possibly due to convergence, the morphology of the two groups is similar. The body is generally globose, with cuticular striations that are sometimes modified into patches of scale-, furrow-, or tooth-like structures. The mouthparts and legs are usually short and stubby. Pretarsal suckers may be present, incomplete, or absent on all legs, and the tarsi may terminate in one or two chitinous spurs. Somatic setae are generally few, unmodified, and quite short. Knemidokoptids have a distinctive anterior dorsal shield marked by a pair of strongly sclerotised, longitudinal, paramedial apodemes running to the base of the mouthparts. Males (but not females) also may have a median posterior dorsal shield, and their first pair of epimeres is fused into a midventral Y-shape. The first epimeres in females (and immatures) may be free or joined by a transverse apodeme into a V- or U-shape. The ovipore is a transverse slit or a three-valved, inverted Y-shape, and the anus is terminal or posterodorsal. Males may or may not have adanal suckers. Most species occur, sometimes worldwide, only on various wild birds in which they may cause clinical knemidokoptic mange; however, species in three genera are of concern for domesticated and cage birds.

Knemidokoptes mutans commonly burrows in the epidermal layers of the skin on the feet and legs of chickens, turkeys, and pheasants, causing a crusty mange known as 'scaly leg.' If untreated, lameness, distortion, or loss of digits may result. The first epimeres of female *Knemidokoptes* are free; legs I and II each have two terminal spurs, but no ambulacrum occurs on any leg; the ovipore is transverse; the anus is dorsal; and the body has a mid-dorsal patch of cuticular scales. Females are 350–450 μm, and males are less than 240 μm long. As in other knemidokoptids, legs of males are longer than those of females, and all of them terminate in a small, long-stalked sucker. A second, similar species, *K. pilae*, infests the face, cere, and legs of budgerigars, leading to a condition known as 'scaly face.' These mites are slightly smaller than *K. mutans*, and both species probably occur worldwide on their respective hosts.

Picinemidicoptes laevis infests columbid birds, including the domestic pigeon, sometimes leading to clinical mange. In females, the first epimeres are fused in a U-shape; each leg has an empodial stalk only, and legs I and II end in one spur each; the ovipore is transverse; the anus is terminal; and the dorsal cuticular striae are unbroken by scales.

Neocnemidocoptes gallinae may infest the skin of the back, head, neck, abdomen, and upper legs of chickens, geese, and pheasants, causing intense pruritus. Feathers in these areas may fall out,

break, or be plucked by the host, leading to a condition known as 'depluming itch.' Affected skin, especially on the neck, may become scaly, thickened, and wrinkly. Although depluming itch is less common worldwide than scaly leg, it may be more damaging and even fatal. Female mites are 340–440 μm long, but males subtend about 210 μm. The first epimeres of female *Neocnemidocoptes* are free; the tarsi each end an empodial stalk only, and one spur terminates each of the anterior two pairs of legs; the ovipore is transverse; the anus is dorsal; and the dorsal somatic cuticle is transversely striate but without scales. Two other, smaller *Neocnemidocoptes, N. columbicola* and *N. columbigallinae*, infest columbiform birds in limited circumstances and possibly might cause pathology in domestic pigeons.

2.1.5. Miscellaneous families

Eight remaining astigmatan fur and feather mite families contain a variety of mange mites that are generally of minor significance due to their limited host ranges or relatively mild clinical effects on their hosts.

Three families of mammal parasites are worthy of note. Atopomelidae comprises over 400 species in nearly 50 genera of fur mites with known hosts in 14 mammalian orders, mostly marsupials in the Southern Hemisphere. The body plan is variable, but most are soft, slightly elongate, flattened or cylindrical, and the legs usually have some flattened segments for grasping the host's hairs to the mite's ventral surface, which often is ridged in the coxal areas of legs I and II. *Chirodiscoides caviae* probably occurs worldwide on guinea pigs, but it has been reported commonly only in Asia and Europe, where it sometimes causes severe pruritus and alopecia to laboratory animals. Listrophoridae is another family of fur mites comprising 170 species in about 20 genera found on nine mammalian orders, mostly rodents and mostly in the Northern Hemisphere. These are somewhat soft, elongate, cylindrical mites with various cuticular striae, spines, and punctate shields, including a sclerotised tegmen dorsally covering the mouthparts. They cling to the host hair-shaft bases by means of a pair of ridged flaps projecting ventrally from the area between the first pair of legs. *Lepoacarus gibbus* is a common listrophorid that sometimes causes mange in domestic and laboratory rabbits, and *Lynxacarus radovskyi* lives on several wild felines and the domestic cat, where mild, scurfy mange sometimes results. Myocoptidae is a nearly cosmopolitan family containing six genera and 60 species of skin-feeding, hair-clasping mites that occur on rodents and marsupials. Myocoptids are generally oval-shaped and dorsoventrally flattened. The cuticle may be extensively striate, scale-covered, or denticulate in females, whereas male cuticles are generally less ornate and more heavily sclerotised. Host hairs are grasped by robust, highly modified legs III and IV in females and legs III in males. *Myocoptes musculinus* is probably the most ubiquitous ectoparasite of laboratory mice. Infestations are usually benign, but stressed or compromised mice may suffer alopecia, erythema, pruritus, and traumatic dermatitis (myocoptic mange.) Another, smaller myocoptid, *Trichoecius romboutsi*, occasionally occurs on laboratory mice, along with *M. musculinus* or other mites, but its role in clinical mange is unclear.

Five families of mites from the skin and feathers of birds deserve mention. These are classified among 36 astigmatan mite families in three superfamilies loosely known as feather mites (Gaud & Atyeo, 1996). Thousands of species of feather mites live on or inside the feathers or skin of nearly every kind of bird worldwide in generally commensal relationships. In rare and unexplained circumstances, the commensal status of nearly any kind of feather mite may transition to that of a parasite, leading to negative consequences for the host. Some entire families of nominal feather mites (e.g. Cytoditidae, Laminosioptidae) have become true parasites with distinct associated pathologies, even mange (e.g. Knemidokoptinae). A few species in other families are more prone than is usual to cause debilitation or injury to their hosts. In the Analgidae, *Megninia cubitalis*, *M. ortari*, *M. hologastra*, and *M. ginglymura* occur on domestic chickens and may cause depluming behavior and economic losses (Gaud *et al*., 1988). *Dermoglyphus elongatus* (family Dermoglyphidae) occurs on caged canaries, and *Dubininia melopsittaci* (family Xolalgidae) occurs on budgerigars, and excessive presence of each mite species may engender depluming and associated skin lesions in the respective hosts. Members of the families Dermationidae and Epidermoptidae (Epidermoptinae) generally feed on the skin or in the feather follicles of their bird hosts, placing them very close to being parasitic. Domestic poultry are hosts to *Rivoltasia bifurcata* and *Epidermoptes bilobatus* from the two respective families, and each mite has occasionally been associated with pityriasis (epidermoptic mange) in chickens (Baker *et al*., 1956).

2.2. Prostigmata

With over 19,000 named species classified into approximately 130 families, prostigmatan mites as a group exhibit tremendous morphological and biological diversity, making generalisations about them difficult. However, all of the prostigmatan mange mites belong in either of two superfamilies, Cheyletoidea (comprising seven families) and Myobioidea (one family). Together, these eight families include nearly 1,100 named mite species, but there are hundreds of undescribed species, as well. The anterior mouthparts in this group may be variously modified by palpal segment elaboration or reduction and by basal cheliceral fusion and extension into elongate, needle-like stylets used to pierce the host's tissues for feeding. Some prostigmatan mange mites have paired, elongate, dorsal respiratory peritremes above the mouthparts. The body usually is elongate, sometimes very much so, and usually soft and thin-skinned, but sometimes with sclerotised plates. Adults usually have eight legs that vary in length and morphology according to the habits of the family, but they each usually terminate distally in a pair of pretarsal claws and a linear empodium that often is equipped with numerous sticky hairs. Proximally, the legs may articulate with simple coxal fields or sclerotised somatic apodemes. The ovipore is a longitudinal, usually mid- or posteroventral slit, whereas, the genital pore in males is dorsal and sometimes equipped with a long aedeagus.

2.2.1. *Demodecidae*

The demodecids comprise more than 150 species of parasitic mites in seven genera from hosts in 11 mammalian orders. *Demodex* is the only genus of importance for domestic hosts, and it contains at least 70 named species plus many more that are unnamed and undescribed. Although other genera display their own unique features, adult *Demodex* are elongate, spindle-shaped, or vermiform mites, 250–850 μm long, that live in the host hair follicles, sebaceous glands, Meibomian glands, and occasionally in epidermal pits. They have short anterior mouthparts with two-segmented palps and retractable needle-like stylets used to puncture surrounding host tissues and feed on predigested cellular fluids. The normally four pairs of legs are usually short, stumpy, composed of three segments each, and terminate distally in paired pretarsal claws, usually with a linear empodium. Coxal fields occupy much of the anteroventral surface of the body where the legs attach. The palps or one pair of legs of some stages of some species may be greatly elongated or otherwise modified, primarily as holdfast organs. The very thin cuticle of the body and appendages is all but devoid of setae, but the opisthosoma is usually transversely striate. Befitting the confines of their narrow follicular or glandular habitats, the immature stages, including the eggs, of *Demodex* spp. are usually spindle-shaped or elongate oval, sometimes extremely so. *Demodex* species are very host specific, only rarely inhabiting more than one species of congeneric mammal host. However, it is not uncommon for a host species to harbour two to four different species of parasitic *Demodex*. With the exception of *Demodex gatoi* (which can be transferred between cats of any age), transfer between hosts occurs only by very close contact between individuals (most probably mother to neonate), making transmission between animal species or from animals to humans very unlikely. Their very thin cuticles mean that demodecids cannot survive away from their hosts for more than a few hours.

Although *Demodex* mites frequently infest the skins of 100% of the individuals of their respective host species, their presence is usually without noticeable consequence for the hosts. On occasion, because of stress or other poorly understood factors, resident mite populations explode in numbers that result in a pathological condition known as demodectic mange. Healthy feral animals almost never suffer from demodectic mange, and laboratory or domesticated hosts are the usual victims (Nutting, 1985). Clinical signs may range from presence of small skin papules, to large nodules, to extensive hair loss. Although rare, severe or generalised cases may lead to mites invading the host circulatory system, secondary bacterial skin infection, and even death. Among domestic animals, clinical disease (sometimes called 'red mange') is most often seen in dogs (*Demodex canis* and *D. injai*), but swine, (*D. phylloides*), goats (*D. caprae*), horses (*D. caballi*), sheep (*D. ovis*), cats (*D. cati* and *D. gatoi*), cattle (*D. bovis, D. tauri,* and *D. ghanensis*), and rabbits (*D. cuniculi*) occasionally develop demodectic mange. Humans are normal hosts for two species of *Demodex* (*D. folliculorum* and *D. brevis*).

2.2.2. *Psorergatidae*

Worldwide, fewer than 100 species of these small parasitic skin mites are described in three genera (Giesen, 1990) (treated as subgenera of *Psorergates* by some authors). Known hosts are

in eight mammalian orders, mostly rodents and bats. Adult psorergatids are about 100–200 μm long, generally circular in outline, and dorsoventrally flattened. The cuticle is very thin, finely striate, and a large, punctate, lightly sclerotised shield covers most of the dorsum. The short anterior mouthparts have stylet-like chelicerae and two-segmented palps, each of which ends in a stout, claw-like seta. There are no dorsal peritremes. The four pairs of moderately long legs are radially attached ventrally, have five segments each, and terminate distally in paired pretarsal claws but no empodium. The femur of each leg often bears a sturdy, retrorse spur ventrally. Psorergatids have relatively few setae, including a few on the mouthparts, five or six pairs on the dorsal shield, one small ventral pair, one or two long pairs on posteroventral body lobes, and less than 10 on each leg. The eggs are almost round and large, nearly two-thirds the size of the mature female. They are deposited in hair follicles or in epidermal pits made by the female. Immature stages are much like adults but smaller, with only six legs for larvae and all legs greatly foreshortened. Transfer from host to host is accomplished directly by motile adult mites, which then move selectively to less-keratinised areas of the host skin, frequently about the head, neck, and the back. There, they invade the hair follicles or burrow body-sized pits into the epidermis, feed by puncturing cell walls with their stylets, and reproduce. Psorergatid mites rarely survive off the host for more than a day.

Psorergatid infestations on healthy wild hosts and most domestic animals are generally low and of little consequence. Sometimes, however, populations of a few species may explode, particularly on sheep and laboratory mice, producing psorergatic mange. Skin damage from activities of adult mites usually is mild and only slightly irritating, but their progeny, from egg nests cut into the dermis, may enlarge these pockets into fluid- or keratin-filled papular lesions that may rupture and cause inflammation and other host immune responses (Nutting, 1985). Psorergatid mange mites of concern occur in two genera, *Psorobia* (with four pairs of marginal setae on the dorsal shield) and *Psorergates* (with three pairs of such marginal setae). Infestations of *Psorobia ovis*, the sheep itch mite, are most troubling in older animals and cause the hosts to rub, scratch, and bite at the wool in the most irritated areas, giving the fleece a ragged, tufted appearance. Powdery scurf sometimes may be present, as well. The life cycle of *P. ovis* takes about five weeks, the condition spreads slowly and inconsistently through a flock, and detection of infestations often is difficult. A similar mite, *P. bos*, occurs widely on cattle, but it seems to have little pathological effect on hosts. *Psorobia cercopitheci*, from Africa (and a similar undescribed Asian species), occasionally cause mange in colonies of laboratory primates. The laboratory mouse is subject to papular lesions on the head and neck and auricular mange caused by *Psorergates simplex* (Yunker, 1973). Incidence of these mites in some mouse colonies may be as high as 80 per cent. Another *Psorergates* mite, *P. muricola*, has been found on five different rodent species, including *Mus musculus*, and *Psorergates rattus* occurs on *Rattus norvegicus*; whether either of these mites infests or damages laboratory rodents is unknown.

2.2.3. *Cheyletidae*

This family of approximately 375 species comprises mostly free-living predator mites and about 100 species parasitic on birds and mammals. The parasites are arranged into approximately 15 genera, with about one-third of the species on mammals and the rest on birds. Although a number of the genera contain species capable of causing limited pathology in their hosts, only a few members of the genus *Cheyletiella* are of concern as mange mites on domestic animals. *Cheyletiella* mites are 300–530 μm long, elongate rhomboidal, and distinguished by a strongly striate cuticle with one (females) or two (males) large dorsal shields. A number of moderately long, simple or barbed setae occur in distinctive patterns on the body, mouthparts, and legs. The anterior mouthparts are large, with short piercing stylets and especially robust, five-segmented palps, each of which terminates in a strong, curved claw-like seta that is lined with weak, ridge-like teeth on the inner margin. Prominent M-shaped peritremes occur on the dorsal surface of the mouthparts. The four pairs of legs are long and strong, and each terminates distally in a linear empodium equipped with a double row of sticky hairs. Although almost all other cheyletiellids also have paired pretarsal claws on each leg, none occurs in *Cheyletiella*. A small sensory organ (solenidion) occurs on the middle segment (genu) of each leg I, and its shape is (statistically) distinctive for each species (Bronswijk & de Kreek, 1976). Females lay their eggs singly and attach them to host hairs near the skin using a finely woven mass of threads. Transmission between hosts is primarily by close contact, but phoresy on ectoparasitic insects is a possibility, as well.

For many years, the identities of the various pathological *Cheyletiella* spp. were confused under the single name *C. parasitivorax* (Smiley, 1970), and these mites were mistakenly thought of as predators on other parasitic mites. However, *C. yasguri* (on dogs), *C. blakei* (on cats), and *C. parasitivorax* (on domestic rabbits) are now separately known to be the cause of mange, and any of the three may sometimes afflict humans in close contact with infested hosts, leading to severe dermatitis, pruritus, and other signs of cheyletiellosis for them, as well. The mites move easily among the host hairs on the keratin layer of the skin, periodically attaching to the surface by means of the palpal claws and puncturing cells of the epidermis with their stylets to engorge on predigested host fluids. The disease is similar in all three domestic hosts and usually is most evident on the back, shoulders, and neck. However, clinical signs are generally mild and not very distinctive or definitive. They may include scruffy hair coat, inflammation, occasional pruritus, alopecia, and almost always, hyperkeratosis. The barely visible, moving mites in the fur of the host and the abundant, powdery white scurf associated with cheyletiellic mange have engendered for it the alternative name 'walking dandruff.'

2.2.4. *Myobiidae*

Myobiids are small (to 900 μm), soft, elongate rectangular, somewhat dorsoventrally flattened fur mites known from five orders of mammals worldwide. More than 450 species of myobiids have been identified, at least half of them from bats. The cuticle is generally transversely striate, without sclerotised shields, and dorsally usually bears 12 to 16 pairs of setae, many of which are expanded, leaf-like, and longitudinally striate. The anterior mouthparts are small, with simple two- or three-segmented palps, cheliceral stylets, and dorsal peritremes. The legs, especially the first pair, are strong and highly modified for grasping host hairs, one or two at a time. They terminate distally in large pretarsal claws but no empodium; sometimes one of the two paired claws on a leg is greatly reduced or absent. The apparatus for clasping hairs are characteristic and consist of various combinations of modified leg segments and setae in the form of spurs, hooks, bosses, ridges, and grooved surfaces. Nymphal and larval myobiids generally resemble their respective adults except for size. Myobiid eggs are usually attached by the females with an adhesive secretion to the bases of the host's hairs. Larvae may actually enter the hair follicles to feed on host fluids issuing from punctures made with the stylets. Nymphs and adults feed at the surface of the host skin in the same way, sometimes even puncturing capillaries and imbibing blood. The life cycles of myobiids are generally brief (ca. 14 days), and the mites freely move between host individuals. Myobiid infestations on wild mammal hosts are usually low in intensity and of little consequence (Nutting, 1985), but on laboratory rodents, they frequently expand greatly and cause intense pruritus and hair loss known as myobiic mange.

Both *Myobia musculi* and *Radfordia affinis* occur on the laboratory mouse and its wild progenitor, the house mouse, and each may cause pathology in laboratory animals. The two mites are superficially similar in appearance, but differ in many minute details, the most readily observed of which is the number of pretarsal claws present on the second leg; there are two in *Radfordia* and one in *Myobia*. *Radfordia ensifera* infests the Norway rat and the laboratory rat, sometimes causing mange in the latter. Whereas, both pretarsal claws on leg II in *R. ensifera* are of equal size, the posterior claw in *R. affinis* is smaller than the anterior one.

2.2.5. *Syringophilidae*

Over 350 species of these very host-specific quill mites have been discovered on a wide variety of bird hosts worldwide, but less than half have been named and described, while probably thousands of unknown species remain extant. The body is elongate (about 500–950 μm) and cylindrical in keeping with the infestation site within the quills of the host. The cuticle is thin, striate, and without sclerotised plates, but a variety of usually long setae arise from its surface, particularly at the posterior end. M-shaped peritremes arise above the mouthparts, which are equipped with stylets and simple, linear palps. The legs are short, stubby, and terminate distally in paired claws and haired empodia. Sclerotised epimeres occur in coxal fields I and II. While residing in the quill shafts, syringophilids puncture the quill walls with their stylets to feed on fluids from the surrounding feather follicle tissues.

Two species of quill mites from domesticated hosts sometimes occur in large numbers and cause serious irritation and severe feather loss that might be confused with knemidokoptic mange; *Syringophilus columbae* parasitises domestic pigeons and *S. bipectinatus* occurs in the quills of

chickens. Modern poultry production methods that physically separate chick broods from laying hens have been very successful in breaking the chain of passage for *S. bipectinatus* from one host generation to the next, all but eliminating the depluming problem except in more traditional production settings. Two other described quill mites, *Picobia polonica* from chickens and *P. khulkhshani* from pigeons, have not yet been associated with host feather loss.

C. REQUIREMENTS FOR VACCINES

There are no commercial vaccines for mange, but researchers are working on them (Liu *et al.*, 2014). Experimentally, inoculation with antigens from *Psoroptes ovis* and *S. scabiei* has reduced the severity of mange in immunised sheep and rabbits. This introduces the future possibility of controlling the effects of mange without the use of acaricides (Burgess *et al.,* 2016).

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NB: At the time of publication (2022) there were no WOAH Reference Laboratories for mange (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.10.7.

SALMONELLOSIS* [1]

SUMMARY

Description of the disease: *Salmonellosis is an infectious disease of humans and animals caused by bacteria of the genus* Salmonella*. Salmonellae are aetiological agents of diarrhoeal and systemic infections. They often cause subclinical infections and may be shed in large numbers within the faeces of clinical cases and carrier animals resulting in contamination of the environment. Infection in food animals often leads to contamination of meat, eggs, milk and milk products. Salmonellosis is one of the most common and economically important food-borne zoonotic diseases in humans. The disease has been recognised in all countries and non-typhoidal* Salmonella *appears to be most prevalent in areas of intensive animal husbandry, especially in pigs, intensively reared calves and poultry.*

The disease can affect all species of domestic animals; young animals and pregnant and lactating animals are the most susceptible to clinical infections. Enteric disease is the commonest clinical manifestation, but a wide range of clinical signs, which include acute septicaemia, abortion, arthritis and respiratory disease, may be seen. Many animals, especially pigs and poultry, may be infected but show no clinical illness. Such animal species have a central role in relation to the spread of infection between flocks and herds and as sources of food contamination and human infection.

Fowl typhoid and Pullorum disease, poultry diseases caused by host-specific Salmonella biovars*, are addressed in chapter 3.3.11.*

Detection of the agent: *Diagnosis is based on the isolation and identification of the organism either from tissues collected aseptically at necropsy or from faeces, rectal swabs, environmental samples, food products and feedstuffs. Prior or current infection of animals by some serovars may also be diagnosed serologically. When infection of the reproductive organs or abortion occurs, it is usual to culture fetal stomach contents, placenta and vaginal swabs and, in the case of poultry, embryonated eggs or ovary/oviduct.*

Salmonellae may be isolated using a variety of techniques that may include pre-enrichment to resuscitate and multiply sub-lethally damaged organisms, enrichment media that contain inhibitory substances to suppress competing organisms and selective plating agars to differentiate salmonellae from other enterobacteria. Alternative methods such as polymerase chain reaction and immunological detection of Salmonella *antigens can also be used, according to legislative requirements.*

Various biochemical, serological and molecular tests can be applied to the pure culture to provide a definitive confirmation of an isolated strain. Salmonellae possess antigens designated somatic (O), flagellar (H) and virulence (Vi), which may be identified by specific typing sera, and the serovar may be determined by reference to the antigenic formulae in the White-Kauffmann–Le Minor scheme. Many laboratories may need to send isolates to a reference laboratory for the identification of the serotype and further characterisation. Alternative serotyping methods, include various methods based on molecular approaches, such as microarray or whole genome sequencing, which are increasingly used. Phage-typing schemes are also available for some serovars, but are less commonly used than in former years, having been largely superseded by molecular methods.

1 Although certain diseases caused by *Salmonella* are included in some individual species sections of the WOAH List, this chapter covers several species and thus gives a broader description.

Serological tests: *Serological tests should be conducted on a statistically representative sample of the population and results are not always indicative of active infection. In the laboratory, the tube agglutination test is the method of choice for export and diagnostic purposes for samples from all species of farm animals. Enzyme-linked immunosorbent assays are available for some serovars and may be used for serological diagnosis and surveillance, especially in poultry and pigs.* Salmonella *vaccination may compromise the diagnostic value of serological tests.*

Requirements for vaccines: *Inactivated and live vaccines are available commercially. Inactivated vaccines usually contain oil or alhydrogel adjuvants to improve their efficacy.*

A. INTRODUCTION

Salmonellosis is an infectious disease of humans and animals, clinically characterised by acute or chronic enteritis, septicaemia or abortion. Animals may be infected without being overtly ill. Salmonellae are primarily intestinal bacteria and may be shed continuously or intermittently within the faeces, resulting in contamination of the environment (Barrow & Methner, 2013).

1. Classification and nomenclature of the agent

The genus *Salmonella* consists of two species: *S. enterica* and *S. bongori* (Grimont & Weill, 2007). *Salmonella enterica* is divided into six subspecies, which are distinguishable by certain biochemical characteristics and susceptibility to lysis by bacteriophage Felix O1. These subspecies are:

	Original subgenera		Current nomenclature
•	Subspecies I	=	subspecies *enterica*
•	Subspecies II	=	subspecies *salamae*
•	Subspecies IIIa	=	subspecies *arizonae*
•	Subspecies IIIb	=	subspecies *diarizonae*
•	Subspecies IV	=	subspecies *houtenae*
•	Subspecies VI	=	subspecies *indica*

For the serovars of *S. bongori*, the symbol V was retained to avoid confusion with the serovar names of *S. enterica* subsp. *enterica*.

Strains of *Salmonella* are classified into serovars on the basis of extensive diversity of lipopolysaccharide (LPS; O antigens) and flagellar protein (H antigens) in accordance with the White-Kauffmann–Le Minor scheme (Grimont & Weil, 2007); currently more than 2600 serovars are recognised, but there are also a number of stable monophasic variant clones of several serovars, and the list of serovars has not been updated for over 10 years. The most common serovars that cause infections in humans and food animals belong to subspecies *enterica*. The serovars of the other subspecies are more likely to be found in cold-blooded animals and in the environment, but are occasionally associated with human disease. Some serovars of subspecies *arizonae* and subspecies *diarizonae* may cause disease in turkeys and sheep and others may be carried by reptiles and amphibians. In accordance with the White-Kauffmann–Le Minor scheme, only serovars of subspecies *enterica* bear a name, e.g. *S. enterica* subsp. *enterica* serovar Enteritidis, or *S. enterica* serovar Enteritidis, or, in short, *S.* Enteritidis. Serovars of other subspecies of *S. enterica, monophasic variants* and serovars within *S. bongori* are designated only by their antigenic formula (e.g. *Salmonella* IV 48:g.z51).

Changes to serovar classification may occur when different O antigens are expressed due to colony form variation or lysogeny by bacteriophage(s) or when different flagellae are expressed as a result of phase variation or deletions or mutations in genes coding for flagellae or expression mechanisms.

2. Description and impact of the disease

The course of infection, the clinical signs, the post-mortem findings and epidemiological patterns vary according to the serovar and the animal species involved. Most serovars can cause disease in a wide range of animal species (Jajere, 2019), but some serovars are host-specific, e.g. *S.* Typhi in humans and *S.* Abortusovis in sheep. Other serovars are host-adapted e.g. *S.* Choleraesuis in pigs and *S.* Dublin in cattle. Host-specific and host-adapted serovars often cause septicaemic disease. In poultry, Pullorum disease or bacillary white diarrhoea and fowl typhoid

are used to describe the host-specific infections caused by *S.* Gallinarum, biovars Pullorum and Gallinarum, respectively. Fowl typhoid and Pullorum disease are covered in detail in chapter 3.3.11.

In humans, young children, the aged and people who are immunologically compromised or taking proton pump inhibitors are most susceptible to salmonellosis. The disease can affect all species of domestic animals; young animals and pregnant animals are particularly susceptible. A wide range of clinical signs, including acute septicaemia, acute or chronic diarrhoea, respiratory disease, abortion, and arthritis, may be seen. Chicks and turkey poults of less than 1 week of age are highly susceptible to *Salmonella* infection and may occasionally exhibit clinical signs including anorexia, adipsia, depression, ruffled feathers, huddling together, somnolence, dehydration, white diarrhoea and pasted vents with considerable mortality as a result, but even in young poultry, subclinical infection is most likely. In calves, septicaemic infection with the host-adapted *S*. Dublin serovar occurs mainly at 2–6 weeks of age. The calves are dull, pyrexic, anorexic, have diarrhoea with blood and mucus in the faeces, may have pneumonia or necrosis of distal extremities, and often become quickly dehydrated and die if appropriate treatment is not given in a timely manner. In pregnant cows, infection with *S*. Dublin is a common cause of abortion. Pigs infected with the host-adapted *S*. Choleraesuis may show signs of septicaemia that can result in death without any preceding clinical signs. However, more commonly, clinical signs of affected pigs are anorexia, high fever, lethargy, shallow cough, difficulty in breathing and cyanotic extremities. *Salmonella* Typhimurium is also a common cause of salmonellosis in cattle and weaned pigs.

Many animals, especially poultry and pigs, but also cattle and sheep, may be infected but show no clinical illness (Jajere, 2019). Such animals have a central role in relation to the spread of the infection between and within flocks and herds. Salmonellosis has been recognised in all countries, but non-typhoidal infection appears to be most prevalent in areas of intensive animal husbandry, especially of poultry and pigs (Jajere, 2019).

3. Zoonotic potential, biosafety and biosecurity requirements

Human salmonellosis is one of the most common and economically important zoonotic diseases. The Centers for Disease Control and Prevention (CDC) estimates that salmonellosis causes more than 1.35 million illnesses each year in the United States of America, with more than 26,500 hospitalisations and 429 deaths (CDC, 2021). A recent study in Australia reported that in 2015, there were nearly 91,000 non-typhoidal salmonellosis cases, of which over 4300 were hospitalised and 19 patients died, at a total societal cost of over 100 million US dollars (Ford *et al*., 2019). The most common cause of infection with *Salmonella* is eating contaminated foods including raw or undercooked eggs or egg products, poultry and pig meat, contaminated fresh fruit and vegetables and soft cheeses made from unpasteurised milk.

Salmonella can also be spread to people through contact with infected birds, livestock, reptiles, amphibians, rodents, dogs and cats. These animals may carry the bacteria even when apparently healthy. Many serovars, including some that are host-adapted such as *S*. Choleraesuis and *S*. Dublin, have been shown to cause serious disease in humans. Abattoir workers, animal attendants and veterinarians may be infected directly during the course of their work when in contact with infected animals. Laboratory personnel may also acquire the infection if safe working practices are not implemented. All laboratory manipulations with live cultures or potentially infected or contaminated material must be performed at an appropriate biosafety and containment level as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

4. Differential diagnosis

Signs in young birds with generalised salmonellosis closely resemble those seen in Pullorum disease, fowl typhoid and those of other acute septicaemic illnesses caused by a wide variety of bacteria including *Escherichia coli.* In all avian species arthritis caused by *Salmonella* infection may be mistaken for synovitis or bursitis caused by other infections. Septicaemic salmonellosis in pigs caused by *S*. Choleraesuis may be mistaken for hog cholera. Septicaemic salmonellosis in calves or weaned pigs may be confused with colibacillosis, although the latter disease occurs usually at a younger age. The acute enteric form of salmonellosis in calves may resemble coccidiosis. Abortions in sheep may be caused not only by *S.* Abortusovis, but also by *Chlamydia abortus, Campylobacter* spp., *Coxiella burnetii, Brucella ovis* or other pathogens.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of salmonellosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[(a)]						
Salmonella isolation	+++	+++	+++	+++	+++	–
Rapid alternative methods, e.g. PCR	+	+	+	+	+	–
Detection of immune response						
SAT	++	–	++	–	+	++
ELISA	++	–	+++	+	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction-based tests; SAT = serum agglutination test; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent detection methods applied on the same clinical sample is recommended.

1. Detection of the agent

The frequency of sampling and the type of samples obtained will depend largely on the objectives of the testing programme, monitoring or international trade regulations, clinical findings, level of detection or precision of prevalence estimates required, cost and availability of sampling resources and laboratory facilities. General guidelines on the collection, submission and storage of diagnostic or survey samples, the sample size, the information to be sent with the samples and the packaging and transportation of samples are described in Chapters 1.1.2 and 1.1.3 of this *Terrestrial Manual.*

Individual samples for bacteriological tests are collected as aseptically as possible and in the case of clinical disease or routine monitoring, samples should be collected before any antibiotic treatment has commenced. Clinical samples are preferably collected during the acute phase of the disease or as soon as possible after death. In the case of flocks of poultry or other avian species, environmental samples, such as naturally pooled faeces, litter and dust or drag or boot swabs from floor surfaces, or large, moist fabric swabs may be the most cost-effective way to identify infected flocks, and can also be useful for other types of livestock unit. In dairy herds, milk filters can be a useful sample type. For smaller animal species, it may be preferable to submit a representative number of sick or recently dead animals to the laboratory. Host-adapted serovars are usually more difficult to isolate from faeces so if these are suspected, infected tissues should be cultured where possible.

Particular attention should be given to the isolation of salmonellae from animals with subclinical infection, as these may only shed bacteria intermittently and in low numbers. An increased sample size, increased number of samples representing more individuals, combined in some cases with pooling of samples to reduce the cost, and repeat sampling can provide an increased diagnostic sensitivity, but pooling can also reduce detection if the sample prevalence or concentration of target bacteria is low. As bacteria are usually clustered in faecal samples, a thorough mixing of the sample before culture may also increase the sensitivity of the procedure. Bacteriological and also serological methods may be used to identify infected flocks or herds and infected individual animals.

1.1. Culture

There are numerous methods for isolation and detection of *Salmonella* in use world-wide. Some of the more common methods are described below. The culture techniques and media that may work best in a particular diagnostic situation depend on a variety of factors, including the *Salmonella* serovar, source

and type of specimens, animal species of origin, experience of the microbiologist, and availability of selective enrichment and selective plating media.

All culture media should be subjected to quality control and must support growth of the *Salmonella* from a small inoculum in the presence of a relevant sample matrix or competing flora. The routine use of a reference strain in parallel with routine samples may lead to cross-contamination of samples if careless techniques are used; therefore, a rare serovar with typical growth characteristics that are similar to the highest priority target strains should be used. It is also possible to use strains with antimicrobial resistance or other markers, such as fluorescence.

The increasing application of external quality assurance programmes has led to greater use of international standard methods, such as ISO 6579-1:2017 (as amended in 2020). The modified semi-solid Rappaport–Vassiliadis (MSRV) agar-based method has been validated for faecal and food, feed and environmental samples and is the required method for detection of *Salmonella* from primary animal production and optional for food or feeding stuffs. The basis of the standard method is pre-enrichment in buffered peptone water, followed by mandatory selective enrichment on MSRV agar for samples of primary production, whereas for food and feedstuffs the prescribed selective enrichment is Müller–Kauffmann tetrathionate broth (MKTTn) and Rappaport–Vassiliadis-soya (RVS) broth or MSRV, then isolation is carried out on xylose-lysine-deoxycholate (XLD) agar and an additional plate medium of choice, which is based on a different biochemical indicator. MSRV agar has been shown to be highly effective for detection of motile salmonellae. Diagnostic methods and assays should be validated as described in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*.

1.1.1. Pre-enrichment media

The number of salmonellae in faeces from asymptomatic animals, environmental samples, animal feed and food is often low, and sometimes damaged, thus it is necessary to use pre-enrichment media, such as buffered peptone water to maximise detection. This may allow the small numbers of salmonellae, which may otherwise be killed by the toxic effect of selective enrichment media, to multiply, and it may help to resuscitate salmonellae that have been sub-lethally damaged, e.g. by freezing, heating, desiccation, or exposure to biocides or organic acids. If acid-treated samples are being tested, it can be advantageous to increase the buffering capacity of the pre-enrichment medium. For highly absorptive samples or fresh faeces and intestinal contents, increasing the ratio of broth to sample weight can improve detection. For intermittent shedder animals, it is advantageous to test at least three consecutive faecal samples.

1.1.2. Selective enrichment media

Enrichment media are liquid or semi-solid agar media containing additives that selectively permit salmonellae to grow while inhibiting the growth of other bacteria. Examples of selective enrichment media are tetrathionate, as in Müller–Kauffmann broth, selenite cystine, brilliant green broth, RVS broth and MSRV agar. Some additives, however, can be relatively toxic to certain serovars of *Salmonella,* e.g. selenite inhibits *S.* Choleraesuis, and brilliant green is toxic to many strains of *S.* Dublin. Elevated temperatures have also been used to increase the selectivity, with a temperature of 43°C being used in some laboratories, although this may be inhibitory with some media, e.g. tetrathionate.

With Rappaport–Vassiliadis at 43°C temperature-sensitive strains, especially *S.* Dublin, are inhibited and 41.5°C is recommended for incubation of RVS broth-based media and MSRV agar. Selective motility enrichment media such as MSRV agar are commonly used to increase the sensitivity of the *Salmonella* isolation procedure. Use of at least two selective enrichment media is recommended for maximum recovery, and detection of mixed serovar contamination, with one incubated at 37°C and the other at a suitable higher temperature. The formulation of the medium, which may vary between suppliers, or even between batches in some cases, temperature and duration of incubation, and the volume of the samples used to inoculate the medium, may all serve to influence the isolation rate, and these variables should always be taken into account. The selectivity of these media is based on the motility of the organism, the presence of malachite green dye and novobiocin, and a high concentration of magnesium chloride. The semi-solid medium allows motility to be detected as halos of growth spreading within the agar from the site of inoculation. If there is no such growth, the sample can often be considered to be negative for *Salmonella* without further plating. Non-motile strains however cannot be detected using semi-

solid selective enrichment. Additives, such as ferrioxamine E, may be added to selective enrichment media to enhance isolation of *Salmonella* from iron or nutrient-limited samples such as eggs, water or soil or antibiotics such as novobiocin may be added to suppress most Gram-positive organisms or other Gram-negative bacteria, such as *Proteus*. Specific antibiotics can be added to enhance the isolation of antimicrobial resistant *Salmonella* strains.

1.1.3. Selective plating media

These are solid, selective agars that permit differential growth to varying degrees. They inhibit growth of bacteria other than *Salmonella* and give information on some of the principal differential biochemical characteristics – usually non-lactose fermentation and hydrogen sulphide (H_2S) production. The results are read after 24 and 48 hours of culture at 37°C. Salmonellae form characteristic colonies on such media that are usually distinguishable from the colonies of other bacteria on the plate, with the possible exceptions of *Proteus, Pseudomonas, Citrobacter* and *Hafnia*. Lactose-fermenting salmonellae may occasionally be isolated and H_2S production may be variable. Such atypical strains may be more effectively detected when semi-solid motility media are used for selective enrichment. Plates such as desoxycholate-citrate agar (DCA), brilliant green agar (BGA) or bismuth-sulphite agar can be used, but these are subject to a higher frequency of false-positive colonies. *Salmonella* Abortusovis is a slow-growing serovar and it is usual to incubate plates for up to 72 hours and to use non-selective blood agar, or minimally selective MacConkey agar. A wide range of chromogenic agars are also available for the selective isolation of salmonellae. Many of these may aid differentiation of suspect colonies, especially from faecal samples, but must be validated for the sample matrices, culture systems and serovar range targeted as sensitivity can be poor in some circumstances, e.g. for host-adapted or some *arizonae* or *diarizonae* serovars. Certain chromogenic agar media, may, however, be more efficient for detection of biochemically atypical salmonellae.

1.1.4. Example test procedures for isolation of *Salmonella* from food, feedstuffs, faecal and environmental samples

i) Add a 10–25 g sample to at least ×10 volume of buffered peptone water at ambient temperature. (NB: for many host-adapted serovars and some *arizonae* serovars, it is preferable to add the sample to selective enrichment medium, such as selenite cysteine broth, and to test tissue samples where possible [including by direct plating]; see culture method for *S.* Gallinarum (biovars Gallinarum and Pullorum) in Chapter 3.3.11 *Fowl typhoid and Pullorum disease.*)

ii) Incubate in pre-warmed buffered peptone water for 16–20 hours at 34–38°C.

iii) Inoculate 15–20 ml MSRV agar in a 90 mm diameter Petri dish with 0.1 ml incubated buffered peptone water, preferably as three separate drops.

iv) Inoculate 10 ml Müller–Kauffmann tetrathionate broth with 1 ml incubated buffered peptone water.

v) Incubate MSRV at 40.5–42.5°C and tetrathionate broth at 34–38°C. The higher end of the temperature range for both pre-enrichment and selective enrichment is recommended for improved isolation of *Salmonella* from faecal and intestinal samples, or environmental samples with a complex flora. The lower part of the range may be more suitable for some food/feed and dry environmental samples.

vi) After 24 and 48 hours of selective enrichment, plate out MSRV by taking 1 µl loop of material from the edge of the turbid growth zone and streaking over one plate of chromogenic agar or BGA plus novobiocin and one plate of XLD agar.

vii) Plate out 10 µl of tetrathionate broth on one plate of chromogenic agar or BGA plus novobiocin and XLD agar.

viii) Incubate plates at 34–38°C for 21–27 hours.

ix) Check up to five suspect colonies (red/pink with reddening of the medium on BGA, red with black centre (or occasionally translucent red in the case of H_2S negative strains on XLD agar) biochemically, using composite media such as TSI, LDC and urea, or commercial biochemical test kits, and by slide agglutination with polyvalent 'O' (A-S) and poly 'H' (phase 1 and phase 2 antisera). Confirm to the serogroup level by using specific 'O' group antiserum. The combination of the biochemical and serological

results can provide confirmation of *Salmonella* spp. Sero-grouping alone is not sufficient to rule out false positives because of cross-reactions e.g. by *Citrobacter* or *Enterobacter* spp. Composite biochemical tests, polymerase chain reaction (PCR) or matrix assisted laser desorption ionisation time of flight (MALDI-ToF) mass spectrometry can also provide confirmation of *Salmonella* spp.

x) Subculture strongly suspect colonies that do not agglutinate with poly H antisera on to non-selective media then repeat testing. If a strong poly 'O' and poly 'H' agglutination can be obtained, this is sufficient for presumptive confirmation. Biochemically and serologically confirmed isolates can then be submitted to a reference laboratory for serotyping.

1.2. Quantification methods

Salmonella from infected tissues can be enumerated by direct plating, but most probable number (MPN) techniques are necessary for faecal, feed or environmental samples. A miniaturised MPN method has been described and listed by ISO, 2012 (ISO/TS 6579-2: 2012). Furthermore, quantitative real-time PCR methods have also been developed (Zhang *et al*., 2020).

1.3. Identification of suspect colonies

Suspect colonies are subcultured onto selective and non-selective agars to ensure the absence of possible contaminants, such as *Proteus* spp. If there is an abundant pure growth, suspect colonies may be tested by slide agglutination with polyvalent *Salmonella*-typing antisera (Ellis *et al.,* 1976). In some cases, the suspect colony may not agglutinate or may auto-agglutinate and it is necessary to use biochemical tests to confirm the identity. These tests can be performed using peptone water sugars or commercial systems or composite media (such as triple sugar iron agar [TSI]). It is particularly important to ensure that *Salmonella* cultures used for determination of antimicrobial resistance of live vaccine testing are not mixed with other organisms such as *Pseudomonas* that are more likely to be multi-resistant or to mask auxotropism. MALDI-TOF is also an acceptable and rapid method for identification of *Salmonella* (Dieckmann & Malorney, 2011).

The determination of the O factor(s) and the H antigen(s), and in special circumstances the Vi antigen (present in *S.* Typhi, *S.* Paratyphi C and *S.* Dublin), is performed by direct slide agglutination or tube agglutination using specific antisera. In the case of biphasic organisms, it is necessary to determine both H phases, by the use of phase inversion – this involves passage through semi-solid agar containing antiserum to the known phase. Screening is facilitated by the availability of antisera directed against several factors, which can be pursued further by the use of monovalent typing sera. More details on serotyping of *Salmonella* are described in ISO/TR 6579-3:2014 . While many laboratories can identify the more common serovars, it is usually necessary to use the facilities of a reference laboratory to confirm the identity of an isolate or to conduct phage typing, if serovar-specific typing phages are available, and for genetic characterisation. Additional biochemical or PCR tests may be necessary to identify some serovar variants, e.g. d-tartrate fermentation, which can be used to differentiate *S.* Paratyphi B var. Java (d-tartrate +) from *S.* Paratyphi B. Isolates should also be tested for their sensitivity to a range of antimicrobial agents as there is increasing concern about the emergence of new multiple resistant strains harbouring (transferable) resistance genes to cephalosporins, colistin and fluoroquinolones (Antonelli *et al*., 2019; Jajere, 2019). Live vaccine strains are also commonly identified by antimicrobial resistance markers or biochemical changes such as auxotrophism or roughness, as well as by commercial PCR kits or published PCR assays.

1.4. Immunological and nucleic acid recognition methods

Numerous alternative *Salmonella* detection methods are in use and some are commercially available. Conventional or real-time quantitative PCR, sometimes including simultaneous identification of key serovars (Heymans *et al*., 2018; Zhang *et al*., 2020), loop-mediated isothermal amplification (LAMP) methods (Yang *et al.,* 2018), enzyme-linked immunodiagnostic assays (Cetin *et al*., 2019) are available. Many of these methods have not been fully validated for faecal and environmental samples, although some progress has been made (Malorny & Hoorfar, 2005; Heymans *et al*., 2018) and in the EU it is possible to validate alternative methods for statutory use by following ISO16140-2, via an authorised certified organisation. These rapid methods are more suited to analysis of human foodstuffs, where inhibitors of the PCR reactions and competing or cross-reacting organisms are not as problematic as for

faeces. Optimisation of PCR detection necessitates suitable DNA extraction techniques and controls to detect inhibition, which may reduce the sensitivity of the test in some cases (Jensen *et al*., 2013; Kanki *et al*., 2009). One of the main advantage of these methods in comparison with the cultural ones is their rapidity, and they can be a valuable diagnostic tool in test and release of batches of *Salmonella*-free food and animal feedstuffs. The rapid methods are usually more expensive than conventional culture, but can be economically viable for initial screening of materials where a low prevalence of contamination is expected or where materials, such as feedstuffs, are held pending a negative test. An enrichment/IMS method linked with enzyme-linked immunosorbent assay (ELISA) or PCR can identify most *Salmonella* contamination within 24 hours (Wang *et al*., 2018). As currently none of the rapid methods has been shown to be suitable for direct detection of low numbers of *Salmonella* in samples, non-selective or selective enrichment stages are usually required (Oliveira *et al*., 2003). Typically this introduces more steps and operator time in the detection procedure. There are many variations and developments in rapid methods for *Salmonella* detection, but none has been shown to satisfactorily replace culture in all circumstances.

In contrast, molecular methods for serotyping or subtyping *Salmonella* isolates are increasingly widely used for outbreak investigation and source attribution (Munck *et al*., 2020). Some kits using these methods are suitable for use in small laboratories that lack the facilities of a reference laboratory (Diep *et al*., 2019) and one is registered by WOAH. Multiplex PCR or whole genome sequencing-based methods may be used to identify specific *Salmonella* serovars (Maurischat *et al.,* 2015a) or to distinguish live vaccine strains from *Salmonella* serovars infecting the flock or herd (Maurischat *et al*.,2015b; Tang *et al*., 2019). A new ISO standard (ISO 16140-6:2019) covers validation of (sub)typing methods and is a prerequisite for such methods to be used to replace *Salmonella* serotyping in statutory EU control programmes. It is important that kits used have been fully validated in accordance with chapter 1.1.6. Kits should preferably be selected from those listed on the WOAH Register of diagnostic kits[2].

2. Serological tests

2.1. Serological identification of infected animals, flocks and herds

A number of serological tests have been developed for the diagnosis of *Salmonella* infections in animals. In poultry, the whole blood test, which uses a stained antigen, and the serum agglutination test (SAT) have been used successfully for over 50 years for the identification of flocks infected with *S.* Gallinarum (biovars Gallinarum and Pullorum) (see Chapter 3.3.11). Because *S.* Enteritidis possesses the same group D somatic antigen as *S.* Gallinarum and is thought to originate from a common ancestor (Thomson *et al*., 2008), the whole blood test and related tests can be used for the diagnosis of *S.* Enteritidis infection, but the sensitivity is low. Other tests, such as the ELISA (Feld *et al*., 2000) have been developed for the diagnosis of *S.* Enteritidis and *S.* Typhimurium infections in poultry and for other serovars in farm animals. The ELISA has been used effectively to identify serologically *S.* Dublin carrier cattle and can be applied to bulk milk for screening dairy herds. An ELISA that includes somatic antigens from a mix of serovars ("mix-ELISA") is used in several countries on serum or tissue fluid released by freezing then thawing muscle samples to detect *Salmonella* infections in pigs. A similar test can be used to detect antibodies to *S.* Enteritidis and *S.* Typhimurium in egg yolk from unvaccinated commercial laying flocks. Several ELISAs are available as commercial kits, but their performance can be variable (Van der Heijden, 2001). There is a need for standardisation of their use and, to this end, panels of control sera are available commercially from Denmark[3] and the Netherlands[4].

2.2. Factors affecting serological diagnosis

1. Serological methods should be used to identify infected flocks/herds rather than to identify infected individual animals, although repeated herd tests can be used as an aid to selective culling of chronic carrier animals. Serological tests are normally designed to detect a limited range of *Salmonella* serovars or serogroups.
2. It is well recognised that some animals with a positive serological response may no longer be infected with *Salmonella*, and in countries with a low prevalence of salmonellosis specificity issues

2 https://www.woah.org/en/what-we-offer/veterinary-products/diagnostic-kits/the-register-of-diagnostic-kits/
3 Statens Serum Institut, Copenhagen, Denmark (www.ssi.dk)
4 GD, Deventer, the Netherlands (www.gddeventer.com)

mean that most positive results will be false. Animals that are actively shedding salmonellae may be serologically negative in the early stages of disease and some individual infected animals never seroconvert. Animals that are serologically positive may have ceased to shed salmonellae although circulating immunoglobulin concentrations may remain high, especially in latent carrier animals, but other animals on the farm may still be shedding intermittently. Serologically negative animals may result from a recent infection and shedding before immunoglobulin production is maximal, or infection with less invasive serovars. Animals that have been infected recently would, in all probability, eventually be detected serologically by an appropriate monitoring programme throughout the life of the flock/herd but there are often cost limitations to the application of effective monitoring programmes.

3. Newborn animals are immunologically immature and do not respond serologically to the somatic LPS antigen until 2–3 weeks of age. They do, however, produce a serological response to the flagellar protein antigens. Cattle may be unresponsive until about 10–12 weeks of age, and suckling pigs may fail to develop an immune response or have an antibody response that reflects maternal immunity. Differential responses involving different antibody classes (IgM, IgA, IgG) can be used in pigs to help differentiate recent infection from infection that occurred some time ago, but this is often not useful for herd testing where individuals are usually at different stages of infection. Most tests are based on IgG and raised antibody levels typically appear 1–3 weeks after infection and last 2–3 months.

   Chickens may also acquire anti-*Salmonella* antibodies passively via antibodies in egg yolk; this may indicate an infected or vaccinated parent flock. Mammals can acquire maternally derived antibodies via the colostrum.

4. Immunisation has been used for many years to aid control of certain *Salmonella* infections in farm animals, and if diagnostic serology is to be used, it is necessary to differentiate the vaccine response from that of actual infection. Many live vaccines given orally do not provoke a significant serum antibody response in the majority of animals, but there may be occasional exceptions that are difficult to interpret. Injectable killed vaccines used for control of *Salmonella* in chickens may produce a very prolonged antibody response, depending on the adjuvant used. No true DIVA (detection of infection in vaccinated animals) test is readily available for specific identification of the antibody response to vaccination, but in the case of *S*. Gallinarum (biovars Gallinarum and Pullorum), combined use of a LPS and flagella-based ELISA can help exclude the possibility of a false positive reaction in a flock vaccinated for *S*. Enteritidis.

5. The effect of antibiotic therapy on the serological response remains unclear. Some workers found reduced titres following therapy whereas others found no effect. Serology, however, may be a more useful diagnostic technique for salmonellosis than culture if antimicrobial therapy has been used.

6. Over 2600 different *Salmonella* serovars exist. Depending on the antigen and test used, serological cross-reactions between different serovars may occur, e.g. *S.* Typhimurium, *S.* Abortusequi, *S.* Gallinarum and *S.* Enteritidis. In some cases cross-reactions may also occur as a result of exposure to organisms other than *Salmonella*.

7. In poultry, egg yolk may be tested for immunoglobulins to *Salmonella,* and eggs may provide a method to screen flocks. This approach is used for monitoring commercial laying flocks in Denmark. In cattle, milk may be tested for anti-*Salmonella* antibodies to screen dairy herds.

8. The use of filter-paper discs for serum collection obviates the necessity to separate serum. The discs also provide long-term storage and reduce transport costs to the laboratory. The sensitivity of the test may be slightly reduced compared with tests carried out on fresh serum.

2.3. The whole blood test

The whole blood test provides a rapid test for fowl typhoid and Pullorum disease that can be used on the farm. The sensitivity of the whole blood test is low and in inexperienced hands false-positive and false-negative results may be recorded. For a detailed description of the whole blood test, see chapter 3.3.11.

2.4. Rapid slide agglutination test

Serum (0.02 ml) is mixed with polyvalent crystal-violet-stained antigen (0.02 ml). The tile is rocked gently for 2 minutes, after which the test is read. The test components are stored at 4°C and must have reached room temperature before being used.

Test sera should be free from contamination and haemolysis. It may be helpful to centrifuge serum samples that have been stored for any period of time.

If nonspecific false-positive reactions are suspected, positive/suspicious sera may be retested with the tube agglutination test or ELISA.

2.5. Serum agglutination test

The SAT is relatively insensitive, and many older animals may have low levels of agglutinins in their sera caused by enterobacteria other than *Salmonella*. Single samples are of little diagnostic value except for initial screening on a herd basis. Paired samples are needed as the minimum requirement for confirmation of active infection. The test is relatively simple; the antigens can be readily prepared and expensive equipment is not necessary. The SAT can be adapted to the microtitre format and can be used to determine somatic and flagellar titres. It is advisable to use standard sera and other confirmatory methods for quality control of the purity and immunogenicity of SAT antigen preparation(s) that are not dependant on sera produced from those antigens. This method has been used for identification of exposure to various *Salmonella* serovars, e.g. *S.* Typhimurium, *S.* Enteritidis, *S.* Dublin, *S. diarizonae* in turkeys, and *S.* Abortusequi.

2.5.1. Preparation of somatic antigen

i) Plate out the *Salmonella* culture from the appropriate stock culture onto a blood agar base (BAB) plate, or other suitable medium, for single colony growth. Incubate overnight at 37°C (±2°C).

ii) Select a smooth colony and carry out a slide agglutination test to ensure that the required somatic antigen is present.

iii) Using a sterile loop, inoculate a nutrient agar slope in a universal container from the selected colony.

iv) Incubate the culture for 8–12 hours at 34–38°C.

v) Using a Pasteur pipette, wash off the culture, preferably inside a safety cabinet, with approximately 2 ml of absolute alcohol, and transfer into a sterile universal container.

vi) Leave the antigen for 4–6 hours at room temperature to enable the alcohol to kill the bacteria and detach flagella.

vii) Spin the universal container in a bench-top centrifuge for 5 minutes at 1000 ***g***. Pour off the liquid and add enough phenol saline to make the antigen up to an opacity equivalent to Brown's tube No. 2 (approximately 10^8 colony-forming units/ml) or other appropriate standard.

viii) Carry out standard titration with known serum to ensure that the antigen is positive for the required factor.

ix) Store in a refrigerator at 4°C until required.

2.5.2. Preparation of flagellar antigens

i) Plate out the appropriate *Salmonella* stock culture on to a BAB plate, or other appropriate medium. Incubate overnight at 34–38°C.

ii) Passage in semi-solid agar (about 0.3%) in a Craigie's tube, or other suitable container, to induce optimum expression of the appropriate flagellar antigen. If the serovar is biphasic, H antiserum corresponding to the phase to be suppressed is added to the agar.

iii) Use slide agglutination to check that the *Salmonella* is in the required phase. If this is correct, inoculate a loop of culture into 20 ml of nutrient broth. Incubate for 12–18 hours at 34–38°C for optimum growth. (If the phase is incorrect, re-passage through semi-solid agar.)

iv) Pipette 250 µl of 40% formaldehyde into the antigen suspension (use gloves and work in a safety/fume cabinet), and leave overnight.

v) Test the antigen by SAT using the appropriate typing serum.

2.5.3. Test procedure

i) It is easiest to screen the sera at a dilution of 1/20; 0.25 ml of antigen is added to 0.25 ml of serum pre-diluted to 1/10 in normal saline.

ii) The tests are incubated in a water bath at 50°C for 24 hours in the case of somatic antigens and for 4 hours for the flagellar antigens. The dilution and time of incubation will vary depending on the antisera that are used.

iii) Sera that give a positive reaction are then diluted from 1/20 to 1/320 and retested with the appropriate antigen.

2.6. Enzyme-linked immunosorbent assays for *Salmonella* Enteritidis

Two main basic systems are available for detection of IgG (IgY) specific for *S.* Enteritidis*:* the indirect ELISA and the competitive 'sandwich type' ELISA (Barrow& Methner, 2013).

The indirect ELISA involves the use of a detecting antigen coated on to the wells of a microtitre plate. After the application of a blocking reagent to reduce nonspecific binding, test samples are applied to the wells. Specifically bound antibody in the sample is detected by an antibody/enzyme conjugate. A variety of antigens, including LPS, flagella, SEF14 fimbriae, outer membrane proteins and crude whole cell antigen preparations have been used.

The competitive sandwich ELISA employs a specific reagent – a monoclonal antibody (MAb) or polyclonal antibody – for coating antigen to wells. This is then followed by a pure or crude antigen preparation. Test samples are applied followed by conjugated antibody, which will not bind to the antigen if the sample contained specific antibodies. The assay time can be shortened by adding both test sample and conjugate together. MAbs have been prepared for LPS, flagella and SEF14 for *S.* Enteritidis.

There are advantages and disadvantages to both systems. The indirect assay is simpler and reagents are available for all *Salmonella* serovars of chickens, turkeys, ducks and mammalian hosts. The competitive ELISA can be applied to all animal species and in general shows higher specificity. However, reagents are not available commercially for most serovars. There are also some affinity problems and it may be less sensitive than the indirect assays. In the field, both systems have produced false-positive reactions and in some cases screening with an indirect LPS ELISA may be followed by confirmation with a flagellar competitive ELISA. This combination has been used to differentiate *S.* Enteritidis field infection from a vaccinal response to *S.* Gallinarum 9R vaccine, which lacks flagellar antigens.

Both types of assay may be used with serum, egg yolk or reconstituted dried blood eluted from filter paper discs. A mix-ELISA (or meat-juice ELISA), is used in Denmark and other countries to detect *Salmonella* infections in pigs (Van der Heijden, 2001). This ELISA contains the 'O' LPS antigens 1, 4, 5, 6, 7 and 12, from *S.* Typhimurium and *S.* Choleraesuis, which enables it to detect serologically up to 95% of the *Salmonella* serogroups found in pigs in most European countries. Group D antigens have also been added to some ELISA kits. Serum is used to screen breeding and multiplying herds, whereas for pigs in the abattoir, the assay is usually performed on the tissue fluid ('meat-juice') that is liberated when a frozen 10 g muscle sample is thawed. This approach is used in most countries, but serum collected from the major blood vessels of the viscera can provide more sensitive and specific results.

With some ELISAs differentiation can be made between infections produced by *Salmonella* serovars from different serogroups. Some cross-reaction can occur between groups B and D and other invasive serovars. There is, however, usually a greater antibody response when LPS from the homologous serovar is used in the ELISA. The optimal method for choosing a 'cut-off' absorbance value, above which sera are designated as having come from an *S.* Enteritidis-infected flock, without producing an unacceptable level of false-positive tests, has not yet been decided on and agreed upon internationally, and in the EU,

ELISA testing is less commonly used because of the extensive use of vaccination against *S.* Enteritidis and *S.* Typhimurium, particularly in laying hen and broiler breeder flocks.

ELISAs are readily adapted to automation and hence to large-scale testing programmes. A major problem is that expensive equipment is necessary and many of the reagents are also expensive. Several commercial ELISA kits for *S.* Enteritidis, *S.* Typhimurium and Group B/C mix-ELISAs are available. An ELISA kit for *S.* Abortusovis has recently been added to the WOAH Register of diagnostic kits[5]; there is a need for a similar validated test for *S.* Abortusequi.

Ideally these should be validated by international ring trials before adoption for surveillance purposes.

An example of a validated in-house ELISA is the one developed at the WOAH Reference Laboratory at APHA Weybridge[6].

The requirements are given below.

2.6.1. Equipment

PVC plates; appropriate pipettes and measuring cylinders; ultrawash microtest plate washer; ELISA plate reader; test filter of 405–410 nm and reference filter of 630 nm.

2.6.2. Antigen

i) Phenol-extracted *S.* Enteritidis LPS is available commercially. This is reconstituted in 1 ml deionised water and stored at –20°C in 100-µl aliquots in phosphate buffered saline (PBS), pH 7.2, at a concentration of 2.5 mg/ml. For use, the antigen should be thawed in coating buffer at the appropriate concentration.

ii) The LPS antigen can also be prepared by the technique of Westphal & Luderitz (1954) and standardised as to its carbohydrate concentration by the method of Gerhardt (1981), and adjusted to 1000 µg/ml.

2.6.3. Serum and conjugate diluent

Add bovine serum albumin (BSA) (2 g) and Tween 20 (0.05 ml) to PBS (100 ml). (Alternatively, powdered milk [1 g] can replace the BSA.) Store at 4°C and make fresh solutions every week.

i) Coating buffer

Add sodium carbonate (1.59 g) and sodium bicarbonate (2.93 g) to deionised water (1 litre) and adjust to pH 9.6. Store at 4°C and renew every 2 weeks.

ii) Substrate buffer

Make a 10% (v/v) solution of diethanolamine in deionised water. The diethanolamine should be pre-warmed to 37°C before dispensing, and the pH of the solution should be adjusted to pH 9.8 with 1 M hydrochloric acid. Store at 4°C and renew every 2 weeks.

iii) Enzyme conjugate

Goat anti-chicken immunoglobulin conjugated to alkaline phosphatase or other species anti-chicken globulin. Store at 4°C diluted in diluent at the appropriate concentration and renew every week.

iv) Enzyme substrate

Dissolve one tablet of *p*-nitrophenyl phosphate disodium (5 mg) in substrate buffer (5 ml) no earlier than 30 minutes before dispensing, and store in the dark.

5 https://www.woah.org/en/what-we-offer/veterinary-products/diagnostic-kits/the-register-of-diagnostic-kits/
6 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

2.7. Standards

i) Positive control antiserum prepared by intramuscular inoculation of four 1-week-old specific pathogen free (SPF) chickens with an inoculum containing 10^6 *S.* Enteritidis. The serum is subsequently obtained 3–4 weeks later when antibody titres are maximal.

ii) Negative control serum A from four 1-week-old SPF birds.

iii) Negative control serum B from 58 1-week-old breeders known to be free from *Salmonella* infections. Pool the sera and store in 100 µl volumes at –20°C.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Many inactivated vaccines are used against salmonellosis caused by different serovars in various animal species, including a combined *S.* Enteritidis and *S.* Typhimurium vaccine for use in poultry, and a more recent vaccine that also includes *S.* Infantis. Inactivation is usually achieved by either heating or the use of formalin and an adjuvant, such as alhydrogel or mineral oil. Live vaccines have also been used in a number of countries; these include the semi-rough strains, such as 9R for fowl typhoid and HWS51 for *S.* Dublin infections (Mastroeni *et al*., 2001). Other attenuated vaccines include auxotrophic and 'metabolic drift' mutants, which are used to prevent *Salmonella* infections in farm animals in Europe, particularly for *S.* Enteritidis and *S.* Typhimurium in poultry and *S.* Typhimurium in pigs. Mutant vaccines attenuated rationally by molecular biological gene-deletion techniques have been developed for poultry and other species; these include aroA mutants and strains with mutations in the genes encoding adenylate cyclase (cya) and the cyclic adenosine monophosphate receptor protein (crp) (Redweik *et al*., 2020). Attenuation of live vaccines is essential to limit intestinal replication and persistence in the animals and environment, ideally without influencing the immunogenicity, but such attenuation is unlikely to have no impact on the vaccinal response (Redweik *et al*., 2020). Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements. Most vaccines are produced as highly industrial commercial processes and are regulated by national veterinary medicines regulatory approval authorities. Smaller quantities of emergency herd vaccines or autogenous vaccines are produced by laboratories and vaccine manufacturers, but each production has to be specifically approved.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

For killed or live vaccines, the bacterial strain should be an organism as closely related to currently circulating field strains as possible. It should be carefully chosen from cases of severe clinical disease, and virulence and antigen production should be assessed. It is best to evaluate a panel of potential strains in this way before testing the final selection. The final vaccinal strain should be identified by historical records and characterised by stable phenotypic or genetic markers. Live vaccinal strains should be marked by stable characters allowing distinction from wild strains. Markers, such as resistance to antimicrobials, for example rifampicin, or auxotrophism, may be used. Attenuation of virulence should be stable and preferably obtained by two independent defined mutations. The stability of live vaccine strains can be verified by regular checks using a sensitive molecular fingerprinting technique, preferably whole genome sequencing.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

i) Sterility and purity

The vaccine strain must be checked as follows:

a) Staining of a smear of bacterial suspension on a glass slide using the Gram stain.

b) Homogeneity of culture on non-selective media.

c) Metabolic requirements as indicated by biochemical tests.

d) Detection of molecular markers, serotype and phage type, if applicable.

e) Agglutination with specific antiserum.

f) The vaccine culture and any adjuvants, preservatives or other materials must be microbiologically sterile and non-toxic at the concentrations used.

ii) Safety

The LD_{50} (50% lethal dose) or ID_{50} (50% infectious dose) may be determined in mice or preferably signs of more mild adverse reactions should be checked in the target species. Ten times the field dose of live vaccine or twice the dose for killed vaccines must be given to the target species at the recommended age and by the recommended route. The animals are observed for absence of adverse reactions. Stability and non-reversion to virulence after serial passages in susceptible species should be shown for live vaccines. It is also necessary to consider repeat vaccination. Live vaccine should be shown not to persist for long in vaccinated animals and in the environment where they are farmed, or be transmitted to milk or eggs that may be consumed, and the method of application should not present a hazard to operators. Live vaccines should not be used in commercial laying flocks during the laying period unless eggs are processed by heat treatment.

iii) Efficacy

Laboratory experiments and field trials should be used to show that the vaccine is effective. The laboratory experiments consist of vaccination–challenge tests in the target species at the recommended dose and age. The efficacy data can also be used as the basis for a batch potency test. Field trials are more difficult to undertake with respect to testing efficacy because of difficulties with standardising the challenge and providing appropriate controls.

iv) Environmental aspects

Live vaccine strains should be tested for their ability to persist in the environment and infect non-target species such as rodents and wild birds that are likely to be exposed. Prolonged survival of some live vaccines in faeces and litter may present an unacceptable environmental hazard when the material is removed from the animal houses.

2.2. Method of manufacture

2.2.1. Procedure

The seed culture is propagated and maintained using suitable media for growth of *Salmonella*. The media used should not contain serum or animal tissues (unless permitted by national regulations). Culture may be on solid medium, in Roux flasks, or in liquid medium, in which case large-scale fermentation equipment may be used. Iron limitation or low temperature incubation on minimal media may enhance LPS antigen production by the vaccine strain.

Vaccine must be produced in suitable clean rooms to which only approved personnel have access. Care must be taken to avoid cross-contamination between areas where live organisms are processed and other areas. Contamination from operators or the environment must be avoided and vaccine preparation should take place in a separate area from diagnostic culture work. Operators must not work with vaccine whilst ill and must not be subject to immunosuppressive conditions or medications. Personnel must be provided with suitable protective clothing in production areas and in animal rooms.

Seed-lot cultures are prepared from the primary seed-lot, and the number of passages is dependent on the validation of the process. The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture and incubation on a shaker at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation. Alternatively, the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In the case of live vaccines, the suspension is diluted in PBS, pH 7.0, and may be freeze-dried.

The time of inactivation of dead vaccines should be at least 33% more than that taken to reduce the viable number to an undetectable level. The inactivation process must be applied to the whole volume of the vaccine cell harvest and confirmed by a sensitive culture method.

Preservatives, excipient for lyophilisation, stabiliser for multidose containers or other substances added to or combined with a vaccine preparation must have no deleterious effect on the immunising potency of the product.

2.2.2. Requirements for substrates and media

All chemicals and growth media used should be guaranteed to be fit for purpose and checked by the use of suitable controls.

2.2.3. In-process controls

The following points require attention:

i) Visual control of the suspension, homogeneity by Gram stain, culture on non-selective medium.

ii) Slide agglutination with specific antisera.

iii) Titration of bacteria by turbidimetry or plate count.

iv) Test of effective inactivation (killed vaccine) by plating on non-selective medium or use of a medium that gives optimum chance of recovery e.g. production medium with neutralisation of the inactivating compound.

v) Titration of viable bacteria (living vaccine) before and after lyophilisation.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use are found in chapter 1.1.9 of this *Terrestrial Manual*.

ii) Safety

A laboratory test that has previously shown a correlation with safety in the target species may be used to determine the absence of deleterious effects on vaccinated animals. Each batch should be tested in the target species at the recommended age and route, using at least twice the field dose for killed vaccines and ten times the dose for live vaccines. Observations are made on any adverse effects on the demeanour and health of the vaccinated animals and an assessment is made of tissue reactions at the injection site.

iii) Batch potency

Potency is tested using vaccination–challenge assay in mice or other species, including (if practicable) the target species and immunological response in target species. Many *Salmonella* vaccines are intended for use in poultry so these should be used for potency and safety tests.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

Certain killed vaccines may occasionally cause abortion in pregnant animals because of their LPS content, and likewise live vaccines should be used with caution in pregnant animals. It is often necessary, however, to vaccinate pregnant animals to provide maternal immunity for their offspring. It may be useful to include endotoxin assay in the safety test programme so that the levels can be compared with those shown to be safe in the double-dose tests. Vaccines may also cause swelling at the site of injection, particularly if an oil-emulsion adjuvant is used.

i) Target and non-target animal safety

Killed vaccines are assessed in a double-dose test, and live vaccines are assessed in a test using ten times the dose, ideally in the target species. Live vaccines should be proven to be harmless in relevant non-target species that could be exposed to vaccine excreted by vaccinated animals.

ii) Reversion to virulence for attenuated/live vaccines

Live vaccines shall be shown in replication tests in target species to not revert to virulent strains during a suitably large number of replications. Mutations, especially undefined mutations, should be shown to be stable and checks on stability can be made by sensitive molecular fingerprinting methods or sequencing. Although the risk is small it is wise not to use live vaccines in a country where the organism in the vaccine has been eradicated.

iii) Environmental consideration

Live vaccines should not be able to replicate in the environment or persist for more than a short period.

2.3.2. Efficacy requirements

i) For animal production

The duration of immunity is likely to vary considerably between products, vaccination regimes and individual vaccinated animals. Immunity to *Salmonella* is normally serovar or serogroup specific. Consultation among colleagues suggests that most killed vaccines will provide some protection for 6 months, while some live vaccines given by injection may elicit stronger immunity, which may persist for 1 year or more. It should be remembered, however, that a strong challenge such as that associated with continuously occupied farms or infected rodents may overwhelm vaccinal immunity and commercial live vaccines may be attenuated to reduce environmental survival in a way that reduces the immune response. There may also be problems with ensuring effective oral administration with live vaccines or accuracy of injection with killed and live injectable vaccines. The *Salmonella* vaccines are intended to limit the extent of clinical disease in the case of ruminants, pigs and *S.* Gallinarum in poultry. If possible, the potency test should relate to the efficacy of the vaccine in the target species, and suitable criteria should be applied for passing batches. It may be possible to assess killed vaccines by the O-H antibody response produced, although it should be remembered that serum antibodies are only part of the host's protective mechanism against *Salmonella.* Alternatively, the potency of the vaccine may be assessed by its effect on challenged vaccinated animals compared quantitatively and statistically with unvaccinated controls.

ii) For control and eradication

Vaccines for *Salmonella* are not capable of eradicating infection from herds or flocks but can increase the threshold for infection, reduce the level of shedding of the organism and reduce vertical transmission in poultry that results in contamination of hatching or table eggs. Vaccination is therefore an aid to other eradication and control measures such as culling, all in-all out production, biosecurity and farm hygiene.

iii) *Stability*

Information is lacking on the stability of killed vaccines. Stability is affected by storage conditions and by the presence of contaminating microorganisms growing in the product. Chemicals with antimicrobial activity, such as thiomersal, phenol or crystal violet are often included as preservatives in killed bacterial vaccines. The stability is assessed by potency tests repeated at appropriate time intervals. The stability of live vaccines can be assessed by performing counts of the number of viable organisms repeated at appropriate time intervals, and genotyping tests to identify genetic changes during fermentation production. It is recommended that live vaccines that contain *Salmonella* serovars that are not endemic in a particular region should not be used for control of other serovars .

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NB: There are WOAH Reference Laboratories for salmonellosis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for salmonellosis

NB: FIRST ADOPTED IN 1991 AS SALMONELLOSIS (*S. ABORTUS OVIS* AND *S. EQUI*) AND SALMONELLOSIS (*S. TYPHIMURIUM* AND *S. ENTERITIDIS*). MOST RECENT UPDATES ADOPTED IN 2022.

CHAPTER 3.10.8.

TOXOPLASMOSIS

SUMMARY

Description of disease: *Toxoplasmosis is a zoonotic infection of animals caused by the protozoan parasite* Toxoplasma gondii*. It has the capacity to infect all warm-blooded animals and, while infection does not cause clinical illness in the majority of animal species, in some it causes acute life-threatening disease and in others, particularly sheep and goats, it may manifest itself as a disease of pregnancy by multiplying in the placenta and fetus. In these latter animals it can result in the abortion or the birth of weak lambs/kids, which may be accompanied by a mummified fetus. Characteristically, in these cases, the placental intercotyledonary membranes are normal, but white foci of necrosis, approximately 1–3 mm in diameter, may be visible in the cotyledons. Microscopically, these foci appear as areas of coagulative necrosis that are relatively free of inflammation. Inflammation, when present, is nonsuppurative.* Toxoplasma *tachyzoites are seen only rarely in association with these foci, usually at the periphery of the lesion. Examination of the brain may reveal focal microgliosis. The lesions often have a small central focus of necrosis that might be mineralised. Focal leukomalacia in cerebral white matter, due to anoxia arising from placental pathology, is often present. Focal microgliosis is more specific, as leukomalacia reflects placental damage but may occur in other pathological conditions where the placenta is compromised, including, though rarely, ovine chlamydiosis. Infection in pigs may cause severe fetal losses in pregnant sows, but more usually is mild and unnoticed. Acute fatal infections affect New World monkeys, marsupials and certain other animals.*

Identification of the agent: Toxoplasma gondii *is an obligate intracellular parasite that has a sexual cycle in Felidae and a two-stage asexual cycle in all warm-blooded animals. It predominantly comprises three molecular lineages (I, II and III). In the acute phase of infection, tachyzoites multiply in cells to cause varying degrees of tissue destruction and, in fatal cases, tachyzoites may be demonstrated in ascitic fluid or in lung impression smears. With the onset of an immune response, tachyzoites are transformed into bradyzoites that multiply slowly in cells to produce tissue cysts. In aborting sheep, goats and pigs,* T. gondii *is often difficult to find in tissue sections, but is more likely to be seen in sections of brain and placenta. Its identity can be confirmed by immunohistochemistry, while the polymerase chain reaction may be used to identify parasite DNA in tissues. Isolation of* T. gondii *from samples is expensive and slow but, if required, is best achieved by inoculation of mice with tissue homogenate derived from fetal brain or placenta. The sexual life-cycle of the parasite takes place exclusively in epithelial cells of the feline intestine and can result in the excretion of large numbers of oocysts in the faeces. Oocysts may remain viable in the environment for many months.*

Serological tests: *The dye test is the longest established serological method, and in many ways represents the 'gold standard', at least in humans. The dye test uses live, virulent* Toxoplasma *tachyzoites, a complement-like 'accessory-factor' and test serum. When specific antibody acts on the tachyzoites, the latter do not stain uniformly with alkaline methylene blue. The test has proven unreliable in some species. In addition, as live* Toxoplasma *is used, the test carries a potential risk of human infection as well as being expensive to conduct. The dye test is used in only a few laboratories in the world. The indirect fluorescent antibody (IFA) test is safer, gives titres comparable with the dye test and can be used to differentiate IgM and IgG antibodies. The direct agglutination test and the latex agglutination test are both relatively rapid and neither requires complex laboratory facilities. The enzyme-linked immunosorbent assay requires more sophisticated laboratory equipment but can process large numbers of samples and does not rely on human interpretation for the result.*

Requirements for vaccines: *A vaccine composed of live* T. gondii *tachyzoites is available commercially for use in sheep in certain European countries and New Zealand. The vaccine is supplied as a concentrated suspension of tachyzoites with an approved diluent and delivery system. The vaccine must be maintained and handled strictly according to the manufacturers' instructions as it has a very short shelf life.*

A. INTRODUCTION

Toxoplasma gondii is a zoonotic, obligate intracellular protozoan parasite that has the capacity to infect all warm-blooded animals including birds. While infection does not cause clinical illness in the majority of animal species, in some it causes acute life-threatening disease and in others, particularly sheep and goats, but also pigs, it manifests itself as a disease of pregnancy by multiplying in the placenta and fetus. Acute, potentially fatal, infections are recorded in New World monkeys (Cunningham *et al*., 1992), marsupials (Canfield *et al*., 1990) and certain other animals (see below). Worldwide reports of clinical and subclinical infections in domestic and wild animals were summarised in two books (Dubey, 2010; Dubey & Beattie, 1988). In acute cases clinical signs may include lymphadenopathy, hepatomegaly, interstitial pneumonia and neural signs. At post-mortem examination lymph nodes, spleen and liver may be enlarged and the latter may have pale foci. In sheep, goats and pigs a primary infection established during pregnancy may result in apparent infertility or in stillbirths and abortion, according to the stage of pregnancy at which infection was initiated. In a typical case of abortion, a ewe or doe infected in mid-gestation produces a stillborn lamb/kid a few days earlier than the predicted end of pregnancy. The aborted fetus is often accompanied by either a weak sibling or a 'mummified' fetus (Buxton, 2000). The ewe/doe remains clinically normal. In such cases, placental cotyledons are typically speckled with white foci around 2–3 mm in diameter while the intercotyledonary membranes appear normal. Infection in early pregnancy, when the fetus has only a rudimentary immune system, results in fetal death and resorption. In this case the mother may present as barren, which in turn can mimic a flock/herd infertility problem. Mothers that become infected in late pregnancy would be expected to produce infected but clinically normal offspring. Following an infection, either during or outside of pregnancy, the parasite would not be expected to cause abortion in any subsequent pregnancy. While recent research has questioned this conclusion (Duncanson *et al*., 2001; Williams *et al*., 2005) the majority of current thinking tends towards the view that recrudescence of a persistent infection during pregnancy leading to repeat abortions is not normally a significant occurrence (Dubey, 2010; Rodger *et al*., 2006). Infection in pigs may cause severe fetal losses in pregnant sows but under modern intensive farming conditions, when contamination of the housing and feed by *T. gondii* oocysts is minimal or absent, infection would generally be expected to be at a very low incidence and cause only mild or unnoticed signs of infection (Lind & Buxton, 2000). However when pigs are maintained outdoors under extensive systems, they are much more likely to encounter oocysts, and so infection would be expected to be more common (Dubey, 2010; Kijlstra *et al*., 2004).

Toxoplasma gondii is an obligate intracellular parasite that has a two-stage asexual cycle in warm-blooded animals and a sexual cycle in felidae. The parasite comprises three genetic lineages (I, II and III) in the main, with type II and III being associated with disease in animals while type II is the predominant form identified in human disease (Howe & Sibley, 1995; Khan *et al*., 2006). In the asexual cycle, the two developmental stages are the rapidly multiplying tachyzoite and the slowly multiplying bradyzoite. In acute infection, tachyzoites actively penetrate host cells where they multiply causing the cell to rupture and release organisms locally and into the bloodstream. As the host develops immunity, the parasite retains its overall size and shape but transforms into the bradyzoite stage and multiplies more slowly within tissue cysts to establish a persistent infection. These microscopic tissue cysts are present most frequently in brain and skeletal muscle and represent the quiescent stage of the parasite within the host. Viable tissue cysts within muscle (meat) are a significant source of human infection. In animals that succumb to acute infection tachyzoites may be demonstrated in ascitic fluid or in lung impression smears as well as in tissue sections of the liver and other affected organs.

The sexual cycle occurs in enteroepithelial cells of the feline definitive host and results in the production of *Toxoplasma* oocysts. Following a primary infection of a cat, oocysts may be shed in the faeces for several days. The oocysts sporulate in the environment over the next 1–5 days (depending on aeration, humidity and temperature), at which time they become infective. They are very resistant to environmental conditions and may remain infective for a year or more. Sporulated oocysts are 11 × 13 μm in diameter and each contains four sporozoites in each of two sporocysts (Dubey & Beattie, 1988). When a susceptible animal ingests sporulated oocysts the sporozoites are released to penetrate the intestinal lining, become tachyzoites and establish an infection.

In sheep, goats, pigs, horses and humans, tissue cysts may remain for the rest of the life of the individual (Dubey & Beattie, 1988). *Toxoplasma* does not usually cause clinical illness in cattle, camelids or deer but, as noted, can cause fatal disease in New World monkeys, marsupials and certain other animals including hares (*Lepus europaeus; L. timidus*) (Gustafsson & Uggla, 1994), the Pallas cat (Brown *et al*., 2005), the arctic fox (Sørensen *et al*., 2005), some birds and marine mammals (Dubey, 2010). It is suggested that these and other similarly affected animals have had minimal exposure to *T. gondii* in their natural habitat through the ages, making them particularly vulnerable to the parasite.

Abortion in sheep and goats due to *T. gondii* must be differentiated from that caused by other infectious agents, including infections with *Chlamydophila abortus* (see Chapter 3.8.5 *Enzootic abortion of ewes*), *Coxiella burnetii* (see Chapter 3.1.17 *Q fever*), *Brucella melitensis* (see Chapter 3.1.4 *Brucellosis* [Brucella abortus, B. melitensis *and* B. suis]), *Campylobacter fetus* (see Chapter 3.4.4 *Bovine genital campylobacteriosis*), *Salmonella* spp. (see Chapter 3.10.7 *Salmonellosis*), border disease (see Chapter 3.8.1 *Border disease*), and the viruses that cause bluetongue, Wesselsbron's disease and Akabane disease. In pigs, *Brucella suis* (see chapter 3.1.4) may also cause fetal death, mummification and abortion.

1. Human health risks

Toxoplasma gondii readily infects human beings and while infection is relatively common (approximately 30% of the population depending on age and environment), clinical illness is relatively uncommon. Those particularly at risk of developing clinical illness include pregnant women, as the parasite can pose a serious threat to the unborn child if the mother becomes infected for the first time while pregnant, and individuals who are immunosuppressed, such as tissue transplant patients, AIDS patients, patients with certain types of cancer and those undergoing certain forms of cancer therapy. These individuals are at risk of developing acute lethal infection if left untreated. The very young and very old may also be more susceptible. On occasions, people with no apparent immune deficiency may develop an illness characterised by general malaise, fever and lymphadenopathy. The most likely sources of human infection are ingestion of raw or lightly cooked meat containing live *T. gondii* tissue cysts, ingestion of raw or lightly cooked vegetables contaminated with oocysts or exposure to oocysts derived from cat faeces, such as may be encountered in gardens and children's sand pits. Toxoplasmosis is now also recognised to be a water-borne zoonosis (Dubey, 2010). This method of transmission occurs where water treatment is ineffective or non-existent and there is a sizeable local felid population that contaminates surface water with oocysts (Bowie *et al.,* 1997; Dubey, 2004). Linked to this there is now also an appreciation that marine mammals are becoming infected by waters from contaminated land and from untreated urban sewage.

All laboratory manipulations with live organisms should be handled with appropriate measures determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.*

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of toxoplasmosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
PCR	–	–	–	++	–	–
Histopathology	–	–	–	+	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
IFAT	–	–	–	++	++	–
ELISA	–	–	–	++	++	–
DAT/MAT	–	–	–	++	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test;
ELISA = enzyme-linked immunosorbent assay; DAT = direct agglutination test; MAT = modified agglutination test.

1. Identification of the agent

1.1. Histopathology

In animals that die with acute toxoplasmosis, focal mononuclear inflammation with or without focal necrosis may be seen in a number of tissues, including the liver, heart and lungs. The latter may be oedematous. Lymph nodes may have undergone expansion and there may or may not be focal necrosis with or without haemorrhage. Typically *Toxoplasma* tachyzoites may be demonstrable in association with necrosis and inflammation.

In cases of abortion and stillbirth in sheep and goats, affected placental cotyledons typically contain large foci of coagulative necrosis that may have become mineralised with time. Any associated inflammation is characteristically slight and nonsuppurative. Well preserved samples of placental cotyledons may show moderate oedema of the mesenchyme of the fetal villi with a diffuse hypercellularity due to the presence of large mononuclear cells. Sometimes small numbers of intracellular and extracellular toxoplasms are visible, usually on the periphery of a necrotic area or in a villus that is in the early stages of infection. The *Toxoplasma* tachyzoites appear ovoid, 2–6 μm long, with nuclei that are moderately basophilic and located centrally or towards the posterior end.

In the fetal brain primary and secondary lesions may develop. Microglial foci, typically with a necrotic and sometimes mineralised centre and often associated with a mild focal lymphoid meningitis, represent a fetal immune response following direct damage by local parasite multiplication. *Toxoplasma* tissue cysts are only rarely found, usually at the periphery of these lesions. Focal leukomalacia is also common and is considered to be due to fetal anoxia in late gestation caused by advanced lesions in the placentome preventing sufficient oxygen transfer from mother to fetus. Such foci occur most commonly in the cerebral white matter cores, but sometimes also in the cerebellar white matter. Focal leukomalacia on its own suggests placental disease or acute insufficiency but the two types of neuropathological change seen together are characteristic of *Toxoplasma* infection. Confirmation of the identity of *T. gondii*-like structures in tissue sections from such cases, as well as from instances of acute toxoplasmosis, may be achieved by immunohistochemistry that labels intact *T. gondii* or antigenic debris. The method is both convenient and sensitive and is used with fixed tissues (including archive tissues) that may also exhibit a degree of decomposition, where isolation would not be appropriate or possible. The ABC indirect immunoperoxidase method and the peroxidase–antiperoxidase (PAP) technique (Uggla *et al*., 1987) are equally good, and details of the procedure can be found in Dubey (2010).

1.2. Nucleic acid recognition methods

Several polymerase chain reaction (PCR)-based assays have been developed for the detection of nucleic acids from *T. gondii* (Table 1). The main target regions are the B1 repetitive sequence, and the

300-copy 529-bp element the P30 (SAG1) gene and 18S ribosomal RNA (rRNA) (Burg *et al*., 1989; Dubey, 2010; Ellis, 1998; Savva *et al*., 1990). The sensitivity of the PCR is dependent on the copy number of the target sequence (P30: 1 copy; B1: 35 copies; rRNA: 110 repeat units). Customised synthetic DNA oligonucleotides are commercially available (e.g. www.sigma-genosys.co.uk). Recently, the method for amplification of the B1 repetitive sequence has been used to analyse the lens aspirates of congenitally infected human cataract patients (Mahalakshima *et al*., 2007) and was found to be more sensitive than the conventional method used (enzyme-linked immunosorbent assay [ELISA]). However, although the PCR is extremely sensitive, care should be taken if it is the only test available, as in many situations a more reliable diagnosis will be gained if it is used in combination with other diagnostic data.

Recently, a real-time PCR has been developed to allow simultaneous quantification and amplification of DNA. It is very similar to existing PCR methods and can be carried out on 96-well microtitre plates. After each round of amplification, fluorogenic dyes intercalate with the double-stranded DNA and the results, shown on an amplification plot, allow quantification of the parasite DNA in the sample. Real-time PCR has been used to amplify and quantify DNA from the *T. gondii* B1 gene (Costa *et al*., 2000; Lin *et al*., 2000). This quantification of parasite DNA can be used to determine the number of parasites in tissues and fluids, such as the amniotic fluid of patients suspected of being congenitally infected with *T. gondii*. (Nagy *et al*., 2007). The real-time PCR is a highly sensitive and specific method, however it is expensive and requires specialised detection systems and therefore may only be cost-effective in laboratories where analysis of large numbers of samples is carried out.

The following method is a nested form of the PCR, amplifying the B1 repetitive sequence of DNA (Wastling *et al*., 1993). Parasite DNA can be extracted and purified from several tissues, including placenta, the central nervous system, heart and skeletal muscle.

Contaminating red blood cells in tissues are removed by washing in 10 mM Tris/NH_4Cl lysis buffer, pH 7.6, followed by centrifugation at 2000 ***g*** for 15 minutes. DNA is then extracted from the resultant pellet and resuspended in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ containing proteinase K 100 µg/ml and 0.5% Tween 20.

Samples are incubated at 55°C for at least 1 hour, then the proteinase K is inactivated by boiling. The PCR procedure is performed in 50 µl volumes. Amplification of the B1 gene is performed by modifying the procedure described in ref. 1. The reaction mixture contains 10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 40 mM KCl, 0.01% gelatin, 0.1 mM dNTPs, 0.2 µM of each primer (oligonucleotide primers are those described in Bowie *et al*., 1997), two sense primers P1 and P2 and two antisense primers P3 and P4) and 2.5 units of Taq polymerase.

Primary amplification is performed with primers 1 and 4 to give a 193 bp product over 25 cycles of 93°C for 1 minute, 50°C for 1.5 minutes and 72°C for 3 minutes. The amplified product is then diluted 1/20 in distilled water to reduce amplification of non-specific products.

Secondary amplification using nested primers 2 and 3 and the same reaction conditions, is carried out over 15 cycles to give a 94 bp product. The final product is then visualised on 1% agarose gels. Southern blotting, using a labelled probe, can be used to confirm the identity of the B1 PCR products and to distinguish them from non-specific products.

1.3. Oocyst detection in drinking water

Toxoplasma gondii oocysts have been detected in drinking water using the method for the detection of *Cryptosporidium* oocysts (Issac-Renton *et al*., 1998). The method relies on the collection of a large-volume sample of water and passing it through a cartridge filter. Identification of *Toxoplasma* oocysts was by means of inoculation of rodents. However, unlike *Cryptosporidium,* the number of *T. gondii* oocysts in water is very low.

From a public health viewpoint, it is necessary to distinguish *T. gondii* oocysts from oocysts of a related coccidium, *Hammondia hammondi,* also present in cat faeces. *Hammondia hammondi* is non-pathogenic. Bioassays, currently the only definitive way to detect viable oocysts of these parasites, are expensive and only a few laboratories in the world have the facilities to do them. Although DNA detection is considered highly specific, cross reactivity has been observed between *T. gondii* and *H. hammondi*. Recently, *H. hammondi*-specific primers have been published (Walzer *et al.,* 2014).

Detection of DNA from *T. gondii* oocysts may present additional problems because of inhibitors in faecal matter, and difficulty of releasing DNA from the oocysts. A method is detailed below for preparation of oocysts and extraction of DNA.

1.3.1. Reagents

i) PBS: 300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM NaH_2PO_4.

ii) Sodium hypochlorite, aqueous solution, ≥ 4% as active chlorine.

iii) OOC-lysis buffer (pH 9.5): 600 mM EDTA (ethylene diamine tetra-acetic acid), 1.3% (v/v) N-lauroylsarcosine, 2 mg/ml proteinase K.

iv) OOC-CTAB buffer: 2% (w/v) cetyl-trimethyl ammonium bromide, 1.4 M NaCl, 0.2 % (v/v) mercapthoethanol, 20 mM EDTA, 100 mM tris(hydroxymethyl)aminomethane.

v) phenol/chloroform/isoamyl alcohol (25/24/1).

vi) 4 M NaCl.

vii) 96% (v/v) ethanol.I.70% (v/v) ethanol.

1.3.2. Procedure

i) Wash oocysts four times by centrifugation (1100 ***g*** for 7 minutes without the use of the brake) in 15 ml PBS in a 15 ml centrifugation tube.

ii) Incubate oocyst pellet and the remaining contaminants (up to 0.5 ml) in 2 ml 5.75 % sodium hypochlorite (30 minutes at 37°C).

iii) Add double-distilled H_2O up to 15 ml.

iv) Centrifuge supernatant in a 15 ml tube (1100 ***g*** for 7 minutes without the use of the brake) and mix the pellet with PBS. Wash the pellet three times with PBS (1100 ***g*** for 7 minutes without brake).

v) After a last centrifugation, re-suspend the pellet in 1 ml PBS, transfer into a 1.5 ml reaction tube and spin down (1100 ***g*** for 7 minutes without brake).

vi) Carefully remove as much of the supernatant as possible and apply three freeze–thaw cycles (10 minutes at –20°C followed by 2 minutes at room temperature) to the pellet.

vii) Re-suspend the pellet in 100 µl OOC lysis buffer (45 minutes, 65°C).

viii) Add 400 µl OOC-CTAB buffer (60 minutes, 60°C).

ix) Mix with 500 µl phenol/chloroform/isoamylalcohol (25/24/1) by inverting 50 times. Centrifuge for 7 minutes at 13,000 ***g***.

x) Transfer the supernatant to a fresh tube and mix again with 500 µl phenol/chloroform/isoamylalcohol (25/24/1) by inverting 50 times. Centrifuge for 7 minutes at 13,000 ***g***.

xi) Transfer the supernatant to a fresh tube and add 0.04 volumes of 4 M NaCl and 2–3 volumes of –20°C cold 96% (v/v) ethanol to precipitate DNA (keep at least 20–30 minutes at –20°C).

xii) Centrifuge for 15 minutes at 13,000 ***g***. Decant the supernatant.

xiii) Wash the pellet using 70% (v/v) ethanol and centrifuge for 15 minutes at 13,000 ***g***.

xiv) Discard the ethanol solution and air dry the pellet.

xv) Resolve DNA in double-distilled water for at least 12 hours at 4°C.

xvi) Use 2.5–10 µl aliquots for PCR (see Section 1.2 above).

2. Serological tests

There are several serological tests available for the detection of *T. gondii* antibodies (Table 1). In one type of test the observer judges the given colour of tachyzoites under a microscope, such as with the dye test (DT) and IFA

test. Another depends on the principle of agglutination of *Toxoplasma* tachyzoites, red blood cells or latex particles, as with the direct agglutination test (DAT and modified agglutination test [MAT]) and indirect haemagglutination test (IHA) and latex agglutination (LA) test, respectively. With the ELISA, the degree of colour change defines the quantity of specific antibody in a given solution. The DT, IFA test, DAT and ELISA are outlined below and the IFA test is given in more detail.

The DT (Sabin & Feldman, 1948) is the so-called 'gold standard' serological test for *Toxoplasma* antibody in humans. Live *Toxoplasma* tachyzoites are incubated with a complement-like accessory factor and the test serum at 37°C for 1 hour before methylene blue is added. Specific antibody induces membrane permeability in the parasite so that the cytoplasm is able to leak out and the tachyzoite does not incorporate the dye and so appears colourless. Tachyzoites not exposed to specific antibody (i.e. a negative serum sample) take up the dye and appear blue. The DT is both specific and sensitive in humans, but may be unreliable in other species. In addition, it is potentially hazardous as live parasite is used. It is expensive and requires a high degree of technical expertise. It should be noted that on animal welfare grounds, tachyzoites should be grown in tissue culture rather than in mouse peritoneum whenever possible.

The IFA test (Munday & Corbould, 1971) is a simple and widely used method. Whole, killed *Toxoplasma* tachyzoites are incubated with diluted test serum, the appropriate fluorescent antispecies serum is added, and the result is then viewed with a fluorescence microscope. Fluorescent-labelled antibodies are available commercially for a variety of animal species, the method is relatively inexpensive and kits are commercially available. However, the method requires a fluorescence microscope and the results are read by eye, so individual variation may occur. It may be difficult to find some species-specific conjugates and there is a risk of possible cross-reactivity with rheumatoid factor and anti-nuclear antibodies.

The DAT (Desmonts & Remington, 1980) is both sensitive and specific. Formalinised *Toxoplasma* tachyzoites are added to U-shaped well microtitre plates and dilutions of test sera are then applied. Positive samples will produce agglutination that can be graded, while negative samples will produce a 'button' of precipitated tachyzoites at the bottom of the well. The test is simple and easy to perform although relatively large amounts of antigen are required. Kits are commercially available. It is important to treat sera with mercaptoethanol to avoid false positives due to non-specific IgM. The procedure was modified by Dubey & Desmonts (1987), who called it the modified agglutination test (MAT). The MAT has been used extensively for the detection of *T. gondii* animals in sera of all species of animals and the procedure is detailed below. The MAT may give false negative results in the early stages of infection, or when performed on canine sera. A commercially available latex agglutination test (LAT) is also available but this test is relatively insensitive compared with MAT or IFA.

The original ELISA (Voller *et al*., 1976) uses a soluble antigen preparation made from *Toxoplasma* RH strain tachyzoites (as described below) and layered into wells in a microtitre plate. Test sera (e.g. ovine in origin) are added, followed by an anti-species enzyme-labelled conjugate such as horseradish peroxidase-labelled anti-ovine-IgG. Any attached conjugate causes a colour change in the substrate that is directly related to the amount of bound antibody, and which can be read with a spectrophotometer at the absorbance specific to the substrate used. The assay is simple, can readily test a large number of samples and is easy to perform with the chosen anti-species conjugate. Defined anti-species conjugates, substrates and whole kits are commercially available. However, the assay does require a spectrophotometer. The ELISA is well suited to laboratories required to analyse large numbers of samples.

Recently, a kinetics ELISA (KELA) has been developed (Werre *et al*., 2002). The KELA system measures the rate of reaction between bound enzyme and the substrate solution that leads to development of colour. Three optical densities (OD) are read at 45-second intervals (using the KELA data management program) and the results are reported in terms of slopes. The correlation between the ELISA and the KELA is very high, and therefore, the two tests are very good diagnostic tools, differing only in their convenience of application.

To improve the specificity of the conventional ELISA, assays that use recombinant antigens (Johnson & Illana, 1991) and affinity purified *Toxoplasma*-specific antigens (Lekutis *et al*., 2001) have been developed for use in sheep (Sager *et al*., 2003; Tenter *et al*., 1992) but these tests are not yet routinely used.

Clinically, there is a need to distinguish recent (acute) infections from longstanding (chronic) infections. With the conventional ELISA the detection of *Toxoplasma*-specific IgG and IgM antibodies along with IgA may permit a degree of discrimination between acute and chronic toxoplasmosis. An assay to define avidity of IgG for the P30 antigen of *T. gondii* in sheep has been developed. The avidity was shown to increase over a period of 10 weeks

post-infection (Sager *et al.,* 2003). This test is a good diagnostic tool for discriminating relatively recent from more established infections.

2.1. Preparation of antisera and antigens

Antisera to *T. gondii* and conjugated antisera for use in the IFA test and ELISA, to allow screening of a variety of animal species, may be obtained commercially. International standards for animal sera are not available.

Below are protocols for the preparation of tachyzoite antigen for use in the IFA test and ELISA. Tachyzoites may be grown in tissue culture and retained as whole parasites for the IFA test, or prepared as soluble antigen for the ELISA.

2.2. Preparation of aliquots of a frozen stabilate of *T. gondii* tachyzoites

2.2.1. Test procedure

i) Produce tachyzoites in tissue culture as described.

ii) Centrifuge at 500 ***g*** for 5 minutes and resuspend in Iscove's modified Dulbecco's medium (IMDM) approximately three times.

iii) Add the following solutions to give these concentrations: 10% dimethyl sulphoxide; 20% normal horse serum (free from antibody to *T. gondii*); 70% resuspended tachyzoites to give a final concentration of 1×10^8 tachyzoites/ml.

iv) Allow the preparation to stand on the bench for 1 hour.

v) Dispense into 1-ml aliquots using screw-topped tubes appropriate for liquid nitrogen storage.

vi) Put the tubes into a small container, wrap in thick insulating material and place in –70°C freezer to allow the tachyzoites to freeze gradually.

vii) The next day transfer to liquid nitrogen, keeping well insulated while transferring.

viii) This stabilate may then be used for mouse inoculation or tissue culture growth of the parasite. When removing from storage thaw the sample rapidly in warm water.

2.3. Production of *Toxoplasma* tachyzoites in tissue culture

2.3.1. Test procedure

i) *Toxoplasma gondii* can be grown and maintained in tissue culture by twice-weekly passage in African green monkey kidney (Vero) cells.

ii) Cells and parasite are grown in IMDM supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin and 2% fetal bovine serum.

iii) Tachyzoites are harvested from tissue culture flasks by scraping the cell monolayer using a sterile cell scraper.

iv) Using 25 cm^2 vented tissue culture flasks that have each been seeded with 1×10^5 Vero cells, add tachyzoites at the rate of two tachyzoites per monolayer cell and incubate at 37°C in a 5% CO_2 humidified chamber. Harvest after 3–4 days.

2.4. Preparation of whole tachyzoites for use in the IFA test

2.4.1. Test procedure

i) Produce 4×10^7/ml suspension of RH strain *T. gondii* tachyzoites in PBS.

ii) Add formaldehyde (40%) to give a final concentration of 0.2% (v/v).

iii) Incubate at 4°C overnight and divide into aliquots in suitable sealed tubes and store frozen until required.

2.5. Production of soluble antigen for ELISA

2.5.1. Test procedure

i) Produce a suspension of RH strain *T. gondii* tachyzoites in PBS.

ii) Centrifuge at 2000 ***g*** for 15 minutes, retain the pellet and resuspend it in nine times its volume of distilled water.

iii) Rupture the tachyzoites by freezing and thawing three times.

iv) The antigen preparation is then sonicated for 20 seconds at 4°C at an amplitude of 20 microns.

v) Remove any cellular debris by centrifugation at 10,000 ***g*** for 30 minutes at 4°C.

vi) Retain the supernatant and store at –20°C until required. (Protein estimation might be expected to give a value of between 2 and 4 μg/ml.)

2.6. IFA test

The following is a protocol for carrying out an IFA test for anti-*Toxoplasma* IgG antibodies in sheep serum. It only requires minor modifications for testing different species or for measuring IgM antibody.

2.6.1. Test procedure

i) Clean the required number of tissue culture 15-well multitest slides (Flow laboratories) and allow to dry.

ii) Layer 5 μl of a whole tachyzoite preparation on to each well and allow to dry.

iii) Fix in methanol for 10 minutes.

iv) Wash twice for 10 minutes each time in 0.3 M PBS, pH 7.4.

v) Add 5 μl of the given test sheep serum (diluted in PBS) to each well. (Prepare serial dilutions of the test sera, e.g. 1/16, 1/32, etc. up to 1/1024.) Ensure that positive and negative control sera are included in each test as well as a 'PBS-only' sample. Incubate for 30 minutes at room temperature.

vi) Wash twice for 10 minutes each time in PBS.

vii) Add 5 μl of an appropriate dilution of rabbit-anti-sheep IgG conjugated to fluorescein isothiocyanate, diluted in 0.2% filtered Evan's blue dye in PBS, to each well and incubate for 30 minutes at room temperature.

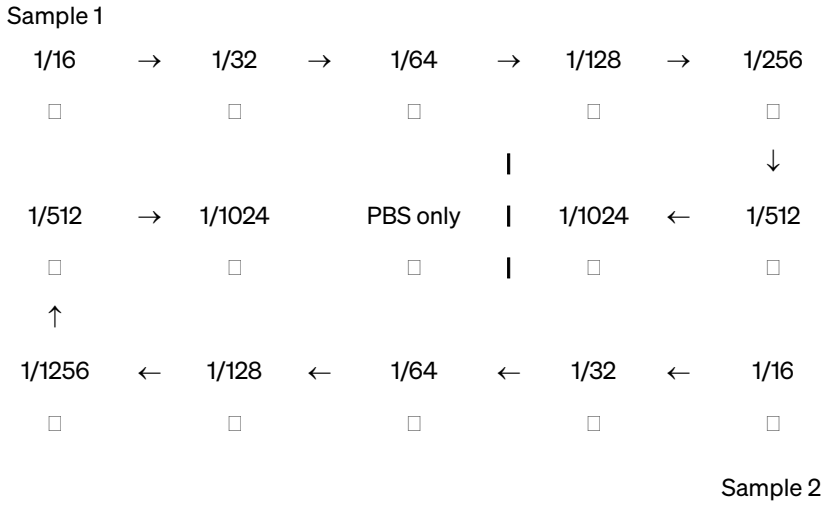
viii) Wash three times for 10 minutes each time in PBS.

ix) Mount the slides under cover-slips with buffered glycerol (nine parts PBS one part glycerol).

x) Examine using a fluorescence microscope, fitted with ×20 and ×40 objective lenses.

With a negative test serum result the whole parasites will appear red due to the autofluorescence of the Evan's blue dye. They may also present with a green fluorescent cap at the parasite pole (nonspecific polar fluorescence). With a positive test serum the parasites will fluoresce red and at least 80% of them within a given well will be surrounded by an unbroken band of green fluorescence. In an adult sheep/goat a positive titre could be defined as ≥1/64 and a negative titre as ≤1/32. For lamb/kid and fetal sera, respective titres could be defined as ≥1/32 and ≤1/16.

An example slide set-up is shown below:



2.7. Procedure for the modified agglutination test

2.7.1. Serum diluting buffer

i) Dissolve 42.5 g NaCl, 1.54 g NaH_2PO_4 (M.W. 120), and 5.4 g Na_2HPO_4 (M.W. 142) in 900 ml deionised water.

ii) Adjust the pH to 7.2. Bring the volume to 1 litre with deionised water.

iii) Store in a refrigerator. This is the 5× stock solution.

iv) Dilute this stock solution 1/5 to give 0.01 M PBS (1 part stock and 4 parts deionised water). PBS should be filtered just before use through a 0.22 μM membrane.

2.7.2. Antigen diluting buffer

i) Dissolve 7.01 g sodium chloride, 3.09 g boric acid, 2.0 g sodium azide in 900 ml deionised water.

ii) Add 24 ml 1 N NaOH and adjust the pH to 8.95.

iii) Bring the volume to 1 litre. This is the stock solution and can be stored at room temperature.

iv) For the working antigen diluting buffer, dissolve 0.4 g bovine serum albumin (BSA) in 100 ml borate buffer. Store at 4°C.

2.7.3. Serum dilutions

i) Dilute serum samples with serum diluting buffer (Section B.2.7.1 above) in small test tubes (1.2 ml in strips of 8 or 12) with a multichannel pipette, starting at 1/25.

ii) Microtitre plates may also be used for making serum dilutions.

2.7.4. Preparation of antigen mixture

i) For each plate, mix 2.5 ml antigen diluting buffer (see Section B.2.7.2 above), 35 μl 2-mercaptoethanol, 50 μl Evans blue dye solution (2 mg/ml water) and 0.15 ml antigen (formalin-fixed whole parasites).

2.7.5. Agglutination procedure

Agglutination is done in U bottom 96 well microtitre plates.

i) Pipette 25 µl antigen mixture to each well immediately after mixing.

ii) Pipette 25 µl serum dilutions into the wells and mix gently with the antigen by repeated pipetting action.

iii) A positive control should be included in each plate. The control should have a titre of 1/200, and two-fold dilutions from 1/25 to 1/3200 should be used.

iv) Cover the plates with sealing tape and incubate at 37°C over night.

v) Read results using a magnifying mirror. A blue button at the bottom of the well means negative. A clear bottom means positive.

C. REQUIREMENTS FOR VACCINES

The only available vaccine is a commercially produced live preparation for sheep, currently licensed for use in the UK, Ireland, France, Portugal and Spain and New Zealand. It consists of tissue culture grown S48 *T. gondii* tachyzoites attenuated by over 3000 passages in mice. The vaccine stimulates effective protective immunity for at least 18 months following a single subcutaneous injection, but as it is unable to produce tissue cysts, sheep are not left with a persistent vaccinal infection. The vaccine has a short shelf life and is a potential risk to immunosuppressed and pregnant female operatives (Buxton, 1993). The vaccine should be stored and used strictly according to the manufacturer's instruction, thus it should not be frozen at any time, should be maintained cool (around 4°C) and not exposed to direct sunlight. The diluent provided should be added to the concentrated suspension of tachyzoites immediately prior to use.

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NB: At the time of publication (2017) there were no WOAH Reference Laboratories for toxoplasmosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 3.10.9.

VEROCYTOTOXIGENIC *ESCHERICHIA COLI*

SUMMARY

Escherichia coli *are normal inhabitants of the gastrointestinal tract of animals and humans. Some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. Since 1977, it has been recognised that some diarrhoegenic strains of* E. coli *produce toxins that have an irreversible cytopathic effect on cultured Vero cells. Such verocytotoxigenic* E. coli (*VTEC*) *belong to over 100 different serotypes.* Escherichia coli *O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC, designated enterohaemorrhagic* E. coli (*EHEC*). *This designation is based on their capacity to cause haemorrhagic colitis and haemolytic uraemic syndrome in humans, their ability to produce verocytotoxins, their ability to cause attaching and effacing lesions on epithelial cells, and their possession of a characteristic large plasmid. In the past two decades, VTEC O157:H7 has risen in importance world-wide as a public health problem. Other non-O157 serogroups, including O26, O91, O103, O104, O111, O113, O117, O118, O121, O128 and O145, have been associated with occasional outbreaks of human disease, and others may be associated with sporadic cases. Ruminants represent the main natural host of VTEC and are generally healthy carriers of the organisms. VTEC have also been isolated from pigs, cats, dogs, chickens and wild birds. Cattle are considered to be the main reservoir of* E. coli O157:H7 *infection for humans. Despite its pathogenicity for humans, infection in animals with* E. coli *O157:H7 is invariably asymptomatic. By contrast, the EHEC serogroups, O26, O111 and O103 may be pathogenic for both humans and animals. The presence of VTEC in animal faeces provides the potential for these organisms to enter the food chain by faecal contamination of milk products, contamination of meat with intestinal contents during the slaughter process or contamination of fruit and vegetables by contact with infected manure. VTEC are also transmitted through contaminated water and by direct contact with infected people or animals.*

Identification of the agent: *Diagnostic procedures for VTEC have been developed, primarily for* E. coli *O157:H7, and seek to overcome the problems of isolating low numbers of target organisms from complex matrices such as animal faeces, food and clinical specimens. Identification of* E. coli *O157:H7 in subclinical animal carriers depends on enrichment of faeces samples in liquid media, usually buffered peptone water with or without the addition of vancomycin, cefsulodin and cefixime, for 6 hours at 37°C followed by immunomagnetic separation using commercially available paramagnetic particles or beads coated with anti-O157 lipopolysaccharide antibody. Beads with bound bacteria are plated on to selective agar, commonly 1% sorbitol MacConkey agar containing cefixime and potassium tellurite, and incubated for 18 hours at 37°C. Non-sorbitol-fermenting colonies are confirmed biochemically as* E. coli *and by serum or latex agglutination as possessing the O157 somatic antigen and/or H7 flagellar antigen. Potential virulence for humans is confirmed by the demonstration of verocytotoxin production by Vero cell assay, enzyme-linked immunosorbent assay (ELISA) or agglutination tests or the demonstration of genes encoding verocytotoxin by polymerase chain reaction. Detection of non-O157 VTEC relies on direct analysis of colonies on nonselective plates by, for example, immunoblotting or DNA probing for verocytotoxin production. Numerous immunological and nucleic acid-based recognition tests have been described to provide a more rapid presumptive diagnosis of VTEC and many are available commercially. Phage typing and pulsed field gel electrophoresis are widely used by reference laboratories for subtyping VTEC O157 for epidemiological purposes.*

Serological tests: *Serological tests are not used routinely in animals to diagnose VTEC infection, but it has been shown that cattle infected with* VTEC *produce serum antibodies to the O157 lipopolysaccharide that can be detected by ELISA.*

Requirements for vaccines and diagnostic biologicals: *No vaccines are currently available for controlling VTEC infections in animals or humans, but a variety of experimental vaccines are being developed.*

A. INTRODUCTION

Escherichia coli are normal inhabitants of the gastrointestinal tract of animals and humans of which only some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. *Escherichia coli* are routinely characterised by serological identification of somatic O, flagellar H and capsular K antigens. However, while some serotypes correlate closely with certain clinical syndromes, differentiation of pathogenic strains from the normal flora depends on the identification of virulence characteristics. Since 1977, it has been recognised that some diarrhoegenic strains of *E. coli* produce toxins that have an irreversible cytopathic effect on cultured Vero cells (Konowalchuk *et al*., 1977). Such verocytotoxigenic *E. coli* (VTEC) have been shown to belong to over 100 different serotypes (Johnson *et al*., 1996a; Strockbine *et al*., 1998). They are also described as Shiga toxin-producing *E. coli* (STEC) due to the similarity demonstrated between verocytotoxins (VT) and Shiga toxins (Stx) of *Shigella dysenteriae* (O'Brien & Laveck, 1983). In the past two decades, VTEC O157:H7 has increased in importance world-wide as a public health problem. *Escherichia coli* O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC, designated enterohaemorrhagic *E. coli* (EHEC). This designation is based on their capacity to cause haemorrhagic colitis and haemolytic uraemic syndrome in humans, their ability to produce VT, their ability to cause attaching and effacing lesions on epithelial cells, and their possession of a characteristic large plasmid (Nataro & Kaper, 1998). Other non-O157 serotypes, including O26:H11, O104:H21, O111:H– and O145:H–, have been associated with occasional outbreaks of human disease, and others still with sporadic cases (Johnson *et al*., 1996a).

Ruminants represent the main natural host of VTEC and are generally healthy carriers of the organisms. VTEC have also been isolated from pigs, cats, dogs, chickens and wild birds; these species can be transiently colonised by the organisms (Beutin *et al*., 1993; Johnson *et al*., 1996a). Surveys have shown that O157 strains normally represent a minority of the VTECs that colonise the intestinal tract of animals. The presence of VTEC in animal faeces provides the potential for these organisms to enter the food chain via faecal contamination of milk, contamination of meat with intestinal contents during slaughter or contamination of fruit and vegetables by contact with contaminated manure. VTEC are also transmitted through water and by direct contact with infected people, animals or animal waste. Contaminated water, used for irrigating or for washing vegetables, can also be source of infection for humans or animals. Cattle are considered to be the main reservoir of *E. coli* O157:H7 infection for humans, although the organism has been isolated from a variety of farmed animals, horses, dogs, rabbits, birds and flies. Despite its ability to cause severe disease in humans (Paton & Paton, 1998), infection in animals with *E. coli* O157:H7 is invariably subclinical. Some non-O157 serotypes, however, are pathogenic for animals and humans and include O26:H11; O103:H2; O111:H– (Bettelheim, 2000; Johnson *et al*., 1996a).

VTEC are also associated with oedema disease in piglets with four serotypes responsible for the majority of outbreaks world-wide, namely O45:K+, O138:K81, O139:K82 and O141:K–. The main virulence factors are a fimbrial adhesin, F18, involved in colonisation and the VT2e toxin, which is responsible for clinical signs. A high degree of genetic relatedness between O101 strains harbouring stx2e genes of human and porcine origin has been demonstrated. The role of pigs as subclinical carriers of STEC in the epidemiology of human disease needs further research.

Because *E. coli* O157:H7 has become the predominant zoonotic VTEC, diagnostic methods have been developed to detect selectively this serotype in human clinical cases (Strockbine *et al*., 1998) and in food sources (Vernozy-Rozand, 1997). For the latter, a validated International Standard detection method is available (EN ISO 16654:2001). In this chapter, however, emphasis will be given to the isolation and identification of O157 and other VTEC from carrier animals (Clifton-Hadley, 2000).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

1.1. Samples

In most cases, samples taken from animals for VTEC isolation will be faeces collected for surveillance purposes or as part of an epidemiological trace-back exercise following an outbreak of disease in humans. Samples may be taken from the rectum or from freshly voided faeces on the farm or from intestinal contents after slaughter. A variety of VTEC are present in healthy animals and not all are thought to be pathogenic for humans. *Escherichia coli* O157:H7, which is the most significant VTEC in human disease, is carried subclinically in animals. Cattle are thought to be the most important reservoir of this serotype. In an infected herd, only a proportion of the animals will be detectably infected, the organism is usually present in carriers in low numbers and is shed intermittently in faeces. Shedding is influenced by the age of the animals, diet, stress, population density, geographical location and season (Meyer-Broseta *et al*., 2001). Some animals are thought to contribute disproportionately to transmission of infection and have been termed "super-shedders" (Matthews *et al*., 2006). Isolation rates may be improved by taking faeces samples in preference to rectal swabs, by increasing the sample size, by increasing the number of individuals sampled and by repeat sampling. Use of recto-anal mucosal swabs is reported to improve detection of colonised as distinct from transiently infected cattle (Rice *et al*., 2003). Precautions should be taken to avoid cross-contamination of samples in transit and at the laboratory. Samples should be kept cool and cultured as soon as possible after collection.

1.2. Safety

Care should be exercised when handling VTEC-positive samples as the infective dose capable of causing severe human infection may be low (possibly 100 organisms for VTEC O157:H7) and laboratory-acquired infections have been reported (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

1.3. Isolation

1.3.1. Liquid enrichment media

Clinical samples are routinely plated directly on to solid media for isolation of *E. coli,* but the number of target VTEC organisms in faeces from healthy carriers is usually low and enrichment in liquid media improves recovery. Commonly used enrichment media are buffered peptone water either unsupplemented (which gives good recovery) or supplemented with 8 mg/litre vancomycin, 10 mg/litre cefsulodin and 0.05 mg/litre cefixime (BPW-VCC) to suppress the growth of Gram-positive organisms, *Aeromonas* spp. and *Proteus* spp.; modified trypticase–soy broth (mTSB) supplemented with 20 mg/litre novobiocin or 10 mg/litre acriflavin to reduce the growth of Gram-positive organisms; or modified *E. coli* broth with 20 mg/litre novobiocin (mEC+n). EHEC *E. coli* grow poorly at 44°C. The optimal incubation for bovine faeces to minimise overgrowth by other organisms is 6 hours at 37°C. For meat samples, enrichment for 6 hours at 41–42°C is used and for water and dairy products, 24 hours at 41–42°C. Nonselective pre-enrichment is necessary for the effective recovery of low levels of stressed *E. coli* O157. Enrichment broths should be pre-warmed to prevent cold-shocking the organisms and slowing their initial growth; 24 hours' incubation may increase recovery if the organisms are stressed.

1.3.2. Immunomagnetic separation

Immunomagnetic separation (IMS) has been used as a selective concentration technique to improve isolation of *E. coli* O157:H7 where numbers of the organism are low (Chapman *et al*., 1994). Commercially available paramagnetic particles or beads coated with anti-lipopolysaccharide (LPS) antibody are mixed with an aliquot of incubated broth. Beads with bound bacteria are separated from the supernatant by a magnetic field and after washing are plated on to selective agar and incubated for 18 hours at 37°C to isolate suspect colonies. The technique is serogroup specific. Commercial systems are available for manual or automated separation (Chapman & Cudjoe, 2001). Recovery may be affected by the bead-to-organism ratio

(optimum is 3:1), the enrichment broth used and the problem of nonspecific adsorption of *E. coli* to the magnetic beads (which can be reduced by the use of a low ionic strength solution in the IMS procedure and careful washing). These factors should be taken into account when trying to maximise the sensitivity of the technique for detecting target *E. coli.*

1.3.3. Selective culture for *Escherichia coli* O157

There are no biochemical characteristics that distinguish the majority of VTEC from other *E. coli,* however, the inability of most strains of *E. coli* O157:H7 to ferment D-sorbitol rapidly and their lack of beta-glucuronidase activity can be exploited in the isolation and identification of these organisms. However, the less common sorbitol fermenting and beta-glucuronidase positive *E. coli* O157:H– variants (nonmotile due to lack of expression of the H7 antigen), will not be identified by isolation in such selective media chosen for these biochemical characteristics (Karch & Bielaszewska, 2001). MacConkey agar containing 1% D-sorbitol instead of lactose (SMAC) is a useful and inexpensive medium on which non-sorbitol-fermenting *E. coli* grow as small, round greyish-white colonies. Selectivity is improved by the addition of 0.5% rhamnose, and addition of 0.05 mg/litre cefixime (CR-SMAC) inhibits overgrowth by *Proteus* spp. While fewer presumptive colonies require testing on this medium, rhamnose is an expensive supplement. An alternative modification is the addition of 2.5 mg/litre potassium tellurite in addition to cefixime (CT-SMAC), which has a greater inhibitory effect against non-O157 *E. coli* and other non-sorbitol fermenters, such as *Aeromonas, Plesiomonas, Morganella* and *Providencia,* than against *E. coli* O157 (O'Brien & Laveck, 1983). This is currently the most commonly used medium for isolating *E. coli* O157.

Media containing fluorogenic or chromogenic glucuronides are used to distinguish non-beta-glucuronidase-producing *E. coli* O157:H7 from beta-glucuronidase-producing *E. coli.* Hydrolysis of 4-methylumbelliferyl-beta-D-glucuronide (MUG) by beta-glucuronidase activity produces a fluorescent compound visible under UV light. The addition of 0.1 g/litre 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide (BCIG) to SMAC differentiates white colonies of *E. coli* O157:H7 from green-blue colonies of sorbitol negative, beta-glucuronidase positive organisms. Commercially available chromogenic and fluorogenic media may be found by reference to media catalogues. While advances have been made in improving the selectivity of media for *E. coli* O157:H7, isolation rates, particularly of stressed organisms, may be adversely affected by the additives used. To mitigate against these effects, addition of recovery agents such as 1% sodium pyruvate to tryptone–soy agar or delaying exposure of stressed cells to selective agents can aid recovery of the organism (Blackburn. & McCarthy, 2000).

Sorbitol-fermenting (SF) *E. coli* O157:H– have been isolated from patients with diarrhoea and HUS but the epidemiology of this infection is poorly understood and only rarely has the organism been isolated from animals, including cattle (Lee & Choi, 2006). The majority of SF *E. coli* O157:H– isolates are susceptible to tellurite and cannot be identified on CT-SMAC. Microbiological analysis for this organism is laborious and entails plating IMS-separated organisms onto SMAC and testing individual SF colonies by latex agglutination for the O157 antigen. Alternatively, colony sweeps are tested by polymerase chain reaction (PCR) for the presence of vt_2, *eae,* rfb_{O157} and *sfpA* (see below). Well-spaced colonies from growth positive by PCR are then tested by colony hybridisation with probes for vt_2, *eae* and *sfpA* or colony immunoblot using specific antibody (Karch & Bielaszewska, 2001; Lee & Choi, 2006).

1.3.4. Isolation of other VTEC

Non-O157 VTEC grow well on media that permit the growth of *E. coli,* such as blood agar or MacConkey agar, and the majority can only be differentiated from other *E. coli* by their ability to produce VT. The large number of different VTEC serotypes precludes the use of O-antisera for the routine screening and presumptive identification of colonies on these media. IMS can be used for selective concentration of serogroups O26, O103, O111 and O145 from a pre-enriched sample, as for the O157 strains. These serogroups are the non-O157 VTECs most commonly associated with human disease, and commercially produced beads are currently available.

The inability of O26 strains to ferment rhamnose has led to the recent development of media that may prove to be useful in differentiating O26 *E. coli* from other enteric organisms. The first is rhamnose-MacConkey agar (RMAC) in which the lactose in the MacConkey medium is

replaced by 10 g/litre rhamnose. Addition of 2.5 mg/litre potassium tellurite and 0.05 mg/litre cefixime (CT-RMAC) is said to improve specificity. The second is a chromogenic rhamnose agar incorporating 10 g/litre rhamnose and 0.02 g/litre phenol red in ES coliform agar (an indicator medium for beta-galactosidase activity) to which is added 0.5 mg/litre potassium tellurite and 0.05 mg/litre cefixime. On this medium, O26 colonies are reported to be dark blue to black, other *E. coli* serotypes are green, and enterobacteria other than *E. coli* are green, yellow or colourless.

One potentially useful virulence marker for VTEC is enterohaemolysin production, which causes haemolysis of washed sheep erythrocytes after overnight incubation on blood agar supplemented with calcium. This characteristic is shared by 90% of VT-producing *E. coli* isolated from human infections. However, the finding that a proportion of disease-producing VTEC can be negative for enterohaemolysin production reduces the value of enterohaemolysin agar as a screen.

In most cases, therefore, isolation of VTEC relies on direct analysis of colonies on plates by immunoblotting or DNA probing for VT production to identify colonies for further characterisation. Colonies are first replicated so that positive colonies can be isolated after replicates have been tested. Colonies may be blotted on to suitable membranes (nitrocellulose or nylon) from which replicates are made or picked off into 96-well microtitre plates containing broth for replication before transferring aliquots to appropriate filters. Colonies are then analysed using nucleic acid probes or antibodies to identify any VTEC (Strockbine *et al*., 1998). Hull *et al.* (1993) developed a mitomycin immunoblot assay for detecting VTEC in faeces that was simple enough to use in routine diagnostic laboratories. Serial dilutions of faeces in broth are inoculated on to MacConkey agar plates and incubated overnight at 37°C. Using standard replica plating techniques, growth from the plate yielding approximately 200 colonies is transferred to two 0.45-μm pore-size nitrocellulose filters laid on Syncase agar with 25 ng mitomycin/ml. This medium induces vegetative growth of bacteriophages carrying the genes for VT and enhances toxin expression. (Alternatively, bacterial or faecal suspensions may be plated directly on to the filters.) The plates are incubated overnight at 37°C. After overnight growth, filters are removed from the plates, immersed in a chloroform bath for 15 minutes, then blocked for 1 hour with 5% non-fat milk in 10 mM Tris, 150 mM NaCl, 0.05% Tween (pH 8) (TNT). The filters are incubated for 1 hour in antisera raised against VT1 or VT2, given three 5-minute washes in TNT, then incubated for 1 hour with alkaline phosphatase-conjugated anti-immunoglobulin G followed by three further 5-minute washes in TNT. Any reaction is visualised by colour development with nitroblue and 5-bromo-4-chloro-3-indolyl-phosphate. VT1, VT2 and VT-negative control *E. coli* are tested in parallel. The use of polyclonal antibodies results in some false positives that are eliminated by using monoclonal antibodies. When the use of DNA probes was compared with the use of the mitomycin immunoblot colony assay, it was shown that the results were comparable. The immunoblot assay has the advantage of being simpler to perform than DNA probing. Mitomycin plates have a long shelf life when stored in the dark at 4°C.

Colony immunoblotting or probing are labour intensive techniques and may be better applied to samples that have been screened and shown to be positive for the presence of VT or VT genes by, for example, enzyme-linked immunosorbent assay (ELISA) or PCR.

1.4. Identification and characterisation of suspect colonies

Colonies growing on solid media that are suspected to be VTEC must be confirmed biochemically or genotypically (e.g. by *GadA* PCR) to be *E. coli*. Somatic 'O' and flagellar 'H' antigens are identified serologically. Not all VTEC isolated from animals are thought to be pathogenic for people. Some isolates of *E. coli* O157, particularly from pigs, are non-verocytotoxigenic and nonpathogenic for humans. Diagnosis, therefore, must include the demonstration of known virulence factors in the isolates. These include the verocytotoxins VT1 (Stx1) and VT2 (Stx2) and their genes and an outer membrane adhesion protein associated with attaching and effacing lesions, intimin, which is encoded by the *eae* gene (Law, 2000). For VTEC O157 strains, subtyping methods are available in reference laboratories for epidemiological investigations.

1.4.1. Biochemical tests

VTEC are biochemically similar to other *E. coli.* VTEC O157:H7 strains differ in failing to ferment sorbitol, failing to produce beta-glucuronidase and fermenting raffinose and dulcitol. *Escherichia coli* can be distinguished from *E. hermanii* by lack of growth in the presence of potassium cyanide and failure to ferment cellobiose. *Escherichia hermanii* is positive for both tests. Ninety-eight per cent of *E. hermanii* strains have a characteristic yellow pigment on nutrient agar that is not seen in VTEC. *Escherichia coli* may be confirmed by demonstration of the use of tryptophan and beta-galactosidase activity (see below) or by commercially available biochemical test strips.

1.4.2. Serological tests

Commercial latex kits are available for O157, O26, O91, O103, O111, O128, O145 and H7. Tests should be carried out according to the manufacturer's instructions and should incorporate positive and negative control organisms and control latex. Presumptive diagnosis may also be made using slide or tube agglutination tests with anti-O LPS antiserum (antisera to 181 O–antigens are available). O157 antiserum has been shown to cross-react with other organisms including *E. hermanii* (frequently found in foods), *Salmonella* O group N, *Yersinia enterocolitica* serotype O9 and *Citrobacter freundii,* indicating the need to confirm putative VTEC colonies as *E. coli.* Isolates can be tested for the presence of flagellar antigen (antisera have been raised to 56 H antigens), but this may require passage through motility medium. Some pathogens are nonmotile.

1.4.3. Verocytotoxin production in Vero cell assay (Johnson *et al.*, 1996a)

The Vero cell assay remains a standard method for the confirmation of VT production (see below). Vero cells have a high concentration of globotriaosylceramide (Gb_3) and globotetraosylceramide (Gb_4) toxin-binding receptors in their plasma membranes and will normally detect all variants of VT. The test can be used on faecal suspensions, culture filtrates or live cultures. In mixed faecal cultures, the sensitivity of the assay is increased by treating the suspension with polymyxin B or mitomycin to release cell-associated toxin. While the test is sensitive, it is not available in most routine diagnostic laboratories. It is labour intensive and results can take 3–4 days after the cell culture is inoculated. Where tissue culture facilities are not available, other methods may be used for detecting VT production, including ELISA or agglutination and PCR can detect the *vt* genes. All of these methods are now available as commercial kits.

1.4.4. Subtyping of *Escherichia coli* O157 for epidemiological studies

A variety of methods is available in reference laboratories to help discriminate between strains of *E. coli* O157:H7 to aid epidemiological investigations of outbreaks of human disease (Hopkins & Hilton, 2000; Strockbine *et al*., 1998). These methods vary in technical complexity and more than one technique is required to provide useful differentiation. Techniques include phage typing, biotyping and antimicrobial sensitivity testing (resistance being uncommon in strains from most countries), plasmid profiling, restriction fragment length polymorphism analysis, ribotyping, pulsed field gel electrophoresis (PFGE) and various PCR-based analyses (random amplification of polymorphic DNA; repetitive DNA element PCR; amplified fragment length polymorphism analysis). Of these, only phage typing and PFGE are widely used. Despite some difficulties with interpretation of profiles, PFGE has emerged as the standard method used by public health reference laboratories for subtyping VTEC O157 due to its high level of discrimination and accuracy and reproducibility. It is used in 'Pulsenet', a network of public health laboratories performing a standardised PFGE method that allows comparison of fingerprints held on an electronic database by the Centres for Disease Control and Prevention in the USA (http://www.cdc.gov/pulsenet/). The European Union's 'Enter-net' system for the surveillance of *Salmonella* and VTEC relies largely on phage typing to subtype *E. coli* O157:H7 strains. Use of subtyping of genes for intimin and VT has proved valuable for epidemiological studies and source attribution (Beutin *et al*., 2004; 2007). Subtyping methods for non-O157 serotypes have been less well explored, however, similar molecular approaches to those used for VTEC O157 can be taken.

1.5. Non-culture techniques for detecting VTEC

Although definitive diagnosis of VTEC relies on the isolation and characterisation of pure cultures, cultural methods for VTEC are time-consuming and labour intensive. This has led to the development of a range of immunological and nucleic acid hybridisation tests for rapid identification of O and H antigens, VT or genes associated with VT production in the sample. As the tests have a detection level above the numbers at which the target organism is normally present in the faeces, an enrichment step (preferably nonselective for isolation of injured or stressed bacteria) is required to increase the numbers prior to testing.

1.5.1. Immunological methods

Immunoassays to identify O and H antigens and VT may be used to confirm the identity of the organisms once isolated from clinical, food or environmental samples, while others, including dipstick and membrane technologies, microplate assays, colony immunoblotting, immunofluorescence and ELISA, are used as rapid methods for detecting the presence of potential pathogens in samples prior to isolation, thus shortening the time for a presumptive diagnosis. Most assays for somatic and flagellar antigens are designed to detect the O157 LPS and H7 flagellar antigen. Toxin assays have the advantage of detecting all VTEC. Enzyme immunoassays for O157 and VT, visual immunoassays for O157 and agglutination tests for O157, H7 and VT are available commercially as kits (De Boer & Heuvelink, 2001; Clifton-Hadley, 2000; Nataro & Kaper, 1998; Strockbine *et al*., 1998). Not all have been validated for use with faeces. Specialised reagents in which anti-O157 LPS antibodies are conjugated to fluorescein, peroxidase or phosphatase are also available. Of the enzyme immunoassays, the most commonly used format is a sandwich assay. Antibody is bound to a carrier surface to capture a specific VTEC antigen; following the addition of an appropriate substrate, a second antibody with an enzyme label binds to this antigen and produces a colour reaction. The kits have been validated with specific pre-enrichment protocols and reagents to ensure reproducible results. Some use heat-treated samples thus improving the safety of the test, and some incorporate an automated processing system to screen large numbers of samples. Others are blot ELISAs developed to screen colonies for O157 antigen. The commercial kits have the advantage of being easy to perform in routine laboratories, and tests should be carried out according to the manufacturers' instructions. Kits validated for food and carcase samples or for human clinical samples may lack sensitivity for animal faeces samples. Immunological assays only give a presumptive result, which must be confirmed by isolation and characterisation of the organisms producing the O157 antigen or the toxin. The availability of kits is changing and the WOAH Reference Laboratories should be able to provide the latest information on validated diagnostic kits.

1.5.2. Nucleic acid recognition methods

i) *Colony hybridisation assays*

Colony hybridisation is a useful means of detecting VTEC in mixed culture for further characterisation. DNA probes and synthetic oligonucleotide probes are available labelled with digoxigenin or biotin and therefore suitable for use in routine diagnostic laboratories. Assays have been described to detect *VT* genes, the 60 MDa plasmid in *E. coli* O157 and the *eae* gene individually and in combination (Nataro & Kaper, 1998; Paton & Paton, 1998; Strockbine *et al*., 1998). Hybridisation assays are less sensitive for detecting VTEC in broth cultures or faecal extracts.

ii) *PCR for VT genes and other virulence markers*

Many PCRs are described in the literature for detection of *VT1*, *VT2* and *VT2* variant genes (Nataro & Kaper, 1998; Paton & Paton, 1998; Strockbine *et al*., 1998), and a number of these toxin-typing PCR methods has recently been compared (Ziebell *et al*., 2002). Demonstration of the genes associated with VT-production does not confirm gene expression and hence production of toxin. PCR can be used on pure or mixed plate or broth cultures, and extracts from food or faeces. It can also be used to detect genes in non-viable organisms. As well as its role in diagnosis, PCR has the potential to be used to screen samples for VTEC in epidemiological studies. Amplification of target genes in bacterial DNA extracts from faeces is less successful than from pure cultures, and careful

preparation of the sample is required to improve sensitivity. Faeces contain nonspecific PCR inhibitors and no single method of removing these is ideal. Sensitivity is improved by nonselective enrichment prior to testing, but remains lower than using IMS or the Vero cell cytotoxicity assay. Commercial assays are available.

DNA probes, PCR assays and microarrays have also been developed to detect other genes in VTEC shown to be associated with virulence in humans, including *eae* (encoding for intimin), *ehx* (encoding for enterohaemolysin production), *fliC* (encoding the H7 antigen), O157 *rfb* (encoding O157 LPS), *uidA* (the mutant glucuronidase gene in beta-glucuronidase-negative *E. coli* O157:H7) and *katP* (a gene carried on the large plasmid of *E. coli* O157:H7 encoding a novel catalase peroxidase) (Bekal *et al*., 2003; Nataro & Kaper, 1998; Paton & Paton, 1998; Strockbine *et al*., 1998). A variety of multiplex assays has been developed to detect simultaneously several diagnostic genes. These assays are of value in the characterisation of pure cultures. On mixed populations of bacteria in food or faeces samples, they may have a use in identifying samples to which isolation procedures should be targeted.

1.6. Screening faeces for *Escherichia coli* O157:H7

Escherichia coli O157:H7 is the VTEC of greatest public health concern in most countries. Its carriage in the intestinal tract of healthy animals, particularly cattle, represents a source of direct and indirect infection to humans. Screening relies on cultural techniques designed to overcome the problems of isolating low numbers of organisms, possibly in a stressed state, from a competing background flora followed by identification of suspect colonies and demonstration of known virulence characteristics. These methods are still evolving and the following is a description of the methods routinely employed in one national veterinary laboratory. Suitable precautions should be taken to avoid human infection (see Chapter 1.1.4).

1.6.1. Pre-enrichment

i) Transport faeces in sterile, leak proof, closed containers at 4°C and culture as soon as possible, preferably within 2 hours of collection. Faeces intended for long-term storage should be frozen at –70°C.

ii) Mix faeces at a dilution of 1/10 in warmed buffered peptone water (BPW) in a labelled container.

iii) Incubate at 37°C±2°C for 6 hours.

iv) Include positive and negative control cultures.

1.6.2. Immunomagnetic separation

i) Use of Dynabeads® anti-*E. coli* O157 product 710.04 (Dynal Biotech, ASA, Oslo, Norway) meets the requirements of AFNOR (DYN 16/02-0696 and DIN 10167); it is cited in the USA Food and Drug Administration's Bacteriological Analytical Manual and the Health Canada Compendium of Analytical Methods and is the official method of the Japanese Health Ministry.

ii) Following the instructions of the manufacturers, carry out immunomagnetic separation (IMS) on the pre-enriched samples using the manual (MIMS) or automated (AIMS) method. Care should be taken to mix the beads well before use and to avoid cross-contamination between prepared tubes. If using the manual method, adherence to the instructions for careful washing of the bead–bacteria complexes is essential.

iii) After the final wash, use a micropipette to transfer 50 µl of each bead–bacteria suspension to a labelled sorbitol MacConkey agar plate containing cefixime and potassium tellurite (CT-SMAC) (Zadik *et al*., 1993) taking care to avoid cross-contamination.

iv) Using a sterile swab, spread the drop over one-third to one-half of the plate to break up the complexes. Using a sterile 10 µl loop, dilute the bead–bacteria complexes further over one quadrant by streaking out at right angles from the previously streaked area. Using a second sterile loop, streak out at right angles from this quadrant into the final unstreaked area of the plate to obtain single colonies. Incubate at 37°C±2°C for 16–18 hours (sorbitol-

fermenting colonies lose colour after this time and may be confused with non-sorbitol fermenting *E. coli* O157). An alternative method for isolating sorbitol-negative colonies is to spread the entire inoculum over the surface of a dry CT-SMAC plate with a sterile bent rod.

1.6.3. Colony identification

i) Pick off up to 10 white, sorbitol-negative colonies per plate and test by O157 latex agglutination following the manufacturer's instructions (include appropriate positive and negative control organisms and latex control).

ii) Subculture agglutination-positive colonies on to solid medium without antibiotics (e.g. 5% sheep blood agar). Streak to obtain single colonies. Incubate at 37°C±2°C overnight.

1.6.4. Confirmation of *Escherichia coli*

i) Inoculate o-nitrophenyl beta-D-galactopyranoside (ONPG) broth. Set up positive and negative controls. Incubate overnight, aerobically at 37°C. *Escherichia coli* produce a positive result indicated by a change to yellow colouration confirming beta-galactosidase activity.

ii) Place a circle of 0.45 µm cellulose nitrate membrane filter paper on to a plate of tryptone bile agar (TBA) using sterile forceps. Use a 1 µl loop to remove a loopful of growth to be tested and inoculate a pea-sized area on the surface of the Millipore filter. Set up positive and negative controls. Incubate at 44°C for at least 17 hours. Transfer the membrane to filter paper soaked with indole reagent for the detection of the use of tryptophan. *Escherichia coli* show a positive reaction indicated by a purple/pink colouration.

iii) A commercial reagent for detection of indole is available. The reagent is placed on to filter paper and a portion of the colony rubbed into the reagent spot. This requires less than 5 minutes and can be backed up by the described test if suspicious colonies appear negative. Indole medium is also available commercially.

iv) Alternatively, use commercially available biochemical test kits to confirm *E. coli*.

1.6.5. Somatic determination (Matthews *et al.*, 2006)

i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 4 ml of Schlecht broth (Ring & Schlecht, 1970). Incubate at 37°C±2°C overnight.

ii) Boil the Schlecht broth for a minimum of 1 hour at 100°C.

iii) Dispense 25 µl of 0.85% saline into wells 2 to 12 of a U-well microtitre plate. Dispense 50 µl of O157 antiserum into well 1. Make a doubling-dilution series of the antiserum to 1/1024, discarding 25 µl after mixing well. Add 50 µl of boiled broth suspension to wells 1 to 12. Cover the plate to prevent evaporation and incubate at 37°C for 6 hours. Use a black background to identify agglutination in the wells.

1.6.6. Vero cell assay

i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 4 ml of Mundell *et al*. (1976) broth. Incubate at 37°C±2°C overnight.

ii) Set up broths with control strains of organisms producing no toxin, thermolabile enterotoxin (LT), cytotoxic necrotising factor (CNF) and verocytotoxin (VT). Incubate at 37°C±2°C overnight.

iii) Dispense Vero cells (African green monkey kidney cells, reference ATCC CCL81, seeding rate 2×10^5/ml) into flat-well microtest plates, 200 µl to each well, 24 hours before inoculation. Incubate at 37°C±2°C in 5% CO_2 for 24 hours.

iv) Add 100 µl of a 400,000 units/ml solution of polymyxin B sulphate in sterile distilled water to each overnight broth culture. Incubate at 37°C±2°C for 5 hours.

vi) Centrifuge the broths at 3000 rpm for 30 minutes.

vii) Remove supernatants into labelled sterile containers (approximately 1.5 ml required).

viii) Place the Vero cell plate on a numbered worksheet to identify each well. Inoculate 10 µl of prepared supernatant into the relevant well of Vero cells. Return Vero cells to the CO_2 incubator and incubate for 3 days.

ix) Examine cells after 24 hours, 48 hours and 72 hours to observe any cytopathic effect. Compare with positive and negative test controls. With VT-positive samples, the cell sheet becomes disintegrated with blackened, shrivelled cells observed between 24 and 72 hours.

1.6.7. Multiplex PCR for VT1, VT2 and *eae* (Beebakhee *et al.*, 1992; Jackson *et al.*, 1987; Strockbine *et al.*, 1988)

Multiplex PCR is used to confirm the presence of virulence determinants using primers as shown below:

Target gene	Accession no.	Primer sequence	Nucleotide position	Amplicon size (bp)
VT1	M19437	F (5'-CGC-TCT-GCA-ATA-GGT-ACT-CC-3')	287–306	256
		R (5'-CGC-TGT-TGT-ACC-TGG-AAA-GG-3')	522–541	
VT2	X07865	F (5'-TCC-ATG-ACA-ACG-GAC-AGC-AG-3')	623–642	185
		R (5'-GC-TTC-TGC-TGT-GAC-AGT-GAC-3')	788–807	
eaeA	X60439	F (5'-GC-TTA-GTG-CTG-GTT-TAG-GAT-TG-3')	271–293	618
		R (5'-CCA-GTG-AAC-TAC-CGT-CAA-AG-3')	871–890	

i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 1 ml of Luria-Bertani broth. Set up three appropriate control broths. Incubate at 37°C±2°C overnight.

ii) Boil the broths for 15 minutes at 100°C. Remove from waterbath and allow to cool.

iii) Prepare master mix for 48 µl per sample containing:

1 × Saiki buffer (50 mM KCl; 10 mM Tris, pH 8.5; 100 µg/ml gelatin); 3 mM $MgCl_2$; 0.5 U Taq polymerase; 25 pmoles of each primer (forward and reverse primers for *VT1*, *VT2* and *eaeA*); 0.2 mM each of dATP, dCTP, dGTP and dTTP.

iv) Mix by inverting tubes and dispense 48 µl into each PCR reaction tube.

v) Add 2 µl of boiled culture (crude DNA extract) to the bottom of each reaction tube (include three control extracts and a media blank).

vi) Run the PCR using cycling parameters of initial denaturation at 94°C for 2 minutes; 25 cycles of 94°C for 1 minute, 62°C for 1.5 minutes and 72°C for 2 minutes; with a final extension of 72°C for 5 minutes. The reaction is held at 4°C until required for electrophoresis.

vii) Electrophorese 15 µl of each PCR sample on a 1.5% agarose gel in E buffer (10× strength solution made by adding to distilled water in the following order: 109 g/litre Tris, 55.6 g/litre ortho-boric acid, 9.3 g EDTA, made up to 1 litre with distilled water and adjusted to pH 8.0 with 10 ml concentrated hydrochloric acid diluted in distilled water before use). Run 100 bp step ladder molecular weight marker for comparison.

ix) Stain in ethidium bromide and view by transillumination.

x) Inspect control lanes to identify positions of VT1, VT2 and eae amplicons. Compare with bands present in test sample lanes. Record the results.

2. Serological tests

In humans, serodiagnosis of VTEC can be valuable, particularly later in the course of the disease when the causative organism becomes increasingly difficult to isolate from faeces. LPS has emerged as the antigen of choice, and production of serum antibodies to the LPS of a wide range of prevalent serotypes of VTEC has been demonstrated. Serological tests are not used for diagnosis of animal infection with VTEC. However, it has been shown that exposure of cattle to *E. coli* O157:H7 infection results in the production of antibodies against the O157 LPS, which persist for months, demonstrable by the indirect ELISA (Johnson *et al*., 1996b). Cross-reactions have been demonstrated between O157-LPS and the LPS antigens of other bacteria including *E. coli* O55, *Salmonella* spp., *Yersinia enterocolitica, Brucella abortus* and *V. cholerae* non-O1 strains. To reduce cross-reactivity, a blocking ELISA using a monoclonal antibody specific for *E. coli* O157 as the competing antibody has been developed for detection of serum antibodies to O157 antigen in cattle (Laegreid *et al*., 1998). Serum antibodies to VT1, but not to VT2, have been demonstrated in cattle by toxin neutralisation tests in Vero cell assays (Johnson *et al*., 1996b). Other studies have shown greater prevalence of VT1 neutralising antibodies in cattle sera than VT2 which may be explained by the greater prevalence of VT1-producing VTEC in cattle and/or the lesser immunogenicity of VT2.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are currently no vaccines available to control zoonotic VTEC. Various approaches to the immunological control of EHEC infections in humans are being explored (Levine, 1998). These are aimed at preventing colonisation, intestinal disease or the serious sequelae of haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura. They include the use of conjugate vaccines (e.g. O157 polysaccharide linked to the B-subunit of VT1 and VT2 as carrier proteins), live-vector vaccines, toxoid vaccine or passive immunisation with hyperimmune globulin or monoclonal antibodies against VT. However, were an effective vaccine to become available, there is debate about the social, political and economic consequences of widespread vaccination of people against pathogens in their food. As animals, mainly cattle, are thought to be the reservoirs of infection for the human population, a novel strategy being explored is to vaccinate cattle in order to reduce colonisation with pathogenic VTEC and thereby reduce contamination of food and the environment (i.e. to make food safer as opposed to protecting people against their food). One approach is to use a live, toxin-negative colonising strain as an oral vaccine to induce antibodies against surface components, and another is to deliver colonisation factors, such as intimin, as an edible vaccine in transgenic plants (Gyles, 1998).

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NB: There is a WOAH Reference Laboratory for *Escherichia coli* (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for *E. coli*

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2008.

CHAPTER 3.10.10.

ZOONOSES TRANSMISSIBLE FROM NON-HUMAN PRIMATES

SUMMARY

The Terrestrial Animal Health Code (*chapter 6.12*) *requires tests for certain diseases in non-human primates imported for research, educational or breeding purposes. This chapter indicates where to find further information on such tests. It is important to recognise that primate species represent a significant risk of pathogen transmission to humans in contact, including the collection of samples for laboratory testing, and the handling of those samples in the laboratory. Veterinary laboratories should seek advice from medical authorities on the appropriate health protocols that should be followed by staff handling such materials. All laboratory manipulations with live cultures or potentially infected or contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis* (*Chapter 1.1.4* Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities)*.*

In addition to the specific tests required by the WOAH Terrestrial Code *as detailed below, additional information on the health monitoring of non-human primate colonies, including a list of potential zoonotic diseases and the types of tests used for diagnosis, is provided by the Federation of European Laboratory Animal Science Associations* (*FELASA*) (*Balansard* et al., *2019*) *and also by the Committee on Occupational Health and Safety in the Care and Use of Nonhuman Primates* (*2003*)[1].

1. Tuberculosis

The test procedures and preparation of reagents are described in Chapter 3.1.13 *Mammalian tuberculosis* (*infection with* Mycobacterium tuberculosis *complex*)*.* The delayed hypersensitivity skin test in non-human primates is usually carried out by the intradermal injection of at least 1500 units (0.1 ml) of undiluted "mammalian old tuberculin"[2] into the edge of the upper eyelid using a sterile 25–27 gauge needle. Tuberculins prepared for use in humans are not of sufficient potency to elicit a response in non-human primates. Purified protein derivatives (PPD) as described in chapter 3.1.13 may also be used, but are generally considered less sensitive for non-human primates. The animal must be suitably restrained or drug-immobilised. For smaller species such as marmosets, tamarins or small prosimians the test can be carried out in the abdominal skin, but this approach requires handling of animals multiple times. A repeat test by the abdominal route may be used in cases where the palpebral reaction is difficult to interpret. False positive and false negative reactions can occur with the tuberculin skin test (Miller, 2008), but nonspecific responses to tuberculin are more common than either a false positive or a false negative response. Nonspecific responses are usually caused by immunological sensitisation to non-pathogenic *Mycobacteria*, often environmental saprophytes, that result in cross reaction to antigens common to both pathogenic and non-pathogenic *Mycobacteria*. Clarification of false positive, false negative, and nonspecific responses can sometimes be done by a battery of testing including *Mycobacterial* culture of faeces, tracheal, bronchial or gastric lavage fluids; radiography to detect tuberculous lesions; haematology and biochemical screens and culture or polymerase chain reaction (PCR) assay of tissue biopsies. Immunological tests can also be used, amongst which the interferon gamma release assay is most widely accepted for verification of the tuberculin test. The combination of a tuberculin test with confirmation by interferon gamma production would be reasonable first steps for screening. However, in species for which little is known about the immunological responses to *Mycobacterial* infection and for which these

1 http://www.nap.edu/catalog/10713.html

2 Mammalian old tuberculin is available from the Colorado Serum Company, 4950 York St, P.O. Box 16428, Denver, Colorado 80216-0428, United States of America.

tests have not been validated, it can be difficult despite battery testing to determine with confidence the tuberculosis status of a non-human primate.

2. Enteric bacteria (*Salmonella, Shigella, Yersinia and Campylobacter* spp.)

These organisms can be detected by standard bacteriological culture methods on samples of fresh faeces or rectal swabs. Culture techniques for Salmonella are described in Chapter 3.10.7 *Salmonellosis* and for *Campylobacter* spp. in Chapter 3.10.4 *Infection with* Campylobacter jejuni *and* C. coli. Methods for the collection, transport and processing of faecal samples are described by WHO (2003). Methods for culture of *Shigella* are described in Appendix 9. Commercial panels of PCR reagents covering the main pathogens are also available for screening faecal samples.

Enteric species of *Yersinia* include *Y. enterocolitica* and *Y. pseudotuberculosis.* Culture and enrichment are more effective if carried out at lower temperatures (4°C rather than 25°C). Details of culture methods including suitable enrichment media are described by Laukanen *et al.* (2010) and Arrausi-Subiza *et al.* (2014). The latter also describe real-time PCR methods for the identification of culture isolates. A general overview of *Y. enterocolitica* and *Y. pseudotuberculosis* is given by Fredriksson-Ahomaa *et al.* (2007), including biochemical methods for the identification of culture isolates.

3. Hepatitis B

Hepatitis B virus (HBV) is classified in the family *Hepadnaviridae*, and is associated with severe disease in humans, where infection is widespread. Although other hepadnaviruses have been identified, and chimpanzees, gorillas, orang-utans, gibbons and woolly monkeys carry similar viruses to those from humans, transmission to humans has not been reported.

4. Macacine herpesvirus 1 (Simian herpes B virus, Cercopithecine herpesvirus 1)

Macacine herpesvirus affects macaques (*Macaca* spp.) and is associated with fatal laboratory infection of humans. It is lethal to some other Old World species, such as colobus, patas and De Brazza's monkeys (Elmore & Eberle, 2008). The situation in New World monkeys is unclear, since human herpesvirus can be lethal for marmosets, but capuchins may be infected with macacine herpesvirus by contact with macaques without developing lethal infection (Coulibaly *et al.,* 2004; Huemer *et al.,* 2002). Non-human primates that have been in contact with macaques may therefore pose a risk of human infection from macacine herpesvirus. Diagnosis is best done by serology with recombinant macacine herpesvirus antigens (Elmore & Eberle, 2008). PCR has not found widespread use for identification of Macacine herpesvirus 1 infected macaques as only those monkeys actually shedding virus at the time of sampling would test positive; latently infected animals not actively shedding virus would not test positive (Eberle & Jones-Engel, 2017). Specified pathogen free colonies of macaques without macacine herpesvirus are being developed.

5. Simian retroviruses

These include simian immunodeficiency virus (SIV), simian type D retrovirus (SRV), simian T-lymphotropic virus, simian foamy virus and gibbon ape leukaemia virus. They pose a potential risk to humans. Diagnostic procedures for infections in non-human primates rely on serology, virus isolation and PCR. See Murphy & Switzer (2008).

6. Endo- and ectoparasites

Non-human primates should be screened during quarantine for the presence of parasites by standard parasitological techniques, according to the parasite under investigation. Methods for these tests may be found in standard parasitological textbooks (Cogswell, 2007; Smith *et al.,* 2007) or, for specific parasites, the relevant chapter in this *Terrestrial Manual*, such as 3.10.2 *Cryptosporidiosis* and 3.10.8 *Toxoplasmosis*.

7. Other zoonotic pathogens

As well as those infections and infestations referred to above, there is a long list of zoonotic agents that may be carried by various species of non-human primate. Given the close phylogenetic relationship between humans and other primates it must be assumed that most pathogens can transmit zoonotically. Further details including the

likely host species, and a suitable regimen for health monitoring in primate colonies, are given in Balansard *et al.,* 2019). The following table is derived from that publication, but is not exhaustive.

Table 1 should be interpreted in context, taking into account the species of primate, its origin (captive bred or wild caught) and its housing. Animals held in cages where they can come into contact with other species or their excreta can acquire infections from one group to the next, which may be subclinical but would cause an infection risk if they were transferred to another room or centre. Wild-caught animals may harbour additional conditions e.g. yaws (*Treponema pallidum*), depending on their origin. Not all pathogens are relevant: wild caught macaques (and most captive bred ones) are a risk for macacine herpesvirus, but this is NOT a risk for other species that have not been in direct contact with macaques so testing would be pointless and unnecessary. Similarly, based on current research, Marburg virus is a risk in wild-caught African non-human primates, and is unlikely to present a risk to New World primates (but it can infect humans). Yellow fever is unlikely to be a risk in primates of Asian origin, but it can infect humans. An individual risk assessment should be made for each case, and screening applied accordingly. Similarly, when screening animals for infection prior to transferring them to another room, centre or back to the wild, a risk assessment should be made on relevant pathogens for the species and any additional risks from the environment in which they were held. Animals exposed to antibiotics repeatedly, or multi-resistant pathogens during their captivity, may also pose a risk for infecting other colonies and human contacts. In addition, the anthropozoonotic potential for humans to transmit pathogens to non-human primates should be considered.

Table 1. Microorganisms and parasites of current concern in non-human primates

(1) Viruses
Adenoviruses
Ebola virus
Foamy virus
Hepatitis A virus
Hepatitis B virus
Herpes T, Herpesvirus platyrrhinae, Saimiriine herpesvirus 1
Herpesvirus cercopithecus, (SA 8), *Cercopithecine herpesvirus 2*
Herpesvirus saimiri, Saimiriine herpèsvirus 2
Lyssa virus (rabies)
Macacine herpesvirus (formerly B virus, *Herpesvirus simiae*, Cercopithecine herpesvirus 1)
Marburg virus
Monkeypox virus
Papiine herpesvirus 2 (formerly *Cercopithecine herpesvirus 16*)
Simian haemorrhagic fever virus
Simian immunodeficiency virus (SIV)
Simian retroviruses or Simian betaretroviruses (formerly Simian retrovirus, type D (SRV)
Simian T-cell lymphotropic virus-1 (STLV-1)
SV 40
West Nile virus
Yellow fever virus
Zika virus

Table 1.* (*cont.*) *Microorganisms and parasites of current concern in non-human primates

(2) Bacteria
Burkholderia pseudomallei
Campylobacter fetus
Campylobacter jejuni
Leptospira interrogans (various serovars)
Mycobacterium africanum
Mycobacterium bovis
Mycobacterium tuberculosis
Salmonella enteritidis
Salmonella typhimurium
Shigella flexneri
Yersinia pseudotuberculosis
(3) Parasites
Ectoparasites: • Mites • Lice • Ticks
Entamoeba histolytica
Giardia spp.
Plasmodia malariae, vivax
Plasmodium brasilianum
Plasmodium cynomolgi
Plasmodium species
Pneumonyssus simicola
Prosthenorchis elegans
Strongyloides stercoralis
Toxoplasma gondii
Trichuris
(4) Dermatomycosis
Trichophyton

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NB: FIRST ADOPTED IN 2008. MOST RECENT UPDATES ADOPTED IN 2021