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Efficacy of recombinant Marek's disease virus vectored vaccines with computationally optimized broadly reactive antigen (COBRA) hemagglutinin insert against genetically diverse H5 high pathogenicity avian influenza viruses

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ABSTRACT

The genetic and antigenic drift associated with the high pathogenicity avian influenza (HPAI) viruses of Goose/Guangdong (Gs/GD) lineage and the emergence of vaccine-resistant field viruses underscores the need for a broadly protective H5 influenza A vaccine. Here, we tested experimental vector herpesvirus of turkey (vHVT)-H5 vaccines containing either wild-type clade 2.3.4.4A-derived H5 inserts or computationally optimized broadly reactive antigen (COBRA) inserts with challenge by homologous and genetically divergent H5 HPAI Gs/GD lineage viruses in chickens. Direct assessment of protection was confirmed for all the tested constructs, which provided clinical protection against the homologous and heterologous H5 HPAI Gs/GD challenge viruses and significantly decreased oropharyngeal shedding titers compared to the sham vaccine. The cross reactivity was assessed by hemagglutinin inhibition (HI) and focus reduction assay against a panel of phylogenetically and antigenically diverse H5 strains. The COBRA-derived H5 inserts elicited antibody responses against antigenically diverse strains, while the wild-type-derived H5 vaccines elicited protection mostly against close antigenically related clades 2.3.4.4A and 2.3.4.4D viruses. In conclusion, the HVT vector, a widely used replicating vaccine platform in poultry, with H5 insert provides clinical protection and significant reduction of viral shedding against homologous and heterologous challenge. In addition, the COBRA-derived inserts have the potential to be used against antigenically distinct co-circulating viruses and future drift variants.

Abbreviations: BHI, brain heart infusion; BIAH, Boehringer Ingelheim Animal Health USA Inc.; BSL, biosafety level; CEF, chicken embryo fibroblasts; CMV, mouse cytomegalovirus; COBRA, computationally optimized broadly reactive antigen; dpc, days post-challenge; ECE, embryonated chicken eggs; Egypt/14, A/Egypt/N04915/2014 H5N1 HPAI virus; FITC, fluorescein isothiocyanate; FRA, focus reduction assay; GMT, geometrical mean titers; Gs/GD, A/goose/Guangdong/1/1996; HA, hemagglutinin; HI, hemagglutination inhibition; HPAI, high pathogenicity avian influenza; HVT, herpesvirus of turkey; IFA, immunofluorescence antibody; LP, low pathogenic; MD, Marek's disease; MDT, mean death time; OP, oropharyngeal; PBS, phosphate-buffered saline; PFU, plaque-forming units; qRRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction; REC, recombinant expression cassettes; SPF, specific pathogen free; SQ, subcutaneous; Tk/MN/15, A/turkey/Minnesota/12582/2015 H5N2 HPAI virus; USNPRC, U.S. National Poultry Research Center; vHVT-H5, vector HVT with H5 insert; VLP, virus-like particle; WHO, World Health Organization.

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1. Introduction

Since its emergence in 1996 in Guangdong, China, H5N1 high pathogenicity avian influenza (HPAI) viruses of A/goose/Guangdong/1/1996 (Gs/GD) lineage have spread globally infecting domestic and wild birds and occasionally spilling over into mammals, including humans [1–4]. Over time, the H5Nx HPAI Gs/GD lineage has diverged into multiple phylogenetically and antigenically distinct clades and subclades based on the H5 hemagglutinin (HA) gene, and antigenic variants resistant to many vaccine seed strains have emerged [5]. Such genetic and antigenic diversity has created challenges in maintaining relevant H5 seed strains for poultry vaccines [6]. Therefore, the development of a broadly protective H5 influenza A vaccine that can provide coverage for antigenically distinct co-circulating viruses and future drift variants is highly desirable.

Several strategies have been investigated to broaden the repertoire of neutralizing antibodies in inactivated influenza vaccines or other non-replicating vaccine platforms that express influenza HA, including multivalent H5N1 vaccines [7,8], targeting conserved domains of HA [9–12], and synthetic consensus DNA antigen-based vaccines [13–17]. In line with the latter strategy, the previously described methodology termed computationally optimized broadly reactive antigen (COBRA) was utilized to generate antigens with novel H5 HA consensus sequences [18,19]. In previous studies using mice, ferrets, and cynomolgus macaques, COBRA HA antigen virus-like particle (VLP) vaccines protected against lethal challenge with homologous and heterologous H5N1 HPAI virus, showing more efficient viral clearance and broader antibody responses against different clades and sub-clades than monovalent or polyvalent vaccines [18,20,21]. In our recent study, COBRA HA VLP vaccines provided clinical protection in chickens challenged with a lethal dose of homologous H5N1 HPAI virus [22]. However, upon challenge with a drifting variant H5N1 HPAI virus, COBRA HA VLP vaccines provided no or partial clinical protection in chickens, and reduction of virus shedding was suboptimal with both challenge strains [22]. Moreover, the robust HA antibody response elicited by COBRA vaccines against the drifted strain did not translate to protective efficacy upon challenge with this virus [22]. Collectively, these results emphasized the need to further improve antigen consensus sequences and vaccine formulation in order to enhance both clinical protection and reduction of virus shedding upon challenge with antigenic diverse HPAI strains.

The use of a live virus as vector platform could improve COBRA HA efficacy. Immunization using Marek's disease (MD) virus serotype 3, also known as herpesvirus of turkey (HVT), is used worldwide to protect chickens against MD. In addition, HVT has been developed and licensed as a live vectored vaccine to protect against many important viral poultry diseases thanks to its persistent replication in the host, its ability to induce both humoral and cell-mediated immunity, and its relatively easy production and administration, among other advantages [23–27]. *In vivo* recombinant vector HVT with H5 insert (vHVT-H5) have demonstrated promising results against HPAI in poultry [26,28–34]. In particular, laboratory and field studies suggest that vHVT-H5 vaccines protect birds against a wide range of H5 HPAI Gs/GD lineage viruses, in addition to being able to overcome the neutralizing effect of maternally-derived antibodies against H5 [26,28–34]. However, recent reports have demonstrated variable protection with vHVT vaccine in chickens when tested against genetically divergent North American clade 2.3.4.4 HPAI viruses [35,36]. Coupling COBRA and vHVT technologies could improve H5 HPAI virus control in the field. The objective of this study was to evaluate the efficacy of several experimental vHVT-H5 vaccines containing different H5 HA gene inserts against challenge with genetically diverse H5 HPAI Gs/GD lineage viruses.

2. Materials and methods

2.1. Generation of vaccine recombinant viruses

Constructs of live vHVT vaccine containing different full-length H5 HA gene inserts were made as previously described [37] (Figure S1). The wild-type recombinant expression cassettes (REC) containing the codon-optimized full-length H5 HA sequences were driven by either the SV40 promoter (vHVT501) or the mouse cytomegalovirus (CMV) promoter (vHVT510). A modification of the wild-type H5 HA REC contained the same sequence as vHVT510 but glycosylated at residue A156T (vHVT510G). All HA sequences in the wild-type H5 HA REC were derived from A/turkey/Washington/61-22/2014 (H5N2) (GenBank accession number AJM70587.1), an isolate belonging to clade 2.3.4.4A. The COBRA REC contained computationally optimized H5 HA sequences COBRA A, COBRA B, or COBRA C, all driven by mouse CMV promoter. The design and characterization of the COBRA HA antigens have been previously described [18,20,21]. Briefly, the COBRA HA antigens were generated by multiple rounds of consensus generation using HA sequences from H5N1 clade 2 human-origin viruses collected from 2004 to 2006. The polybasic cleavage sites of all H5 sequences were modified to low pathogenic (LP) type. The full-length HA amino acid similarities between H5 inserts and challenge viruses are shown in Table S1.

2.2. Expression analysis of recombinant protein

Each of the constructs were passaged for an average of 12 rounds in chicken embryo fibroblasts (CEF) beyond the pre-master seed (X + 12). The X + 12 material of all recombinant viruses generated was evaluated for expression by indirect dual immunofluorescence antibody (IFA) assay. The virus-inoculated CEF were fixed after 48–72 h of infection with ice-cold 95% acetone for 5 min at room temperature and air-dried for 10 min. After rehydration with phosphate-buffered saline (PBS), the CEF monolayer was incubated with two primary antibodies, chicken anti-H5N2 sera (Charles River, North Franklin, CT, USA) diluted 1:500 and L78 monoclonal antibody against HVT (Boehringer Ingelheim Animal Health (BIAH) USA Inc., Gainesville, GA, USA) diluted 1:3000, at 37 °C for 1 h. After three washes with PBS, cells were incubated with two secondary antibodies, rabbit anti-chicken IgG-fluorescein isothiocyanate (FITC) (Sigma Aldrich, St Louis, MO, USA) diluted 1:500 and donkey anti-mouse IgG-Alexa Fluor 568 (Molecular Probe #A10037, lot#1752099) diluted 1:300, at 37 °C for 1 h. After three washes with PBS, cultures were examined for dual expression of H5 and HVT with a fluorescent microscope using FITC- and tetramethylrhodamine isothiocyanate-filters. The X + 12 material of all recombinant viruses generated was also evaluated for recombinant gene integrity by PCR of REC from viral genome and sequencing.

2.3. Challenge viruses

The influenza A isolates clade 2.3.4.4A A/turkey/Minnesota/12582/2015 H5N2 HPAI virus (Tk/MN/15) (GenBank accession numbers KX351776-83) and clade 2.2.1 A/Egypt/N04915/2014 H5N1 HPAI virus (Egypt/14) (GISAID EPI_ISL_262572) were used as challenge viruses. The Tk/MN/15 virus clusters both phenotypically [38] and phylogenetically [39] with clade 2.3.4.4A Midwest H5N2 viruses and represented the homologous challenge virus in this study. The Egypt/14 is the wild type strain for one of the clade 2.2.1 candidate influenza vaccine viruses, World Health Organization (WHO) [40] and represented the heterologous challenge virus in this study. The full-length H5

amino acid similarity between the homologous and heterologous challenge viruses was 90.5% (Table S1). Working stocks were prepared and titrated in embryonated chicken eggs (ECE) using standard methods [41]. Stocks were diluted to the target dose with brain heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD, USA) with penicillin (2000 units/ml; Sigma Aldrich), gentamicin (200 ug/ml; Sigma Aldrich) and amphotericin B (5 ug/ml; Sigma Aldrich). The viruses were manipulated in biosafety level (BSL) 3 enhanced facilities in accordance with procedures approved by the U.S. National Poultry Research Center (USNPRC) Institutional Biosecurity Committee.

2.4. Animals and housing

One hundred and sixty-eight specific pathogen free (SPF) White Leghorn chickens (SPAFAS flock) were randomized into groups (Table S2). Vaccines were administered at 1 day of age by the subcutaneous (SQ) route in 0.2 ml per bird with a target dose of 2,500 plaque-forming units (PFU). The actual dose given was determined by back titration (Table S2). Groups that received the same vaccine were housed together in negative pressured HEPA-filtered isolators at the animal BSL 2 facilities of BIAH (BIAH USA Inc., Athens, GA, USA) during the vaccination period. Birds were subsequently transferred separately by group to negative pressured HEPA-filtered isolators at the animal BSL 3 enhanced facilities of the USNPRC for the challenge period. Birds had ad libitum access to feed and water throughout the experiment. This study was reviewed and approved by the USNPRC Institutional Animal Care and Use committee.

2.5. Experimental design and sampling

Day-old chickens were vaccinated with vHVT-H5 construct or sham-vaccinated with sterile HVT vaccine diluent (BIAH USA Inc., Gainesville, GA, USA) by the SQ route according to Table S2. Four weeks post-vaccination (28 days of age), all birds were challenged by the intrachoanal route with estimated 6 $\log_{10}$ EID_{50}/0.1 ml of either Tk/MN/15 or Egypt/14. The inoculum titers were subsequently verified as 6.9 and 5.7 $\log_{10}$ EID_{50}, respectively, by back titration in ECE. All the birds were monitored daily for 2 weeks following challenge for clinical signs and mortality. Severely sick birds were euthanized and counted as dead for the next day in mean death time (MDT) calculations. Oropharyngeal (OP) swabs were collected at 2 and 4 days post-challenge (dpc) and placed in 1.5 ml of BHI with antibiotics and antifungal. Serum samples were collected pre-challenge (26 days post-vaccination) and at termination (14 dpc). At 14 dpc, surviving birds were euthanized by cervical dislocation.

2.6. Cross-reactivity: Hemagglutination inhibition and focus reduction assay with diverse H5 antigens

The pre-challenge sera were tested by hemagglutination inhibition (HI) and focus reduction assay (FRA) against a panel of antigens of different clades to indirectly assess protection based on cross-positivity and cross-neutralization, respectively. The HI protocol was adapted from the WHO laboratory influenza surveillance manual [42] with modifications [22]. Sera were pre-treated with receptor destroying enzyme (Denka Seiken, Co., Japan) resulting in a pre-dilution of 1:10 before the HI test. The FRA was performed according to standard procedures [43,44] with modifications [45].

Due to limited available sera, the HI (which requires less sera) was the primary cross-positivity test and was used on 13 antigens, while the FRA (which requires more sera) was the secondary test for cross-neutralization confirmation and was restricted to six antigens. Viruses representing avian and human strains that circulated in Asia, Africa, and North America between 2004 and 2017 were obtained through the Influenza Reagent Resource (Table S3). Viruses were passed once in ECE as per WHO instructions [46]. The HA titer for all viruses was determined using horse erythrocytes, and viruses were aliquoted for single-use applications. Viruses were not available for A/chicken/Egypt/CAL3-RLQP/2017 and A/duck/Egypt/S78-RLQP/2017 strains, and VLP displaying the corresponding HA and neuraminidase were used instead.

2.7. Serology against challenge viruses

The sera collected pre and post-challenge were tested by HI assay to directly assess protection based on antibody levels against H5 antigens specific for each corresponding challenge strain. The antigens were prepared as previously described [47] and the HI assays were performed according to standard procedures [48]. Titers were expressed as $\log_2$ geometrical mean titers (GMT). GMT included only positive serum samples. Sera with titers below 4 $\log_2$ GMT were considered negative and expressed as 2 $\log_2$ GMT for statistical purposes.

2.8. Determination of virus from swabs

Swab samples in BHI were processed for quantitative real-time reverse transcriptase polymerase chain reaction (qRRT-PCR) [49] with modifications [50] to determine viral RNA titers. The standard curves for viral RNA quantification were established with RNA extracted from dilutions of the same titrated stocks of the challenge virus. This is a standard protocol among published veterinary influenza vaccine studies given the high correlation between the quantity of RNA determined by qRRT-PCR and the EID_{50} determined by ECE titration when the same challenge virus stock is used to generate the standard curve [51]. The limit of detection was 2.0 $\log_{10}$ EID_{50}/ml for Tk/MN/15 and 1.7 $\log_{10}$ EID_{50}/ml for Egypt/14.

2.9. Statistical analysis

All statistical analyses were performed using SAS v9.4 (SAS Institute, Cary, NC) and R 3.1.1. All tests were two-sided and statistical significance was declared at p value ≤ 0.05. The proportion of birds that exhibited positive swab results in the vaccinated groups were compared against the challenge control group using a Fisher's Exact test in SAS 9.4 and the prevented fraction was calculated using R 3.1.1. The magnitude of post-challenge swab results in birds on 2 and 4 dpc was compared using the Exact Wilcoxon test. Mitigated fraction and the associated 95% confidence intervals were also calculated using SAS 9.4.

3. Results

3.1. Expression of recombinant viruses

The expression of H5 recombinant protein in each HVT vaccine construct was evaluated by dual IFA assay (Figure S2). An average of 300–400 viral plaques were dual-stained for HVT (parent virus) and H5 HA (recombinant gene) expression. The total number of plaques for each staining (HVT and H5) were compared and similar number of plaques were identified in all cases, confirming that almost all HVT viral plaques also expressed H5 antigen after 12 rounds of passage. Similarly, PCR and sequencing of X + 12 material revealed no mutations in the X + 12 material when compared to the reference sequence, confirming genome integrity of the recombinant viruses.

3.2. Cross-reactivity of antibody responses in vaccinated chickens against diverse Gs/GD viral clades

The pre-challenge sera from vaccinated birds were tested against a panel of avian and human strains (Table S3) to assess cross-positivity, as determined by HI (Fig. 1, Table 1), and cross-neutralization, as determined by FRA (Fig. 2). As expected, chickens injected with adjuvant only (sham) showed no HI positivity against any of the H5 test antigens (Fig. 1, Table 1). In general, chickens vaccinated with COBRA-derived H5 (COBRA A, COBRA B, and COBRA C) showed HI titers ($\geq$3 $\log_2$ HI titer) against almost all clades. However, exceptions were observed for clades 2.3.4 and 2.3.4.4A (only COBRA B elicited HI titers), clade 2.3.4.4B (only COBRA C), clade 2.3.4.4D (none of the COBRA vaccines), and clade 7.1 (only COBRA A). In contrast, chickens vaccinated with wild-type clade 2.3.4.4A-derived H5 (vHVT501, vHVT510, and vHVT510G) exhibited HI positivity only against clades 2.3.4.4A and 2.3.4.4D and moderately against clade 2.3.4.2, with vHVT501 being slightly better. In line with the HI results, COBRA-derived H5 elicited neutralizing antibodies against diverse clades, while wild-type clade 2.3.4.4A-derived H5 had superior neutralizing responses only against clades 2.3.4.4A and 2.3.4.4D (Fig. 2).

3.3. Clinical protection against homologous and heterologous HPAI virus

After challenge, all sham-vaccinated birds showed acute severe clinical disease and death, with an MDT of 2.6 dpc for both viruses (Fig. 3). All vaccinated birds remained clinically healthy during the observation period (14 dpc) with no clinical signs from the vaccination or challenge (Fig. 3). The only exception was one vHVT501-vaccinated and Egypt/14-challenged bird that was euthanized at 6 dpc due to prostration (Fig. 3b).

3.4. Serology against challenge viruses

None of the sham-vaccinated birds had detectable HI antibody titers before challenge (Fig. 4). In contrast, most vaccinated birds had seroconverted against homologous Tk/MN/15 virus prior to challenge, with GMT ranging 3.3 to 5.1 $\log_2$, except for COBRA A (0/10 birds) (Fig. 4a). Similarly, most COBRA-vaccinated birds had seroconverted against heterologous Egypt/14 virus prior to challenge, although GMT were slightly lower (3.0 to 4.6 $\log_2$) and wild-type constructs vHVT501 (2/10 birds), vHVT510 (0/10 birds), and vHVT510G (0/10 birds) had low or no seroconversion rates (Fig. 4b). At termination, 50–100% of the vaccinated birds had homologous Tk/MN/15 antibodies; detectable HI antibody titers were similar or slightly higher (3.5 to 5.9 $\log_2$) than pre-challenge titers, and only COBRA A-vaccinated birds had an anamnestic response (2-fold increase) (Fig. 4a). Heterologous groups with pre-challenge antibodies had slightly higher detectable HI antibody titers at termination, while the three constructs with low pre-challenge seroconversion rates (especially vHVT510G) had mounted an anamnestic response (2- to 3.4-fold increase) at termination (Fig. 4b).

3.5. Virus shedding

All the sham-vaccinated birds excreted high virus titers in oropharynx at 2 dpc following homologous Tk/MN/15 and heterologous Egypt/14 challenges (mean 7.2 and 6.2 $\log_{10}$ EID_{50}/ml, respectively) (Fig. 5). In homologous Tk/MN/15 challenged groups, all vaccine constructs significantly decreased OP shedding titers and number of birds shedding at 2 dpc compared to sham-vaccinated birds, with no significant differences in titers or number of birds shedding among vaccine groups (Fig. 5a). In heterologous Egypt/14 challenged groups, all vaccine constructs significantly decreased OP shedding titers at 2 dpc compared to the sham-

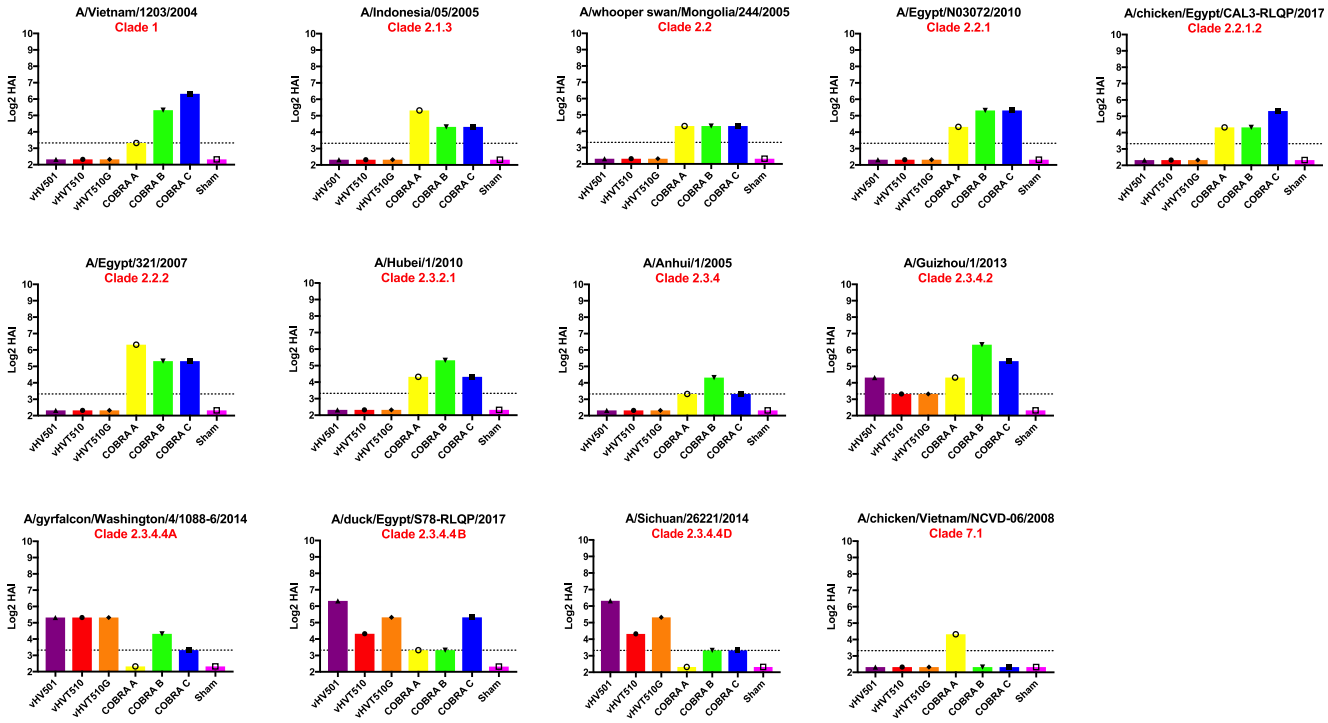

Fig. 1. Hemagglutinin inhibition (HI) cross-reactivity titers. Pre-challenge HI antibody titers were assessed against a panel of 13 antigenically diverse Gs/GD H5 viruses for antigenic cross-positivity reaction. Values are expressed as $\log_2$ mean HI titers. Titers below 3 $\log_2$ were considered negative.

Table 1
Breadth of cross-positivity reaction as measured by HI using pre-challenge sera from vaccinated birds and tested against a panel of 13 viruses from different H5 clades (as per Fig. 1 and Table S3).

Vaccine	Coverage by HI[1]
vHVT501	4/13 (31%)
vHVT510	3/13 (23%)
vHVT510G	3/13 (23%)
COBRA A	8/13 (62%)
COBRA B	10/13 (77%)
COBRA C	9/13 (69%)
Sham	0/13 (0%)

[1] Titers below 4 $\log_2$ GMT were considered negative.

vaccinated birds, but the number of birds shedding was not significantly reduced. Statistically significant differences in mean virus titers were observed among certain vaccinated groups both at 2 and 4 dpc with Egypt/14 virus, with a tendency of COBRA-vaccinated chickens (COBRA A, COBRA B, COBRA C) to shed significantly lower virus titers than wild-type 2.3.4.4A-vaccinated chickens (vHVT501, vHVT510, vHVT510G). One exception was the vHVT510-vaccinated group, which mean virus titers were similar to COBRA-vaccinates. Although the difference in mean virus titers between vHVT510- and vHVT510G-vaccinated chickens at 2 dpc was in the limit of significance ($p = 0.05093$), this difference became significant at 4 dpc (Fig. 5b).

4. Discussion

One of the challenges in developing vaccines for ongoing use in poultry is the genetic and antigenic diversity within the H5 Gs/GD lineage [6]. The wide geographic dispersion of Gs/GD viruses with regional isolation and the long-term utilization of vaccines for the control of H5 AI have been associated with genetic and antigenic drift and the emergence of vaccine-resistant field viruses [52–59]. Thus, vaccines that can protect against antigenically distinct and diverse co-circulating viruses and future drift variants are necessary. The COBRA technology generates synthetic consensus antigen-based vaccines that broaden the repertoire of neutralizing antibodies [18–22], but even with this broadening, their efficacy has encountered some limitations when used in non-replicating vaccine platforms [22]. We conducted a vaccine protection study in chickens using HVT, a widely used replicating vaccine platform in poultry, as a vector. We tested experimental vHVT-H5 vaccines containing wild-type clade 2.3.4.4A-derived HA sequences (vHVT501, vHVT510, vHVT510G) and COBRA HA sequences (COBRA A, COBRA B, COBRA C) derived from clade 2 human-origin viruses with challenge by homologous and genetically divergent H5 HPAI viruses. We directly assessed protection by observing clinical signs and mortality and by measuring reduction in challenge virus shedding from the oropharynx. In addition, we indirectly assessed protection by evaluating cross-reactivity of HI and/or FRA responses on pre-challenge sera against a diverse panel of Gs/GD viruses.

All the tested constructs provided clinical protection against the homologous and a heterologous H5 HPAI Gs/GD challenge viruses. All vaccines were also able to significantly decrease OP shedding titers compared to the sham vaccine. This was especially noteworthy on groups challenged with the homologous strain, which also had significantly fewer birds shedding challenge virus than the sham group. Previous studies with traditional inactivated vaccines indicate that, once they provide protection, their ability to decrease challenge virus replication and shedding from the oropharynx requires a close genetic relationship between vaccine and challenge viruses [47,59–65]. The use of antigenic epitope enhancement computerized techniques such as COBRA, like the increase of immunization doses or the use of immune adjuvants [66,67], has been a strategy in non-replicating vaccine platforms to overcome such limitations, with variable success [18–22]. Here, we note that COBRA-derived constructs, which shared <92% HA amino

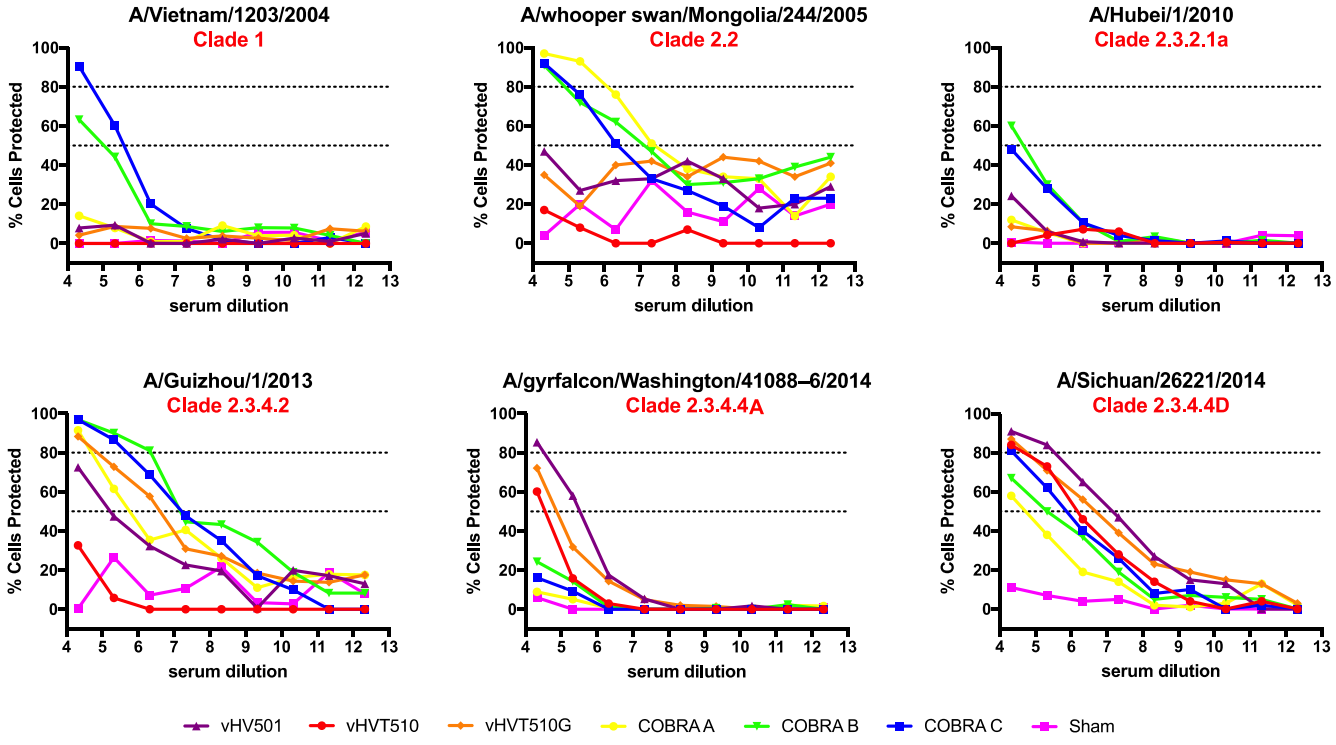

Fig. 2. Focus reduction assay (FRA) cross-neutralization titers. Pre-challenge neutralization titers were assessed against a panel of six antigenically diverse Gs/GD H5 viruses for antigenic cross-neutralization reaction. Values are expressed as percentage of cells protected from viral cytopathic effect at different serum dilutions.

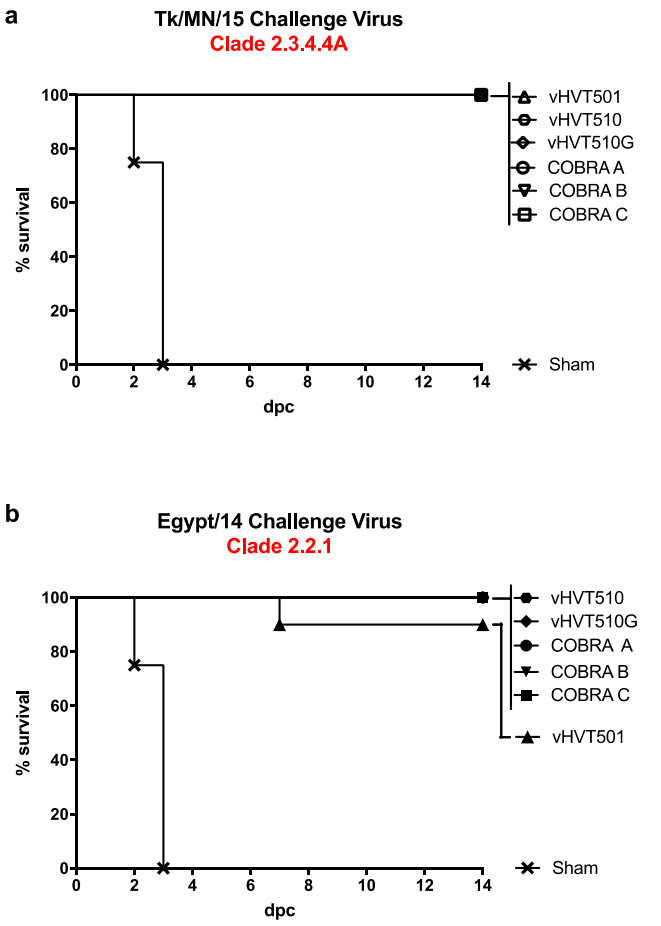

Fig. 3. Survival curves of chickens vaccinated with vHVT-H5 containing one of six different HA inserts or sham-vaccinated, and either challenged with (a) clade 2.3.4.4A A/turkey/Minnesota/12582/2015 H5N2 HPAI virus (Tk/MN/15) or (b) clade 2.2.1 A/Egypt/N04915/2014 H5N1 HPAI virus (Egypt/14).

acid similarity with the homologous challenge virus, provided similar virus shedding reduction than wild-type 2.3.4.4A-derived constructs, which shared almost 99% HA amino acid similarity with the homologous challenge virus. While significant reduction of viral shedding titers was also achieved by all constructs upon heterologous challenge, COBRA-derived constructs did so more efficiently, probably because of their higher amino acid similarity with the heterologous challenge virus (95.2–96%) compared to wild-type 2.3.4.4A-derived constructs (90–90.5%).

In our recent study, two COBRA H5 VLP vaccine constructs provided clinical protection and significant reduction of shedding in chickens challenged with a lethal dose of homologous H5N1 HPAI virus [22]. However, upon challenge with a drifting H5N1 HPAI variant, their effectiveness differed; while both COBRA vaccines significantly decreased shedding titers compared to shams, the COBRA vaccine based on human H5 sequences provided 80% clinical protection, yet the COBRA vaccine based on human and avian H5 sequences did not prevent mortality [22]. Here, we observed better protection from a drift variant than our previous study [22], even though HA amino acid similarity between their homologous and heterologous challenge viruses was greater (93.5%) than ours (90.5%). Possible reasons for our better results compared to our recent study [22] include: i) an improved computerized technique of the COBRA inserts; and ii) the use of the replicating vaccine platform HVT that, in contrast to the non-replicating VLPs, can maintain sufficient antigen doses over time that strongly stimulate both humoral and cell-mediated immunity [23–27]. In addition to these efficacy results, the practicality of HVT as a vaccine platform, i.e. a licensed vaccine already used worldwide against MD, makes this an optimal technology to be coupled with COBRA technology.

When assessing antibody responses against the challenge virus, pre-challenge HI titers and numbers of birds with detectable titers were overall slightly higher when the Tk/MN/15 strain was used for challenge, which likely explained viral shedding results. Similarly, when assessing the breadth of protection, COBRA-derived H5 vaccines elicited a broader protection based on HI and FRA against most of the antigenically diverse Gs/GD viruses than the wild-type 2.3.4.4A-derived H5 vaccines. In contrast, and as expected, wild-type clade 2.3.4.4A-derived H5 vaccines elicited responses mostly against close genetically related clade 2.3.4.4 groups A and D viruses. In line with these results, in our previous study COBRA H5 VLP vaccines elicited broader HI antibody responses than VLP displaying wild-type clade 2.2 HA protein [22]. Our assessment of protection both by challenge virus HI antibodies and by HI cross-positivity and FRA cross-neutralization suggest that vHVT with COBRA H5 inserts are better candidates for vaccine preparedness than wild-type H5 inserts, as they can elicit neutralizing antibodies against drifting clade 2.3.4.4 variants as well as viruses from other Gs/GD clades.

One of the clade 2.3.4.4A H5 insert candidates (vHVT510G) contained a glycosylation at residue A156T of the globular head of the HA, around the receptor binding domain. It has long been recognized that glycosylation can impact HA immunogenicity by masking epitopes [68–72]. Glycosylation at 154–156 residues in particular, which has been observed in avian H5N1 isolates from Egypt and Pakistan [73,74], is associated with higher virus replication efficiency [75–77]. Here, vHVT510 and vHVT510G elicited antibodies that showed similar restricted HI cross-positivity and FRA cross-neutralization among genetically related strains, indicating that glycosylation at 154–156 residues encoded by the vaccine had little impact on the breadth of HI responses against closely related H5 viruses, as previously concluded in a DNA vaccine study in mice [78]. Regarding serology responses against the challenge viruses, we found similar pre-challenge (or primary) responses elicited by vHVT510 and vHVT510G. However, secondary (or anamnestic) responses were greater in vHVT510G-vaccinated birds, especially following heterologous challenge. This suggests that the non-glycosylated construct was more immunogenic than its glycosylated counterpart, as it probably provided a better primary antibody response that was able to more efficiently reduce replication of challenge virus upon heterologous challenge, thus generating less of an anamnestic response.

It should be noted that HI titers are not absolutely predictive of efficacy for a particular H5 strain in chickens [22]. The only bird that succumbed infection lacked pre-challenge HI antibody titers, but many vaccinated survivors lacked HI antibodies as well. This suggests that the presence (>4 $\log_2$ GMT) of pre- and post-challenge HI antibody titers against the challenge virus may be a positive predictor for survival, but HI titers $\leq$ 4 $\log_2$ GMT may not consistently be a negative predictor with antigenic variants. Similarly, previous studies showed HI serology $\geq$8 GMT (i.e., 3 $\log_2$ GMT) [79] or $\geq$10 GMT (i.e., 3.3 $\log_2$ GMT) [61] was associated with protection in challenge studies when the vaccine and field viruses were genetically and antigenically similar [24]. Likewise, another study showed that the lack of antibodies did not predict death [59]. It is apparent that viral vector vaccines do not necessarily produce high levels of antibodies as measured by HI, and protection also derives from cell-mediated immunity, IgA mucosal immunity to uncharacterized influenza viral proteins, or humoral immunity from non-HI antibodies, such as antibodies to conserved regions in the HA stalk or in other viral proteins, as previously observed in studies in poultry [26,29,34–36,59,80–83] and

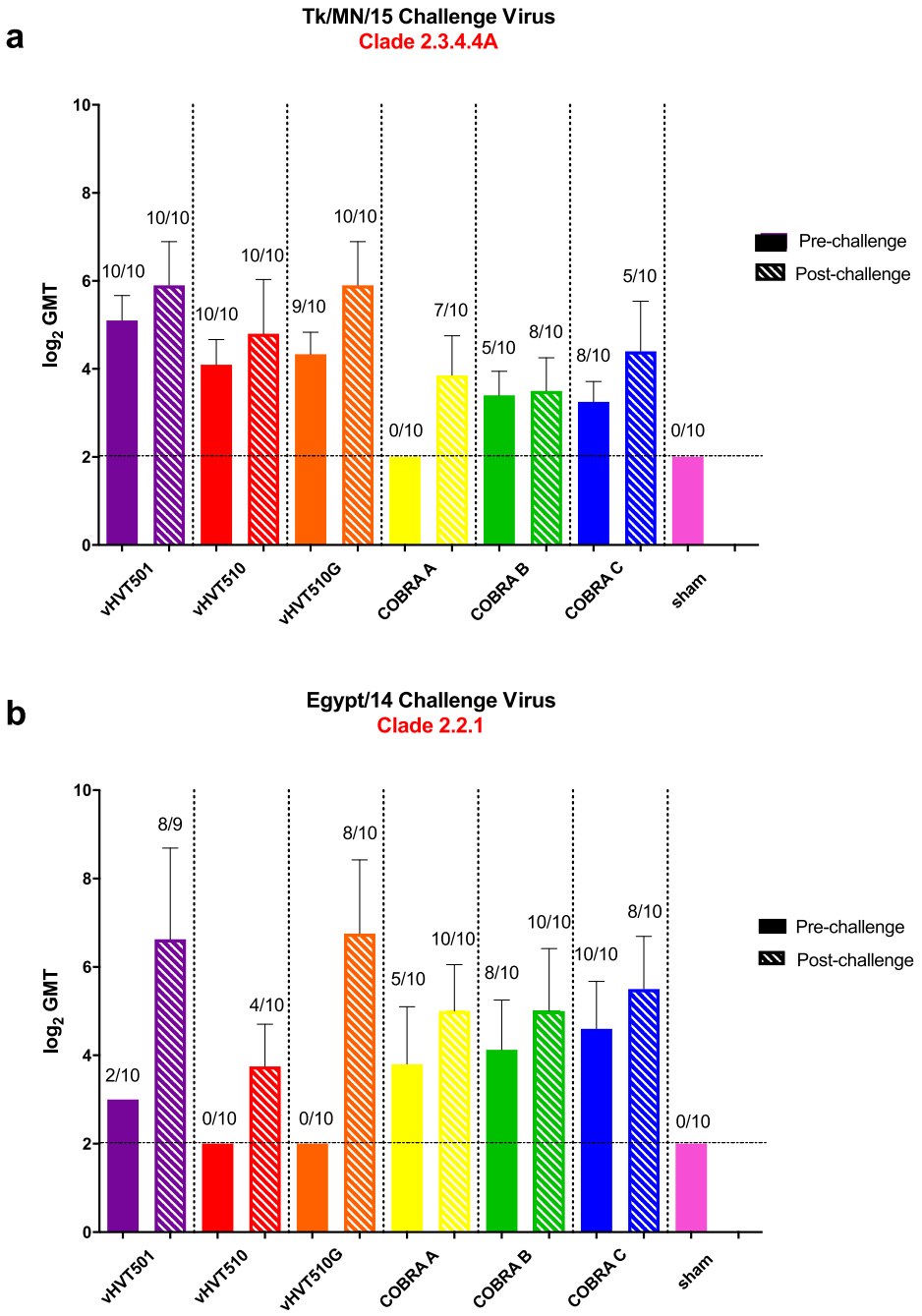

Fig. 4. Serology of chickens vaccinated with vHVT-H5 containing one of six different HA inserts or sham-vaccinated, and either challenged with (a) clade 2.3.4.4A A/turkey/Minnesota/12582/2015 H5N2 HPAI virus (Tk/MN/15) or (b) clade 2.2.1 A/Egypt/N04915/2014 H5N1 HPAI virus (Egypt/14). HI antibody titers against corresponding challenge viruses pre- and post-challenge. Titers are expressed as $\log_2$ GMT. GMT includes only positive serum samples. Samples with titers below 3 $\log_2$ GMT were considered negative. Ratios above the bars indicate the number of birds with HI titers from the total number of birds. All sham-vaccinated challenged birds were either dead or euthanized due to clinical signs before collection of post-challenge serum.

mammals [84–89]. Currently, the HI assay is the established *in vitro* correlate of protection in chicken studies, but the lack of harmonization in vaccine efficacy studies may hamper the establishment of a cut-off protective level of antibodies [24].

In conclusion, the present study demonstrates that the replicating HVT vector vaccine platform with H5 insert provides clinical protection and significant reduction of viral shedding against homologous and heterologous challenge. COBRA-derived constructs prevented virus replication of heterologous challenge more efficiently and elicited broader HI cross-positivity and FRA cross-neutralization antibody responses than wild-type inserts, with a potential for protection against drifting variants. Considering all parameters tested, the COBRA C construct showed the most promising results and will be selected for further investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: AK, MSS, TM, and NP are employees of Boehringer Ingelheim Animal Health. DES declares receipt of a research grant from Boehringer Ingelheim Animal Health.

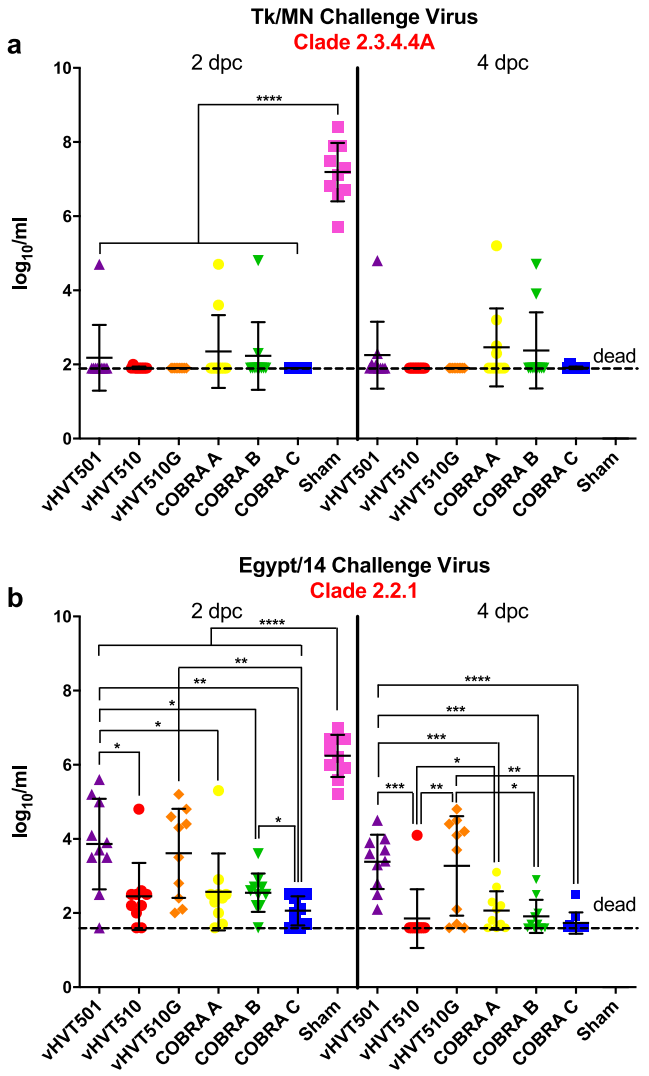

Fig. 5. Scatter plot of oropharyngeal (OP) shedding from chickens vaccinated with vHVT-H5 containing one of six different HA inserts or sham-vaccinated, and either challenged with (a) clade 2.3.4.4A A/turkey/Minnesota/12582/2015 H5N2 HPAI virus (Tk/MN/15) or (b) clade 2.2.1 A/Egypt/N04915/2014 H5N1 HPAI virus (Egypt/14). Shedding titers are expressed as equivalent $\log_{10}$ EID_{50}/ml with error bars included. The limit of detection was 2.0 $\log_{10}$ EID_{50}/ml for Tk/MN/15 and 1.7 $\log_{10}$ EID_{50}/ml for Egypt/14. All samples with titers lower than 2.0 (homologous challenge) or 1.7 (heterologous challenge) were considered negative. For statistical purposes, all the negative samples were assigned the value of 1.9 or 1.6 $\log_{10}$ EID_{50}/ml for Tk/MN/15 and Egypt/14 groups, respectively. Statistical significance was declared at p value $\leq$ 0.05. P values are represented as: * for $p \leq 0.05$; ** for $p < 0.01$; *** for $p < 0.001$; and **** for $p < 0.0001$.

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Author contributions

Conceptualization and funding acquisition (MSS, TM, NP, TMR, DES); animal experiment: design, execution, sample processing, assays, data analysis and curation (KB, MFC, LK, DHL, MSS, DES); vaccine construction and *in vitro* verification (AK, TM); design of vaccine insert (IAN, TMR); *in vitro* neutralization assays, analysis and interpretation (IAN, TMR); writing original draft (KB, DES). Review and editing manuscript (all authors).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.02.075.

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