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Efficacy of multivalent recombinant herpesvirus of turkey vaccines against high pathogenicity avian influenza, infectious bursal disease, and Newcastle disease viruses

Miria F. Criado [a,b,*], Aemro Kassa [c], Kateri Bertran [d,e], Jung-Hoon Kwon [a,f], Mariana Sá e Silva [c], Lindsay Killmaster [a], Ted M. Ross [g], Teshome Mebatsion [c], David E. Swayne [a,*]

[a] Exotic and Emerging Avian Viral Diseases Research Unit, Southeast Poultry Research Laboratory, U.S. National Poultry Research Center (USNPRC), Agricultural Research Service, U.S. Department of Agriculture, 934 College Station Rd, Athens, GA 30605, USA
[b] Department of Pathobiology, College of Veterinary Medicine, Auburn University, 166 Greene Hall, Auburn, AL 36849, USA
[c] Boehringer Ingelheim Animal Health USA Inc., 1730 Olympic Drive, Athens, GA 30601, USA
[d] Unitat mixta d'Investigació IRTA-UAB en Sanitat Animal. Centre de Recerca en Sanitat Animal (CReSA). Campus de la Universitat Autònoma de Barcelona (UAB), Bellaterra 08193, Catalonia, Spain
[e] IRTA. Programa de Sanitat Animal. Centre de Recerca en Sanitat Animal (CReSA). Campus de la Universitat Autònoma de Barcelona (UAB), Bellaterra 08193, Catalonia, Spain
[f] College of Veterinary Medicine, Kyungpook National University, 80 Daehakro, Bukgu, Daegu 41566, Republic of Korea
[g] Center for Vaccines and Immunology, University of Georgia, 501 D.W. Brooks Dr, Athens, GA 30602, USA

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ABSTRACT

Vaccines are an essential tool for the control of viral infections in domestic animals. We generated recombinant vector herpesvirus of turkeys (vHVT) vaccines expressing computationally optimized broadly reactive antigen (COBRA) H5 of avian influenza virus (AIV) alone (vHVT-AI) or in combination with virus protein 2 (VP2) of infectious bursal disease virus (IBDV) (vHVT-IBD-AI) or fusion (F) protein of Newcastle disease virus (NDV) (vHVT-ND-AI). In vaccinated chickens, all three vHVT vaccines provided 90–100% clinical protection against three divergent clades of high pathogenicity avian influenza viruses (HPAIVs), and significantly decreased number of birds and oral viral shedding titers at 2 days post-challenge compared to shams. Four weeks after vaccination, most vaccinated birds had H5 hemagglutination inhibition antibody titers, which significantly increased post-challenge. The vHVT-IBD-AI and vHVT-ND-AI vaccines provided 100% clinical protection against IBDVs and NDV, respectively. Our findings demonstrate that multivalent HVT vector vaccines were efficacious for simultaneous control of HPAIV and other viral infections.

Abbreviations: **ABSL-2**, animal biosafety level 2; **ABSL-3**, animal biosafety level 3; **AIV**, avian influenza virus; **B/BW**, Bursa-body weight; **BHI**, brain heart infusion broth; **BIAH**, Boehringer Ingelheim Animal Health; **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 HPAIV; **EID_{50}**, 50 percent embryo infectious doses; **F**, fusion protein; **FAO**, Food and Agriculture Organization; **FITC**, fluorescein isothiocyanate; **GMT**, geometrical mean titers; **Gs/GD**, A/goose/Guangdong/1/1996; **HA**, Hemagglutinin; **HI**, hemagglutination inhibition; **HPAI**, High pathogenicity avian influenza; **HPAIVs**, High pathogenicity avian influenza viruses; **HVT**, herpesvirus of turkeys; v**HVT**, vector herpesvirus of turkeys; **IACUC**, Institutional Animal Care and Use Committees; **IBD**, infectious bursal disease; **IBDV**, infectious bursal disease virus; **IFA**, indirect immunofluorescence assay; **ILTV**, infectious laryngotracheitis virus; **IRES**, internal ribosomal entry sequence; **LP**, low pathogenic; **MDT**, mean death time; **MDV**, Marek's disease virus; **ND**, Newcastle disease; **NDV**, Newcastle disease virus; **OP**, Oropharyngeal; **PCR**, polymerase chain reaction; **qRRT-PCR**, quantitative real-time reverse transcriptase polymerase chain reaction; **REC**, recombinant expression cassettes; **SPF**, Specific pathogen free; **Tk/Hungary/16**, A/domestic_turkey/Hungary/53433/2016 H5N8 HPAIV; **Tk/MN/15**, A/turkey/Minnesota/12582/2015 H5N2 HPAIV; **TPB**, tryptose phosphate broth; **USNPRC**, U.S. National Poultry Research Center; **vHVT**, recombinant herpesvirus of turkey vectored vaccine; **VLP**, virus-like particle; **VP2**, virus protein 2.

* Corresponding authors at: College of Veterinary Medicine, Auburn University, 166 Greene Hall, Auburn, AL 36849, USA (M. F. Criado) and U.S. National Poultry Research Center (USNPRC), Agricultural Research Service, U.S. Department of Agriculture, 934 College Station Rd, Athens, GA 30605, USA (D. E. Swayne)

E-mail addresses: mfc0034@auburn.edu (M.F. Criado), Aemro.Kassa@boehringer-ingelheim.com (A. Kassa), Kateri.bertran@irta.cat (K. Bertran), junghoon.kwon@knu.ac.kr (J.-H. Kwon), mariana.sa_e_silva@boehringer-ingelheim.com (M. Sá e Silva), Lindsay.Killmaster@usda.gov (L. Killmaster), tedross@uga.edu (T.M. Ross), Teshome.Mebatsion@boehringer-ingelheim.com (T. Mebatsion), birdfluvet@gmail.com (D.E. Swayne).

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1. Introduction

Poultry viral diseases have negative economic and animal welfare impact on the poultry industry worldwide [1,2]. There are no effective treatment options against viral diseases, and vaccines and vaccine programs, when available, vary widely in their effectiveness [2]. Consequently, the prevention and control of these poultry diseases have become challenging, requiring multifaceted programs that must often include several monovalent vaccines administered in several vaccinations to protect against multiple viruses [2,3].

High pathogenicity avian influenza (HPAI), Newcastle disease (ND), and infectious bursal disease (IBD) are among the most significant viral diseases of poultry that utilize vaccination as key part of their control program globally or in many parts of the world [3,4]. Outbreaks of HPAI virus (HPAIV) in poultry and wild birds have had a devastating economic and social impact worldwide [5,6]. According to Food and Agriculture Organization (FAO), only in the first four months of 2022, more than one thousand H5Nx HPAIV outbreaks in animals were reported in Africa, North America, Asia, and Europe [7]. In particular, the H5 HPAIV of A/goose/Guangdong/1/1996 (Gs/GD) Eurasian lineage emerged in 1996 in China and expanded from Asia to the rest of the world [8–11]. The Gs/Gd lineage H5 HPAIV has evolved into multiple clades and subclades by mutations in the hemagglutinin (HA) gene and the cross-protection between different clades is usually low [9].

In addition, Newcastle disease virus (NDV) is a significant worldwide disease of poultry caused by virulent strains of avian orthoavulavirus 1 (former avian paramyxovirus 1) [12]. The NDV is enzootic in multiple countries worldwide and has resulted in at least four panzootic outbreaks since it was first identified in the 1920s, with the fifth panzootic potentially underway in Indonesia, Israel, Pakistan, Eastern Europe, and India [12]. In the last few years, the genotype VII became endemic and spread to many other countries in Asia, Africa, Middle East, and Europe [12]. The introduction of virulent NDV in poultry flocks of NDV-free countries can cause costly outbreaks, as exemplified by the virulent NDV outbreaks reported in California, USA, and neighbouring states in 2018 after 15 years of free-NDV status [13]. The NDV continues to cause economic losses in enzootic countries despite widespread use of vaccination [12]. Finally, IBD is a worldwide immunosuppressive disease of young chickens caused by serotype 1 strains of IBD virus (IBDV) [14]. In addition to high morbidity and mortality, the immunosuppressive effect of the disease predisposes birds to infection by other agents and hampers optimal response to vaccination against other pathogens [14,15].

Vaccination programs have been developed to control all three pathogens, either by eliciting maternal antibodies in hens to be passed through the egg, by directly immunizing the progenies, or both [2–4,16,17]. While routine vaccination against NDV and IBDV is performed virtually worldwide [17–19], routine vaccination against HPAIV has only been used in countries enzootic to the disease [4,17]. Among viral vector vaccines, Meleagrid alphaherpesvirus 1, commonly known as herpesvirus of turkeys (HVT) or a serotype 3 Marek's disease virus (MDV), is the most widely used vector to express protective antigens for avian pathogens [3,17,20–22]. Some advantages of recombinant vector herpesvirus of turkeys HVT (vHVT) vaccines include persistent replication in the host, ability to induce both humoral and cell-mediated immunity, resistance to maternal antibody neutralization (as these viruses are cell-associated), and relatively easy production and administration [3,17,20,21,23–25]. Most of the recombinant vHVT vaccines currently in use express single inserts of foreign genes of AIV, NDV, IBDV, or infectious laryngotracheitis virus (ILTV), thus inducing protection against one pathogen in addition to the highly contagious, lymphoproliferative Meleagrid alphaherpesvirus 1 [3,15,17,24]. Similar to other vaccine vectors, the interference of vHVT vector limits the expression of simultaneous antigens against multiple viruses [26]. However, vHVT double-insert vaccines concurrently expressing antigens of IBDV and NDV, IBDV and ILTV, or NDV and ILTV have been tested or licensed as trivalent recombinant vHVT vaccines [3,17,22,27]. Only one vHVT triple-insert vaccine containing IBDV, ILTV, and H9 HA AIV has been generated, albeit its stability and efficacy *in vivo* remains to be assessed [28]. To the best of our knowledge, there are no vHVT double-insert vaccines expressing the H5 HA gene of AIV, which is the critical antigen of AIV to elicit neutralizing antibodies, in combination with the gene of another pathogen.

In our recent study, vHVT vaccines containing computationally optimized broadly reactive antigen (COBRA) H5 AIV inserts [29,30] were tested in chickens [31]. We used the promising COBRA H5 AIV antigen candidate from our previous study [31] and generated a single-insert (vHVT-AI) and double-insert (vHVT-IBD-AI and vHVT-ND-AI) recombinant vaccines. Our main goal was to evaluate the efficacy of clinical protection, viral shedding, and broadness of neutralizing antibodies in vaccinated chickens against H5 HPAIVs challenges, including clinical protection against IBDV and NDV strains.

2. Materials and methods

2.1. Generation of vector vaccines

Multivalent recombinant vHVT vaccines were generated as previously described [31,32] with modifications (Fig. 1). Briefly, recombinant viruses were generated by *in vitro* recombination in secondary chicken embryo fibroblasts (CEF) co-electroporated with donor plasmids containing recombinant expression cassettes (REC) (Fig. 1) and viral DNA isolated from either HVT strain Fc126 (for vHVT-AI) or vHVT-013 (vHVT-IBD-AI and vHVT-ND-AI).

The plasmid containing REC for vHVT-AI contained COBRA-C H5, HA from H5 HPAI, driven by mouse cytomegalovirus (CMV) promoter [31]. The design and characterization of the computationally optimized COBRA-C H5 antigen have been previously described [30,33,34]. Briefly, the COBRA HA antigen was 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 REC for vHVT-IBD-AI contained the gene for virus protein 2 (VP2) of IBDV driven by mouse CMV promoter and COBRA-C H5 expressed by internal ribosomal entry sequence (IRES) inserted in tandem with the VP2 gene. Similarly, the REC for vHVT-ND-AI contained the gene for fusion (F) protein of NDV driven by mouse CMV promoter and COBRA-C H5 expressed by IRES inserted in tandem with the F gene.

2.2. Molecular characterization of recombinant vaccines

All generated recombinant viruses were evaluated by polymerase chain reaction (PCR). The PCR primers were designed to identify the sequence that should be present only in the recombinant virus and absent from the parent virus vaccine construct (Table S1). In addition, PCR primers were used to amplify the entire REC, including part of the recombination arm, promoter, and recombinant genes. All the final constructs were also confirmed by Sanger sequencing reaction. Briefly, viral DNA was extracted from infected CEF cells by QIA DNeasy Blood & Tissue Kit (Qiagen Inc., Germantown, MD, USA). PCR was performed using 500 ng of DNA with specific primer pairs (Table S1) and OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA, USA), according to manufacturer's protocol. The PCR cycling condi-

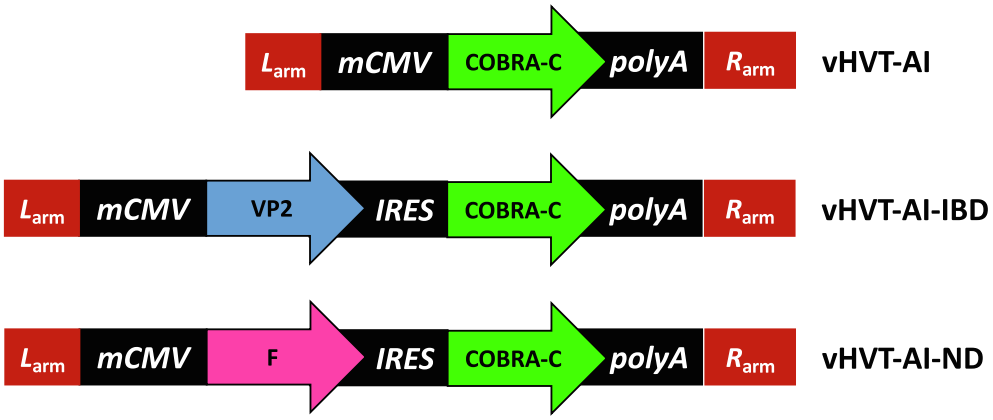

Fig. 1. Schematic representation of the generation of multivalent recombinant HVT vaccines. Recombinant viruses contained recombinant expression cassettes (REC) and viral DNA isolated from either HVT Fc126 (for vHVT-AI) or HVT013 (for vHVT-IBD-AI and vHVT-ND-AI). The vHVT-AI contained COBRA-C H5 protein of HPAIV driven by mouse cytomegalovirus (CMV) promoter. The vHVT-IBD-AI contained virus protein 2 (VP2) of IBDV driven by mouse CMV promoter and COBRA-C H5 protein expressed by internal ribosomal entry sequence (IRES) inserted in tandem with the VP2 gene. Similarly, the vHVT-ND-AI contained fusion (F) protein of NDV driven by mouse CMV promoter and COBRA-C H5 protein expressed by IRES inserted in tandem with the F gene.

tion was 94 °C for 2 mins, followed by 40 cycles of 30 secs at 94 °C, 45 secs at 58 °C, and 1 to 6 mins (adjusted according expected the amplicon size) at 68°; then a final extension at 68 °C for 5 mins.

2.3. Protein expression analysis by indirect immunofluorescence assay

Vaccine constructs were screened for expression of recombinant antigens by indirect immunofluorescence assay (IFA) with antigen-specific antibodies. To obtain a pure population of recombinant virus, the electroporated IFA-positive CEF were recovered, and the population mixture was serially diluted and screened by IFA. The screening procedure was repeated for multiple rounds until pure recombinant virus populations were recovered.

IFA was performed as previously described [31] with modifications. Briefly, the CEFs inoculated with viruses were fixed after 2–3 days post-infection with 95% acetone and incubated with antibodies for VP2 (IBDV), F (NDV), HA (AIV H5 strain) or HVT antigens. For all COBRA-H5 constructs, the expression of HA of AIV H5 and HVT antigens were evaluated using chicken anti-H5N2 sera (Charles River, North Franklin, CT, USA) diluted 1:300 and L78.2 monoclonal antibody against HVT (Boehringer Ingelheim Animal Health (BIAH) USA Inc., Gainesville, GA, USA) diluted 1:3,000, as previously described [31]. In addition, the expression of IBDV VP2 (COBRA H5-IBDV construct) and NDV F (COBRA H5-NDV construct) was evaluated using chicken IBD antisera (Charles Rivers Laboratories, North Franklin, CT, USA) diluted 1:500 and chicken NDV antisera (Charles Rivers Laboratories, North Franklin, CT, USA) diluted 1:300, respectively. After washes with PBS, cells were incubated with secondary antibodies, goat anti-chicken IgY (H + L) Alexa Fluor 568 (Life technologies Corporation, Carlsbad, CA, USA, #A11041) and anti-mouse IgG-fluorescein isothiocyanate (FITC) (Sigma-Aldrich Inc, St. Louis, MO, US, #F2012-1) both diluted 1:300. The protein expression was examined using Nikon Eclipse Ti inverted microscope.

2.4. Animals and housing

Specific pathogen free (SPF) White Leghorn chickens were assigned into groups for HPAIV challenge (Table 1, twelve groups), IBDV challenge (Table 2, five groups), and NDV challenge (Table 3, two groups). Birds were housed in negative pressured HEPA-filtered isolators at the animal biosafety level 2 (ABSL-2) facilities of Boehringer Ingelheim Animal Health (BIAH) USA Inc., Athens, GA for the vaccination and challenge periods. Only the birds that were subsequently challenged with HPAIVs were transferred to negative pressured HEPA-filtered isolators at the animal biosafety level 3 (ABSL-3) enhanced facilities of the U.S. National Poultry Research Center (USNPRC) for the challenge period. These studies

Table 1
Experimental design for chickens vaccinated with multivalent vHVT vaccines and challenged with clade 2.3.4.4A A/turkey/Minnesota/12582/2015 H5N2 HPAIV (Tk/MN/15), clade 2.3.4.4B A/domestic turkey/Hungary/53433/2016 H5N8 HPAIV (Tk/Hungary/16), or clade 2.2.1 A/Egypt/N04915/2014 H5N1 HPAIV (Egypt/14).

Group	Vaccine [1]	H5 HPAIV challenge [2]	Number of birds
1	vHVT-IBD-AI	Tk/MN/15	10
2	vHVT-ND-AI	Tk/MN/15	10
3	vHVT-AI	Tk/MN/15	10
4	Sham-vaccinated	Tk/MN/15	10
5	vHVT-IBD-AI	Tk/Hungary/16	10
6	vHVT-ND-AI	Tk/Hungary/16	10
7	vHVT-AI	Tk/Hungary/16	10
8	Sham-vaccinated	Tk/Hungary/16	10
9	vHVT-IBD-AI	Egypt/14	10
10	vHVT-ND-AI	Egypt/14	10
11	vHVT-AI	Egypt/14	10
12	Sham-vaccinated	Egypt/14	10

[1] The vaccines were administered at 1 day old by the subcutaneous route in 0.2 ml per bird. Sham-vaccinated birds were administered sterile HVT diluent and used as negative controls.
[2] All birds were challenged 4 weeks post-vaccination by the intrachoanal route with Tk/MN/15 at 5.9 $\log_{10}$ EID_{50} (Groups 1–4), Tk/Hungary/16 at 5.9 $\log_{10}$ EID_{50} (Groups 5–8), or Egypt/14 at 6.5 $\log_{10}$ EID_{50} (Groups 9–12).

Table 2
Experimental design for chickens vaccinated with multivalent vHVT vaccine and challenged with STC or Var-E IBDV strains.

Group	Vaccine [1]	IBDV challenge [2]	Number of birds
1	vHVT-IBD-AI	STC	20
2	Sham-vaccinated	STC	20
3	vHVT-IBD-AI	Var-E	20
4	Sham-vaccinated	Var-E	20
5	Sham-vaccinated	TPB diluent	20

[1] The vaccines were administered at 1 day old by the subcutaneous route in 0.2 ml per bird. Sham-vaccinated birds were administered sterile HVT diluent and used as negative controls.
[2] All birds were challenged 3 weeks post-vaccination by the eyedrop route with STC IBDV strain at 1.8 $\log_{10}$ EID_{50} (Groups 1 and 2), or at 4 weeks post-vaccination by the eyedrop route with Var-E IBDV strain at 2.9 $\log_{10}$ EID_{50} (Groups 3 and 4). Group 5 was administered TPB diluent and used as negative control.

Table 3
Experimental design for chickens vaccinated with multivalent vHVT vaccine and challenged with GB Texas NDV strain.

Group	Vaccine[1]	NDV challenge [2]	Number of birds
1	vHVT-ND-AI	Texas GB	20
2	Sham-vaccinated	Texas GB	20

[1] The vaccines were administered at 1 day old by the subcutaneous route in 0.2 ml per bird. Sham-vaccinated birds were administered sterile HVT diluent and used as negative controls.
[2] All birds were challenged 4 weeks post-vaccination by the intramuscular route with Texas GB NDV strain at 4.2 $\log_{10}$ EID_{50} (Groups 1 and 2).

were reviewed and approved by the BIAH USA Inc. and USNPRC Institutional Animal Care and Use committees (IACUC).

2.5. Vaccination

Vaccines were administered at 1 day of age by the subcutaneous route in 0.2 ml per bird (Tables 1 to 3). Sham-vaccinated birds were administered sterile HVT diluent (diluent commercialized by BIAH USA Inc.) and used as negative controls.

2.6. Challenge viruses

The HPAIV challenge stocks were prepared and titrated in embryonated chicken eggs (ECE) using standard methods [35]. 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, St. Louis, MO, USA), gentamicin (200 ug/ml; Sigma-Aldrich, St. Louis, MO, USA) and amphotericin B (5 ug/ml; Sigma-Aldrich, St. Louis, MO, USA). The HPAIVs were manipulated in biosafety level (BSL) 3 enhanced facilities in accordance with procedures approved by the USNPRC Institutional Biosafety Committee. The IBDV and NDV challenge stocks were prepared and titrated in ECE and diluted to the target dose with tryptose phosphate broth (TPB) (Sigma-Aldrich, St. Louis, MO, USA). The IBDV and NDV were manipulated in BSL-2 facilities in accordance with procedures approved by BIAH USA Inc. and IACUC. The vHVT-IBD-AI-, vHVT-ND-AI-, and vHVT-AI-vaccinated birds were challenged against three different HPAIV strains (Table 1): clade 2.3.4.4c (previously termed 2.3.4.4A) A/turkey/Minnesota/12582/2015 H5N2 HPAIV (Tk/MN/15) (GenBank accession numbers KX351776-83), clade 2.3.4.4b (previously termed 2.3.4.4B) A/domestic_turkey/Hungary/53433/2016 H5N8 HPAIV (Tk/Hungary/16) (GISAID accession number EPI859207), and clade 2.2.1 A/Egypt/N04915/2014 H5N1 HPAIV (Egypt/14) (GISAID EPI_ISL_262572). The full-length H5 HA amino acid identity between COBRA C insert and the HPAIV challenges are shown in Table S2.

The vHVT-IBD-AI-vaccinated birds were also challenged against classical STC IBDV strain (EP-1) and variant-E IBDV strain (Var-E; 1084-E CP2 3–14-95) (Table 2). The vHVT-ND-AI –vaccinated birds were also tested against neurotropic, velogenic GB Texas NDV strain (Table 3).

2.7. HPAIV challenge and sampling

Four weeks post-vaccination, all SPF chickens from groups in Table 1 were challenged by the intra-choanal route with estimated target dose of 6 $\log_{10}$ EID_{50}/0.1 ml of HPAIV (Table 1). The inoculum titers were verified as 5.9, 5.9, and 6.5 $\log_{10}$ EID_{50}/dose for Tk/MN/15, Tk/Hungary/16, and Egypt/14 HPAIVs, respectively, by back titration in ECE. Birds were monitored daily for clinical signs and mortality for 14 dpc. 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 antibiotic and antimycotic. Serum samples were collected pre-challenge (26 days post-vaccination) and at termination (14 dpc). At 14 dpc, surviving birds were euthanized following approved protocol by the IACUC.

2.8. IBDV challenge and sampling

SPF chickens from groups 1 and 2 in Table 2 were challenged with STC IBDV strain at three weeks post-vaccination by the eye-drop route with estimated target dose of 2 $\log_{10}$ EID_{50}/0.03 ml (Table 2). The inoculum titer was verified as 1.8 $\log_{10}$ EID_{50}/dose by back titration in ECE. Groups 3 and 4 in Table 2 were challenged with Var-E IBDV strain at four weeks post-vaccination by the eye-drop route with estimated target dose of 2 $\log_{10}$ EID_{50}/0.03 ml (Table 2). The inoculum titer was verified as 2.9 $\log_{10}$ EID_{50}/dose by back titration in ECE. Group 5 received TPB and was used as negative control. Birds were monitored daily for clinical signs and mortality. At 4 dpc, STC IBDV challenged birds were euthanized and necropsied to evaluate gross lesions in the cloacal bursa. At 11 dpc, Var-E IBDV challenged birds were euthanized and necropsied to evaluate bursa-to-body weight (B/BW) ratios.

2.9. NDV challenge and sampling

Four weeks post-vaccination, all SPF chickens from groups in Table 3 were challenged by the intramuscular route, following guidance for NDV vaccine licensure in the US (9CFR 113.329) [36] with estimated target dose of 4 $\log_{10}$ EID_{50}/0.1 ml of Texas GB NDV strain. The inoculum titer was verified as 4.2 $\log_{10}$ EID_{50}/-dose by back titration in ECE. Birds were monitored daily for clinical signs and mortality for 14 dpc, and then euthanized following approved protocol.

2.10. Determination of HPAIV shedding from swabs

OP swab samples collected from HPAIV challenged birds were processed for quantitative real-time reverse transcriptase polymerase chain reaction (qRRT-PCR) [37] with modifications [38] 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 [39]. The lower limit of detection was 1.1 $\log_{10}$ EID_{50}/ml for Tk/MN/15, 1.4 $\log_{10}$ EID_{50}/ml for Tk/Hungary/16, and 1.7 $\log_{10}$ EID_{50}/ml for Egypt/14. For statistical purposes, negative qRRT-PCR samples were assigned a value 0.1 lower than the corresponding limit of detection.

2.11. Serology in HPAIV challenged birds

The sera collected pre- and post-challenge was tested by hemagglutination inhibition (HI) assay to determine humoral antibody levels against H5 antigens specific for each corresponding challenge strain. The antigens were prepared as previously described [40] and the HI assays were performed according to standard procedures [41]. Titers were expressed as $\log_2$ geometrical mean titers (GMT). Samples with titers below 3 $\log_2$ GMT were considered negative and expressed as 2 $\log_2$ GMT for statistical purposes.

2.12. Statistical analysis

Differences in virus shedding and HI titers between vaccinated groups were analyzed with Kruskal-Wallis test and Dunn's Multiple Comparison test with One-way ANOVA using GraphPad software Prism 8 (San Diego, CA). The softwares SAS v9.4 (SAS Institute, Cary, NC) and R 3.1.1 were used to confirm these results and to calculate the B/BW ratios. Statistical significance was declared at p value ≤ 0.05. All data obtained was plotted using GraphPad software Prism 8.

3. Results

3.1. Generation and validation of recombinant viruses

Three recombinant virus vaccines were generated as shown in Fig. 1. The PCR reactions demonstrated that each gene sequence in the generated recombinant viruses resulted in the expected amplicon size (data not shown). In addition, the sequencing reactions confirmed that all gene inserts present in each construct had no changes compared to original sequences (data not shown). The expression of recombinant proteins in each live vector vaccine was confirmed by IFA assay, in which viral plaques were stained for the parent virus (HVT) and the recombinant genes (VP2, F, HA) (Fig. 2).

3.2. HPAIV challenge

3.2.1. Clinical protection

After challenge, all sham-vaccinated birds showed acute severe clinical disease and death, with MDT of 2.6, 3, and 2.4 dpc for Tk/MN/15, Tk/Hungary/16, and Egypt/14, respectively (Fig. 3). All vaccinated birds challenged with Tk/MN/15 remained clinically healthy during the observation period (14 dpc) with no clinical signs from the vaccination or challenge (Fig. 3a). All the Tk/Hungary/16 and Egypt/14 challenged birds remained clinically healthy, except for one vHVT-ND-AI vaccinated, Tk/Hungary/16 challenged bird that was euthanized at 7 dpc due to prostration (Fig. 3b) and one vHVT-ND-AI vaccinated, Egypt/14 challenged bird that was found dead at 7 dpc (Fig. 3c).

3.2.2. Virus shedding

At 2 dpc, all the sham-vaccinated birds had high virus titers in the OP swab samples regardless of the HPAIV challenge used (Fig. 4). The mean viral titers in the sham groups were 7.3, 6.0, and 6.6 $\log_{10}$ EID_{50}/ml for Tk/MN/15 (Fig. 4a), Tk/Hungary/16 (Fig. 4b), and Egypt/14 (Fig. 4c), respectively. All vaccine constructs significantly decreased OP viral shedding titers at 2 dpc compared to sham-vaccinated birds for the three HPAIVs and the vaccinated groups were not significantly different among each other. In addition, the number of vaccinated birds shedding virus also decreased compared to sham groups, and it was significantly lower for vHVT-AI vaccinated birds regardless of HPAIV challenge (Fig. 4) and vHVT-IBD-AI vaccinated birds challenged against Tk/Hungary/16 (Fig. 4b).

At 4 dpc, all sham-vaccinated birds were either dead or euthanized due to clinical signs, except for one Tk/Hungary/16 challenged bird (Fig. 4). Significant differences in viral shedding titers among vaccine groups were only observed at 4 dpc with Tk/MN/15 (Fig. 4a) and Tk/Hungary/16 (Fig. 4b) challenges.

3.2.3. Serology

None of the sham-vaccinated birds had detectable hemagglutination inhibition (HI) antibody titers before challenge (Fig. 5). In contrast, most vHVT-IBDV-AI and vHVT-AI vaccinated birds had HI titers against the corresponding challenge HPAIV prior to challenge (from 8 to 10 birds out of 10 per group) with mean titers that ranged between 3.2 and 4.2 $\log_2$ GMT (Fig. 5). The vHVT-ND-AI vaccinated birds tended to have lower seroconversion rates, especially against Tk/MN/15 (3/10), and lower mean titers (2.5 to 2.9 $\log_2$ GMT) pre-challenge (Fig. 5).

None of the sham-vaccinated birds survived, and post-challenge sera was not collected. All the surviving birds had statically significant higher antibody titers in the post-challenge sera (14 dpc) compared to pre-challenge sera (Fig. 5), except for the HVT-AI vaccinated group challenge with Tk/Hungary/16 that no difference was observed (Fig. 5b). The post-challenge mean HI antibody titers of the vaccinated birds ranged from 4.7 to 6.3, 5.2 to 6.0, and 6.0 to 7.5 $\log_2$ after challenge with Tk/MN/15, Tk/Hungary/16, and Egypt/14, respectively (Fig. 5).

3.3. IBDV challenge

After STC IBDV challenge, 1 out of 20 sham-vaccinated birds showed lethargy at 4 dpc, and 3 out of 20 birds died on 4 dpc. All sham-vaccinated birds had typical IBDV lesions in the cloacal bursa at 4 dpc consisting of *peri*-bursal edema, edema, and/or macroscopic hemorrhages. All the vHVT-IBD-AI vaccinated birds were clinically healthy during the observation period (4 dpc) and lacked typical IBDV lesions in the cloacal bursa.

After Var-E IBDV challenge, all birds remained clinically healthy during the observation period (11 dpc). The mean Bursa-body weight (B/BW) ratios were 0.36 for vaccinated and challenged birds, 0.12 for sham-vaccinated and challenged controls, and 0.52 for sham-vaccinated and sham-challenged controls (Fig. 6). All mean B/BW ratios were significantly different among them.

3.4. NDV challenge

After Texas GB NDV challenge, all the sham-vaccinated birds were found dead at 4 dpc, and all the vHVT-ND-AI vaccinated birds remained clinically healthy and survived the observation period (14 dpc).

4. Discussion

Viral coinfections are frequent in densely populated poultry areas, and vaccines are an essential tool for their control [2–4,16,17]. Since simultaneous administration of multiple single insert vHVT vaccines is not a viable solution [26], the development of multivalent recombinant vaccines that can induce simultaneous protection against multiple avian pathogens is highly desirable. Among the recombinant viral vectors, HVT is the most successful and widely used commercially for the delivery of various immunogenic proteins to protect against poultry diseases such as HPAIV, IBDV, and NDV [3,17,20–22]. Here, we generated vHVT vaccines expressing COBRA H5 AIV antigen alone (vHVT-AI) or in combination with VP2 IBDV antigen (vHVT-IBD-AI) or F NDV antigen (vHVT-ND-AI). Subsequently, we tested the efficacy of these vaccines against experimental challenge with divergent H5 HPAIV of the Gs/GD lineage in SPF chickens. In addition, we also evaluated the clinical protection conferred by challenge against IBDV and NDV. This study did not evaluate the protection against MDV, and additional tests are necessary to confirm it. However, vHVT is a commonly used vaccine to control MDV showing great success at protecting chickens against tumors and mortality [3,15,17,24].

Even though the vHVT vaccine has been established and used for several years, there are still several challenges in developing a vaccine with multiple inserts. One of the major complications is the compatibility between the vector backbone and the inserts

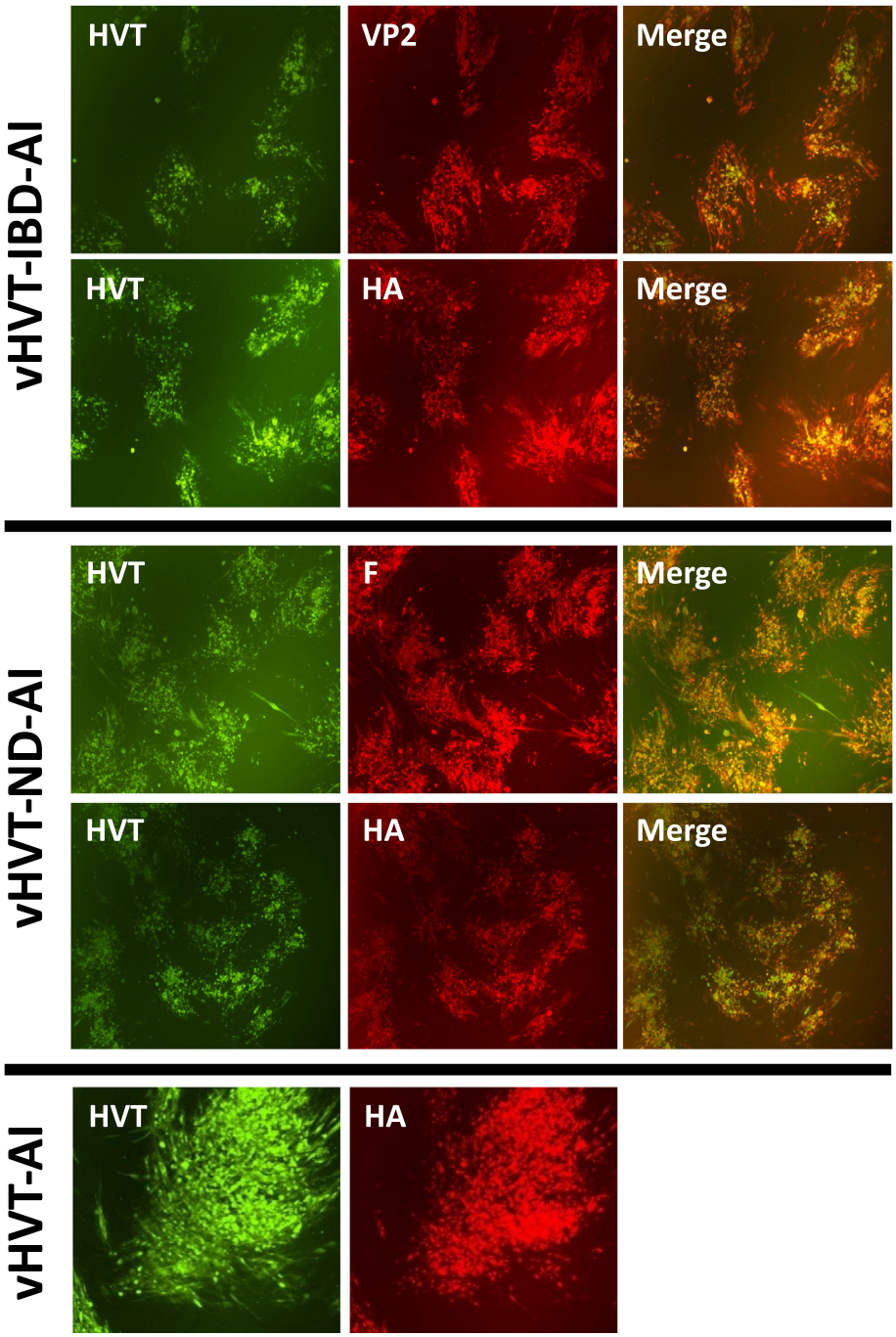

Fig. 2. Indirect immunofluorescence antibody (IFA) assay. Immunofluorescence staining showing the expression of HVT, protein 2 (VP2) of infectious bursal disease virus (IBDV), Hemagglutinin (HA) of H5 avian influenza virus (AIV), fusion (F) protein of Newcastle disease virus (NDV) for all vaccine candidates.

[17,42,43]. Problems can arise due to the chemical incompatibility or genetic mismatch (sequence and location) of the inserts in the vector [42,43]. Any of these complications can affect the vector replication, the expression of the inserts, and/or the production of the complete protein once inside the host, which ultimately can impact the generation of a good immune response against the vector and/or inserts [42]. Therefore, it is crucial to analyze the stability of any viral vector, especially multivalent viral vectors, during the early stages of vaccine development [28]. Here, we confirmed that vHVT vaccine candidates containing H5 AIV insert alone (vHVT-AI) or H5 AIV insert combined with VP2 IBDV insert (vHVT-IBD-AI) or F NDV insert (vHVT-ND-AI) were stable in the expression of both vector and inserts after 12 rounds of amplification in cell culture. Analyzes were confirmed by IFA assay, PCR, and sequencing reactions. Subsequent *in vivo* efficacy studies with these constructs further confirmed protection levels consistent with single vHVT constructs.

Another challenge for a multivalent poultry vaccine is identifying the ideal insert for HPAIV. The control of HPAIV is one of the greatest challenges for the poultry industry. Over time, H5N1 HPAIV Gs/GD lineage diverged into multiple phylogenetically and antigenically distinct clades and subclades based on the H5 HA gene [9]. Consequently, antigenic variants resistant to many vaccine seed strains have emerged [43], which has created challenges

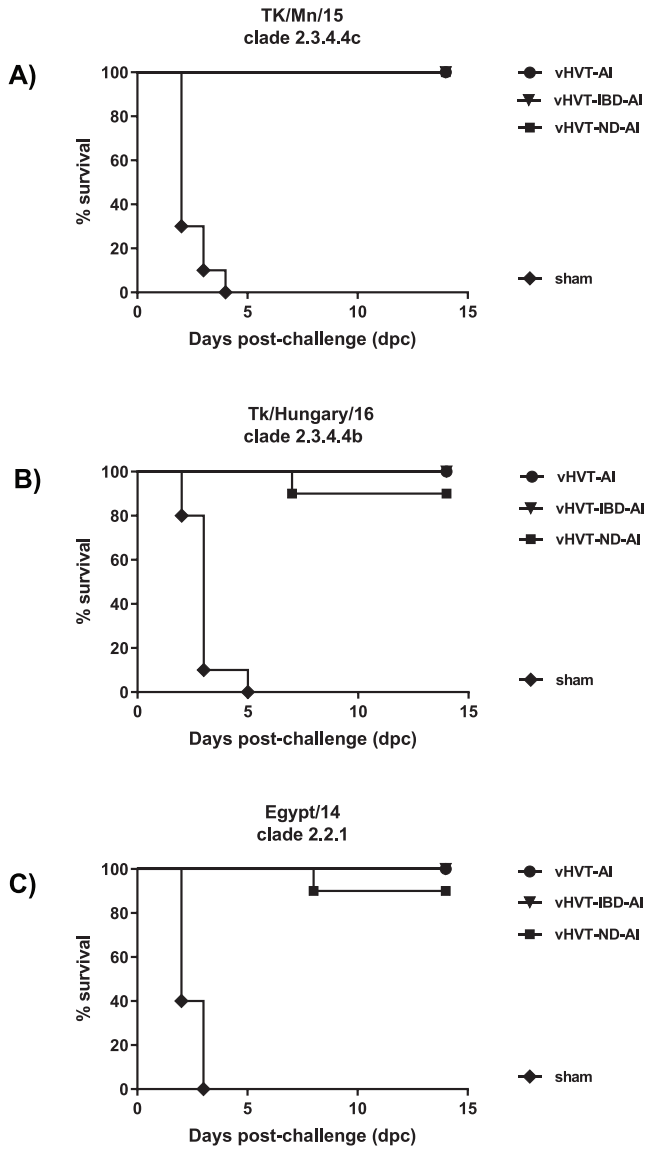

Fig. 3. Survival curves of chickens vaccinated with experimental H5 COBRA-, IBDV-, and NDV-multivalent vHVT vaccines and challenged with a. clade 2.3.4.4c A/turkey/Minnesota/12582/2015 H5N2 HPAIV (Tk/MN/15), b. clade 2.3.4.4b A/domestic turkey/Hungary/53433/2016 H5N8 HPAIV (Tk/Hungary/16) or c. clade 2.2.1 A/Egypt/N04915/2014 H5N1 HPAIV (Egypt/14).

in maintaining relevant H5 seed strains for poultry vaccines [44]. As a result, even vHVT-H5 vaccines which traditionally demonstrated promising results against a wide range of H5Nx HPAIVs of the Gs/GD lineage [45–51] may confer variable protection against genetically divergent HPAIVs [52–54]. COBRA technology, is one of the vaccine strategies in poultry, which generate antigens with novel H5 HA consensus sequences [29,30].

In previous studies in mammals, COBRA H5 antigen virus-like particle (VLP) vaccines protected against lethal challenge with homologous and heterologous H5N1 HPAIV, showing more efficient viral clearance and broader antibody responses against different clades and sub-clades than monovalent or polyvalent vaccines [29,32,33]. In our previous study, COBRA H5 VLP vaccines provided clinical protection in chickens challenged with a lethal dose of homologous H5N1 HPAIV, clade 2.2 (A/whooper swan/Mongolia/244/2005) [55]. However, even if these vaccines elicited a robust HA antibody response, they provided no or partial clinical protection against a drifting H5N1 HPAIV variant, clade 2.3.2.1b (A/duck/Vietnam/NCVD-672/2011), and reduction of virus shedding was suboptimal with both challenge strains [55]. To overcome the limitations of non-replicating vaccine platforms, vHVT vaccines were tested in our recent study in chickens as a vaccine platform for COBRA H5 inserts [30]. While both wild-type H5 and COBRA H5 inserts provided clinical protection and significant reduction of viral shedding against homologous and heterologous H5 HPAIV Gs/GD, COBRA H5 inserts elicited broader antibody responses against antigenically diverse strains [30]. These results suggested that the combination of COBRA technology with the self-replicating vHVT could improve H5Nx HPAIV control in the field.

In the present study, all three vHVT vaccine candidates, being COBRA H5 insert alone (vHVT-AI) or combined with VP2 IBDV insert (vHVT-IBD-AI) or F NDV insert (vHVT-ND-AI), provided 90–100% clinical protection against clade 2.3.4.4c, clade 2.3.4.4b, and clade 2.2.1 HPAIVs. All vaccines were also able to significantly decrease OP shedding titers compared to the sham vaccine and elicit neutralizing antibodies against clade 2.3.4.4 variants and other Gs/GD clade. This was especially noteworthy on groups vaccinated with vHVT-AI and vHVT-IBD-AI, which also tended to elicit high HI titer and had significantly fewer birds shedding challenge virus than the sham group. The vHVT-ND-AI vaccine was less efficient in reducing viral shedding, in line with relatively lower pre-challenge HI antibody titers. Some vaccinated survivors lacked detection of pre-challenge HI antibodies, especially in the vHVT-ND-AI vaccinated groups. This suggests that the presence of pre-challenge HI antibody titers against the challenge virus may be a positive predictor for survival, but HI titers < 3 $\log_2$ GMT may not be a consistent negative predictor with antigenic variants, as previously observed [19,30,54–58]. It is evident that viral vector vaccines do not necessarily produce high levels of antibodies as measured by HI, and protection in poultry 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 [24,46,52,53,57–59]. Future studies are still necessary to evaluate which of these pathways are activated and are essential in protecting birds against the lethal HPAIV challenge when using our vHVT vaccines.

It is also important to highlight that all the surviving birds had significantly higher antibody titers in the post-challenge sera (14 dpc) compared to pre-challenge sera, with similar patterns observed in other studies using the vHVT vaccines with a single insert [25,31]. Thus, it suggests that multivalent vaccines using vHVT did not change the patterns of antibody production. The only exception was HVT-AI vaccinated group challenge with Tk/Hungary/16. In this case, additional experiments with more birds per group may increase the statistical power to observe differences between pre-and post-challenge.

In general, homologous or closely related vaccine virus-challenge virus in the same genetic clade provides the best protection for AIV. In our study, the HA amino acid sequence from the challenge viruses compared to the vaccine COBRA C insert varied between 90.3% and 96.1% (Table S2), with no significant difference in protection between challenges. These results showed no direct correlation between HA amino acid differences and evaluated parameters, such as protection, shedding, or antibody titers. A possible explanation, as shown in a previous study, suggested that specific changes in critical antigenic sites might be a better predictor of protection than the overall sequence identity of the AIV HA protein [60]. Thus, some essential epitopes, alone or in combination, may affect vaccine protection regardless of the clades of the H5Nx challenge viruses [60]. In addition, the COBRA technology overcomes genetic differences in genetic variants detected in the field due to broader antibody responses against different clades and sub-clades, as shown in previous studies [30,33]. Together,

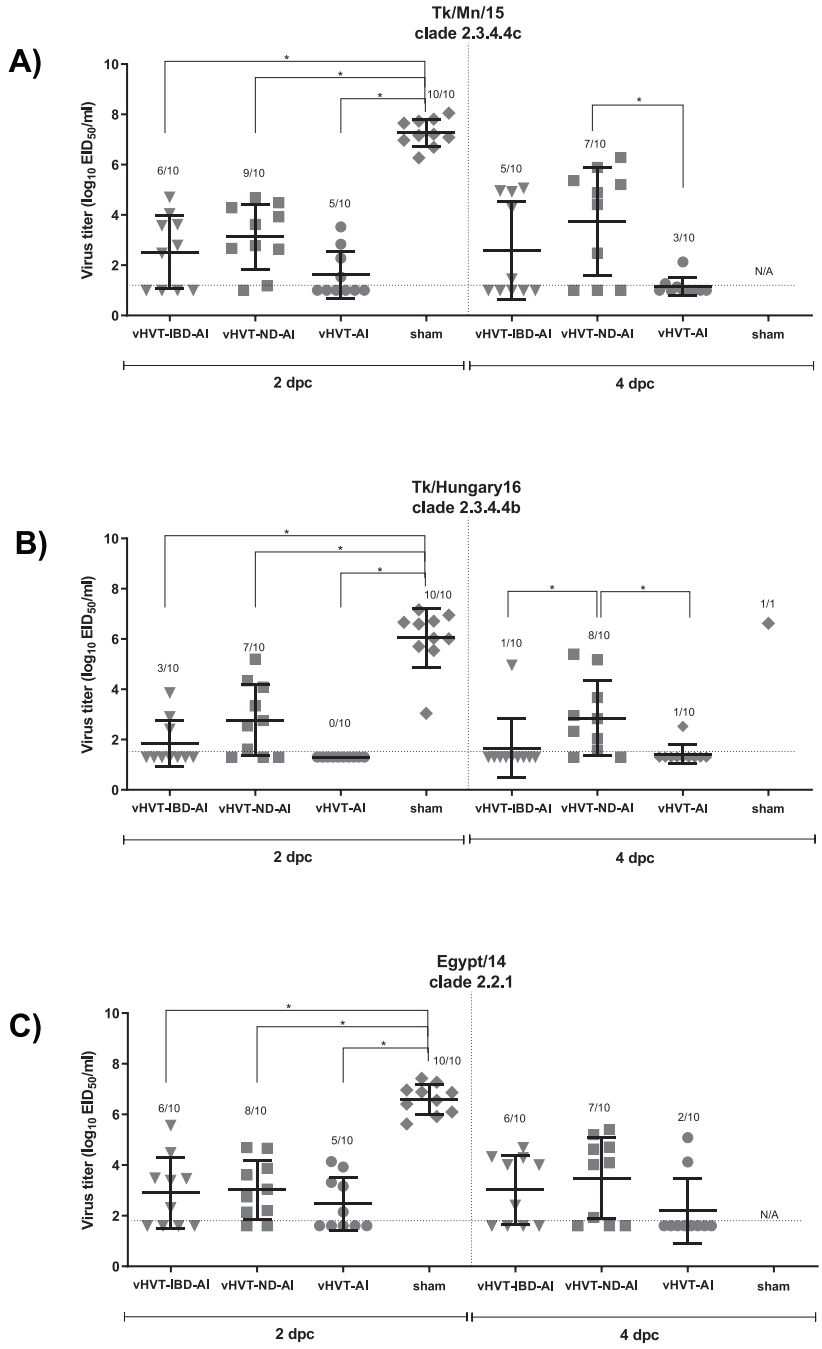

Fig. 4. Scatter plot of oropharyngeal (OP) shedding from chickens vaccinated with experimental H5 COBRA-, IBDV-, and NDV-multivalent vHVT vaccines and challenged with a. clade 2.3.4.4c A/turkey/Minnesota/12582/2015 H5N2 HPAIV (Tk/MN/15), b. clade 2.3.4.4b A/domestic turkey/Hungary/53433/2016 H5N8 HPAIV (Tk/Hungary/16) or c. clade 2.2.1 A/Egypt/N04915/2014 H5N1 HPAIV (Egypt/14). Shedding titers are expressed as equivalent $\log_{10}$ EID_{50} (50 percent embryo infectious doses) /ml with error bars. Ratios above the bars indicate the number of birds with positive qRRT-PCR values from the total number of birds. The limit of detection was 1.1 $\log_{10}$ EID_{50}/ml for Tk/MN/15, 1.4 $\log_{10}$ EID_{50}/ml for Tk/Hungary/16, and 1.7 $\log_{10}$ EID_{50}/ml for Egypt/14. For statistical purposes, negative qRRT-PCR samples were assigned a value 0.1 lower than the corresponding limit of detection. Statistical significance was declared at p value ≤ 0.05.

all of these explain the good efficiency of protection against divergent clades of H5 HPAIV in our study.

The vHVT-IBD-AI vaccine was also tested against classical STC and variant-E IBDV strains. On the one hand, the assessment of protection against classical STC IBDV was performed early (4 dpc) by evaluation of gross lesions in the bursa, which demonstrated acute effects of the classical STC IBDV as well as 100% protection conferred by the vHVT-IBD-AI vaccine. The assessment of protection against variant-E IBDV strain was performed by B/BW ratio calculation at 11 dpc, as previously showed as a useful tool for evaluating the impact of IBDV challenge and vaccination in homogeneous SPF WL chickens [61]. The statistically significant differences between mean B/BW ratios of the vHVT-IBD-AI vaccinated and challenged group compared to the sham-vaccinated and challenged group indicated protection of the bursa. The high levels of clinical protection and bursal integrity following classical and variant IBDV challenges are consistent with previous studies against classical and variant IBDV strains using vHVT expressing VP2 IBDV insert alone or combined with inserts of other pathogens [16,42,61–63]. The vHVT-ND-AI vaccine was also tested against

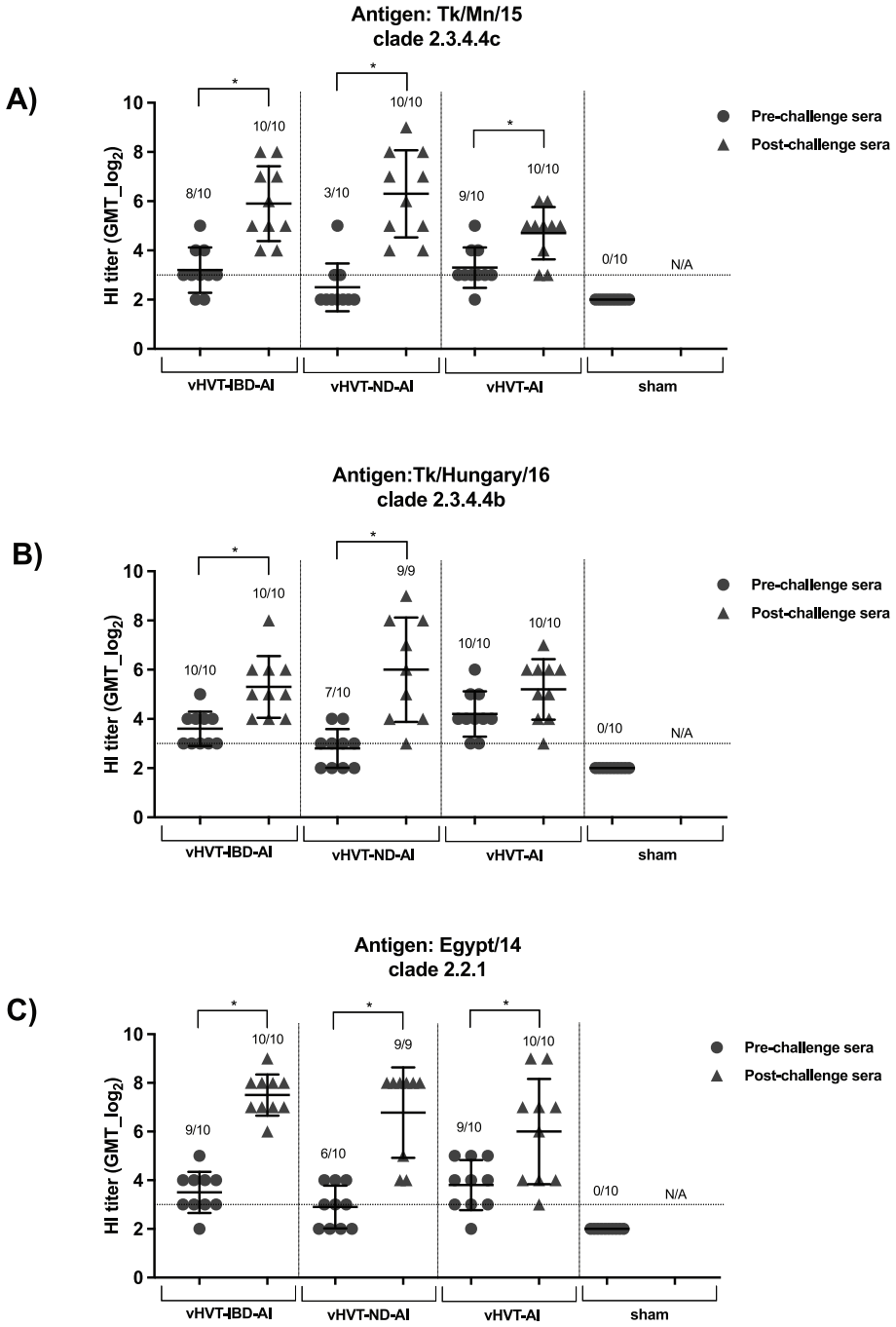

Fig. 5. Serology of chickens vaccinated with experimental H5 COBRA-, IBDV-, and NDV-multivalent vHVT vaccines and challenged with a. clade 2.3.4.4c A/turkey/Minnesota/12582/2015 H5N2 HPAIV (Tk/MN/15), b. clade 2.3.4.4b A/domestic turkey/Hungary/53433/2016 H5N8 HPAIV (Tk/Hungary/16) or c. clade 2.2.1 A/Egypt/N04915/2014 H5N1 HPAIV (Egypt/14). HI antibody titers against corresponding challenge viruses pre- and post-challenge. Titers are expressed as $\log_2$ GMT. Samples with titers below 3 $\log_2$ GMT were considered negative and expressed as 2 $\log_2$ GMT for statistical purposes. Ratios above the bars indicate the number of birds with HI titers from the total number of birds. N/A indicated that sham-vaccinated challenged birds were either dead or euthanized due to clinical signs before collection of post-challenge serum.

velogenic Texas GB NDV strain, which provided 100% clinical protection against lethal challenge. This high level of clinical protection against velogenic NDV is similar to previous studies with vHVT expressing F NDV insert alone or in combination with inserts of other pathogens [16,26,42,64–68].

Vaccines for viral disease in poultry are essential in endemic countries to control infection. Despite several advantages of vHVT vaccines, as with any other vaccine, it has some limitations and should be combined with different strategies to obtain the best outcome. Some vHVT vaccines require four weeks before full immunity can be reached [16,69], which could be a challenge for endemic countries. One important strategy to overcome these issues is a prime-boost vaccination, usually combining initial vaccination with vHVT followed by lived or killed vaccination to increase immunity and protect birds against infection [6,12]. Vaccination should be combined with enhanced biosecurity measures and constant epidemiological surveillance. Biosecurity measures will help to reduce the possibility of introducing and spreading zoonotic infectious agents, providing protection to animals and humans. Finally, epidemiological surveillance helps to identify

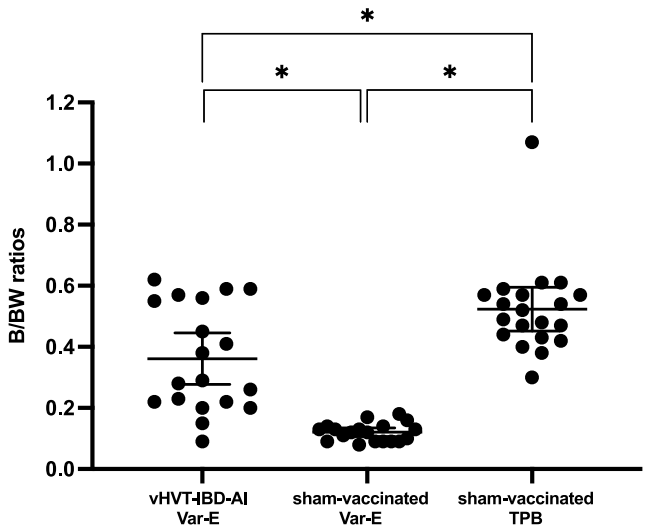

Fig. 6. Bursa-body weight (B/BW) ratios (11 dpc) from chickens vaccinated with vHVT-IBD-AI or sham-vaccinated and challenged with Var-E IBDV strain or sham-challenged with TPB. Statistical significance was declared at p value ≤ 0.05.

emergent variant viruses circulating in the field, develop a plan for disease prevention and control, and consequently improve vaccination effectiveness by identifying ideal targets.

In summary, the main objective of this study was to evaluate the feasibility of multivalent vHVT vaccine to confer multi-level protection against HPAIV challenges, plus efficacy against IBDV and NDV challenges based on clinical protection. Further studies using more discriminating indicators of vaccine efficiency, like virus shedding or antibody titers, are needed for IBDV, NDV and MDV. In conclusion, the present study demonstrates that the replicating vHVT vaccine platform with COBRA H5 insert alone or in combination with VP2 IBDV or F NDV provides clinical protection and significant reduction of viral shedding against challenge with diverse Gs/GD H5 HPAIVs. In addition, HVT vector vaccines with dual inserts also provided clinical protection against classical IBDV, variant IBDV, and virulent NDV. Our findings demonstrate that insertion of multiple genes from two different pathogens within the HVT genome had no adverse effect on the expression, stability, and *in vivo* efficacy against HPAIV, IBDV, and NDV challenges, rendering HVT vector vaccines an efficacious tool for simultaneous control of HPAIV and other viral infections. Therefore, the combination of COBRA and vHVT vaccine vector technologies to generate multivalent vHVT vaccines has a great potential to improve the control of H5Nx HPAIV and other poultry pathogens in the field.

Short title: Efficacy of multivalent vector vaccines against viral infections in poultry.

CRediT authorship contribution statement

Miria F. Criado: Writing – review & editing, Writing – original draft, Methodology, Validation, Conceptualization. **Aemro Kassa:** Writing – review & editing, Resources, Methodology, Conceptualization, Validation. **Kateri Bertran:** Writing – review & editing, Writing – original draft. **Jung-Hoon Kwon:** Writing – review & editing, Methodology. **Mariana Sá e Silva:** Project administration, Writing – review & editing, Writing – original draft, Methodology, Validation, Conceptualization. **Lindsay Killmaster:** Writing – review & editing. **Ted M. Ross:** Writing – review & editing, Resources. **Teshome Mebatsion:** Project administration, Writing – review & editing, Resources, Conceptualization. **David E. Swayne:** Funding acquisition, Supervision, Project administration, Writing – review & editing, Resources, Methodology, Conceptualization.

Data availability

The data that support the findings of this study are provided in the figures and tables of the article. Additional information is available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics

The study was conducted in accordance with guidelines of the Institutional Animal Care and Use Committee (IACUC) at the US National Poultry Research Center, Athens, Georgia (USNPRC; Animal Use Proposal# USNPRC-2020-019) and Boehringer Ingelheim Animal Health US Inc, Athens, Georgia (Animal Use Proposals # 18-39 M-04/19; 18-20 M-03/19; and 17-32 M-10/18).

Data availability

The data that support the findings of this study are provided in the figures and tables of the article. Additional information is available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2023.03.055.

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