

REIVINDICACIONES

1. Una vacuna recombinante que comprende un vector de adenovirus aviar serotipo 9 (FAdV-9) que tiene insertada por lo menos una secuencia de nucleótidos exógena que codifica para por lo menos un antígeno de una enfermedad de aves y que reemplaza la región no esencial del genoma del adenovirus, y un vehículo, adyuvante y/o excipiente farmacéuticamente aceptable, caracterizada porque la secuencia de nucleótidos exógena se localiza entre los nucleótidos 491-2782 y el vector de FAdV-9 comprende la secuencia SEQ ID NO:1.

2. La vacuna recombinante de conformidad con la reivindicación 1, caracterizada además porque el vector de FAdV-9 se encuentra vivo (activo) o inactivado.

3. La vacuna recombinante de conformidad con la reivindicación 1, caracterizada además porque la secuencia de nucleótidos exógena codifica para por lo menos un antígeno de una enfermedad de interés, seleccionado entre influenza aviar, hepatitis con cuerpos de inclusión, laringotraqueitis infecciosa de las aves, enfermedad de Newcastle, infección de la bolsa de Fabricio, bronquitis infecciosa, enfermedad causada por metapneumovirus (MPNV), enfermedad de Marek, anemia infecciosa de las aves, o cualquier otro gen cuyo tamaño permita su inserción en el vector de FAdV-9.

4. La vacuna recombinante de conformidad con la reivindicación 3, caracterizada además porque la secuencia de nucleótidos exógena codifica para un antígeno del grupo que comprende hemoaglutinina (HA) del virus de influenza aviar; fibra y hexón de virus de hepatitis con cuerpos de inclusión, serotipos 4 y 8; glicoproteína B (gB) y glicoproteína D (gD) del virus de laringotraqueitis infecciosa de las aves(LTI);proteínas HN y F del virus de la enfermedad de Newcastle; proteína VP2 de virus de infección de la bolsa de Fabricio; proteína S1 y S2 de virus de bronquitis infecciosa; proteína F de metapneumovirus (MPNV); y proteína VP1, VP2 y VP3 de anemia infecciosa de las aves.

5. La vacuna recombinante de conformidad con la reivindicación 4, caracterizada además porque la HA del virus de influenza aviar se selecciona de por lo menos uno de los subtipos H1, H2, H3, H5, H6, H7 o H9 de dicha proteína.

6. La vacuna recombinante de conformidad con la reivindicación 1, caracterizada además porque la secuencia de nucleótidos exógena comprende un gen que codifica para por lo menos un antígeno de un adenovirus aviar diferente al FAdV-9 utilizado como vector viral.

7. La vacuna recombinante de conformidad con la reivindicación 1, caracterizada además porque la concentración del virus requerida para lograr la respuesta antigénica es de por lo menos de $10^{5.0}$ DICC50%.

8. La vacuna recombinante de conformidad con la reivindicación 7, caracterizada además porque la concentración del virus requerida para lograr la respuesta antigénica es de por lo menos $10^{6.0}$ DICC50%.

9. La vacuna recombinante de conformidad con la reivindicación 2, caracterizada además porque cuando la vacuna se encuentra viva (activa), los vehículos farmacéuticamente aceptables son soluciones acuosas, preferiblemente seleccionadas del grupo que consiste de soluciones acuosas que comprenden estabilizador TPGA (trehalosa, fosfato, glutamato, albúmina); soluciones acuosas que comprenden estabilizador de peptonas; y soluciones acuosas que comprenden leche descremada.

10. La vacuna recombinante de conformidad con la reivindicación 2, caracterizada además porque cuando la vacuna se encuentra inactivada, los vehículos farmacéuticamente aceptables son soluciones acuosas o emulsiones.

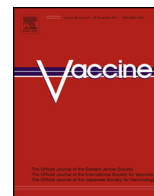
11. La vacuna recombinante de conformidad con la reivindicación 10, caracterizada además porque el vehículo farmacéuticamente aceptable es una emulsión agua-aceite; una emulsión aceite-agua; o una emulsión agua-aceite-agua.

12. La vacuna recombinante de conformidad con la reivindicación 2, caracterizada además porque la vacuna viva o activa está preparada para administrarse

por vía intramuscular, vía intranasal, vía subcutánea, aspersión, nebulización, oral a través del agua de bebida, o *in ovo*.

13. La vacuna recombinante de conformidad con la reivindicación 2, caracterizada además porque la vacuna inactivada está preparada para administrarse por vía intramuscular o subcutánea, preferiblemente por vía subcutánea.

14. La vacuna recombinante de conformidad con la reivindicación 1, caracterizada además porque comprende una vacuna de virus completo contra la enfermedad de Marek.



Suboptimal protection against H5N1 highly pathogenic avian influenza viruses from Vietnam in ducks vaccinated with commercial poultry vaccines



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ABSTRACT

Domestic ducks are the second most abundant poultry species in many Asian countries including Vietnam, and play a critical role in the epizootiology of H5N1 highly pathogenic avian influenza (HPAI) [FAO]. In this study, we examined the protective efficacy in ducks of two commercial H5N1 vaccines widely used in Vietnam; Re-1 containing A/goose/Guangdong/1/1996 hemagglutinin (HA) clade 0 antigens, and Re-5 containing A/duck/Anhui/1/2006 HA clade 2.3.4 antigens. Ducks received two doses of either vaccine at 7 and at 14 or 21 days of age followed by challenge at 30 days of age with viruses belonging to the HA clades 1.1, 2.3.4.3, 2.3.2.1.A and 2.3.2.1.B isolated between 2008 and 2011 in Vietnam. Ducks vaccinated with the Re-1 vaccine were protected after infection with the two H5N1 HPAI viruses isolated in 2008 (HA clades 1.1 and 2.3.4.3) showing no mortality and limited virus shedding. The Re-1 and Re-5 vaccines conferred 90–100% protection against mortality after challenge with the 2010 H5N1 HPAI viruses (HA clade 2.3.2.1.A); but vaccinated ducks shed virus for more than 7 days after challenge. Similarly, the Re-1 and Re-5 vaccines only showed partial protection against the 2011 H5N1 HPAI viruses (HA clade 2.3.2.1.A and 2.3.2.1.B), with a high proportion of vaccinated ducks shedding virus for more than 10 days. Furthermore, 50% mortality was observed in ducks vaccinated with Re-1 and challenged with the 2.3.2.1.B virus. The HA proteins of the 2011 challenge viruses had the greatest number of amino acid differences from the two vaccines as compared to the viruses from 2008 and 2009, which correlates with the lesser protection observed with these viruses. These studies demonstrate the suboptimal protection conferred by the Re-1 and Re-5 commercial vaccines in ducks against H5N1 HPAI clade 2.3.2.1 viruses, and underscore the importance of monitoring vaccine efficacy in the control of H5N1 HPAI in ducks.

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1. Introduction

The Asian lineage H5N1 highly pathogenic avian influenza (HPAI) viruses continue to circulate and cause great economic losses in poultry in Southeast Asia, the Middle East and Africa. The incidence of H5N1 HPAI infections is monitored globally due to their impact on avian health and production, as well as the sporadic zoonotic infections that raise pandemic concerns. Since the first virus isolation in 1996 from a goose in the Guangdong province

in China [1,2], H5N1 HPAI viruses have continued to evolve by point mutations and genetic reassortment events that affect their antigenicity and other phenotypic characteristics [3–6].

Historically, ducks naturally or experimentally infected with HPAI viruses developed only subclinical to mild disease [7–10]. A change was recognized beginning in 2002, when many emerging Asian lineage H5N1 HPAI viruses were shown to cause high mortality in ducks [11–16] however the reason for this increase in pathogenicity has not been clearly determined and appears to be multigenic in nature [17–21]. Although some H5N1 HPAI viruses can cause severe disease in ducks, a proportion of infected ducks remain clinically normal, and both sick and asymptomatic infected ducks shed virus into the environment providing opportunities for outbreaks in any exposed susceptible avian population.

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The duration and amount of virus shed will depend on many factors including the ducks' age and species, and the virulence of the H5N1 HPAI virus strain, with infected surviving ducks commonly shedding virus for more than seven days [22–24]. Asymptomatic infected wild migratory ducks are suspected of contributing to the spread of H5N1 HPAI viruses from Asia to other parts of the world [25–27], whereas free-range and backyard domestic ducks play a major role in perpetuating this virus in agricultural systems and in nature [23,26,28]. Free-range, as well as backyard domestic ducks have been associated with disseminating H5N1 HPAI viruses between premises [26,29–31]. Given the widespread infection of waterfowl with H5N1 HPAI viruses in certain parts of the world, reducing the risk of virus infection in domestic ducks is considered crucial for controlling the spread of H5N1 HPAI [23,32–35]. Vaccination can decrease infection rates and reduce viral shedding among infected animals, especially when enforcement of biocontainment measures is impractical. However, there is insufficient information on the efficacy of H5N1 HPAI vaccination in domestic duck species to guide disease control programs.

Vietnam is one of the countries most affected by H5N1 HPAI and the disease has remained enzootic in poultry since 2003 [36,37]. Vietnam has a highly dense domestic duck population which has been recognized as a primary factor in the spread and persistence of H5N1 HPAI in this country [37–39]. Vaccination of poultry against H5N1 HPAI, including domestic ducks, is part of the disease control strategy in Vietnam [37]. The Re-1 vaccine was used extensively to vaccinate poultry in Vietnam but was replaced in 2008 with the Re-5 vaccine, which contained antigens from a more recent field virus [40]. Despite efforts to control H5N1 HPAI in Vietnam, eradication has not been achieved, due in part to the introduction and spread of new viruses with distinct antigenic properties. The efficacy of vaccines in use should be tested against newly emerging viruses.

The objective of this study was to examine the protective efficacy of two commercial inactivated vaccines used in Vietnam to vaccinate domestic ducks against H5N1 HPAI. Three similar experiments were conducted to assess clinical and virus shedding outcomes in Re-1 or Re-5-vaccinated ducks challenged with different H5N1 HPAI viruses isolated in Vietnam in 2008, 2010 and 2011.

2. Materials and methods

2.1. Viruses and vaccines

The following H5N1 HPAI viruses were obtained from the National Center for Veterinary Diagnosis, Vietnam, and the Centers for Disease Control and Prevention, GA: A/chicken/Vietnam/NCVD-117/2008 (VN117/08) (HA clade 1.1), A/chicken/Vietnam/NCVD-185/2008 (VN185/08) (HA clade 2.3.4.3); A/chicken/Vietnam/NCVD-398/2010 (VN398/10) (HA clade 2.3.2.1.A), A/chicken/Vietnam/NCVD-421/2010 (VN421/10) (HA clade 2.3.2.1.A); A/chicken/Vietnam/NCVD-675/2011 (VN675/11) (HA clade 2.3.2.1.A) and A/duck/Vietnam/NCVD-672/2011 (VN672/11) (HA clade 2.3.2.1.B). The commercial inactivated H5N1 Re-1 and Re-5 vaccines, previously and currently used in Vietnam, produced by Harbin Veterinary Research Institute (People's Republic of China) were used to vaccinate the ducks. The Re-1 and Re-5 viruses were produced by reverse genetics to remove the polybasic amino acid cleavage site of HA. The HA and NA genes of Re-1 were from A/goose/Guangdong/1/1996 (HA clade 0), and those of Re-5 were from A/duck/Anhui/1/2006 (HA clade 2.3.4) [40]. All experiments using H5N1 HPAI viruses were performed in biosecurity level-3 enhanced facilities at the Southeast Poultry Research Laboratory (SEPRL).

2.2. Animal experiments

Three similar experiments were conducted (Table 1). Pekin ducks (*Anas platyrhynchos* var. *domestica*) were obtained at one day of age from a commercial hatchery. Serum samples were collected from 15 ducks in each experiment to ascertain that the birds were serologically negative for antibodies to the NP protein of influenza A viruses as determined by the commercial ELISA test FlockCheck AI (Idexx Laboratories, Westbrook, ME). One-week-old ducks (10 ducks/group) were vaccinated subcutaneously in the nape of the neck with 0.5 ml of Re-1 or Re-5 vaccine. An identical second vaccine dose was given at 14 days of age (experiment I) or at 21 days of age (experiments II and III). These vaccination schedules were based on our previous studies to evaluate responses with two doses of vaccine given before one month of age [41,42]. Additional groups of six to nine ducks in each experiment were not vaccinated and served as challenge controls. At 30 days of age, blood samples were collected, and all ducks were inoculated intranasally with $10^{5.0}$ EID₅₀ of the different H5N1 HPAI challenge viruses. Ducks were observed daily for clinical signs and mortality. Oropharyngeal and cloacal swabs were collected at different days post challenge (dpc) for determining viral shedding. At the end of the experiments (9 dpc for experiment I; 15 dpc for experiment II; 10 dpc for experiment III) blood was collected from all surviving ducks for antibody assays and ducks were euthanized by injection of sodium pentobarbital (5 g/ml). All bird experiments were approved by the SEPRL Institutional Animal Care and Use Committee.

2.3. Serology

Hemagglutination inhibition (HI) tests were performed to determine antibody responses against vaccine and virus challenge in the serum samples collected before challenge and at 9, 15 and 10 dpc [43]. A/goose/Hong Kong/437-6/1999 (H5N1) and A/Anhui/1/2005 (H5N1) were used as HI antigens, serving as surrogate for the homologous viruses from the Re-1 and Re-5 vaccines which are not available from the manufacturer. HI titers are reported as log₂ values, with 3 log₂ being the minimum titer considered as positive.

2.4. Determination of virus shedding

Oropharyngeal and cloacal swabs were collected in sterile brain heart infusion medium and kept frozen at –70 °C. Viral RNA was extracted using Trizol LS reagent (Invitrogen, Calsbad, CA) and the MagMAX AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX) [44]. Quantitative real time RT-PCR (qRT-PCR) was performed as previously described [45] with modifications. Briefly, qRT-PCR targeting the influenza M gene was conducted using AgPath-ID one-step RT-PCR Kit (Ambion) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Calsbad, CA). A reverse primer specifically redesigned for these Vietnam H5N1 viruses was used (5'-TCCTGCAAAGACATCTTCAAGTTTCTGCG-3'). For viral quantification, a standard curve was established with viral RNA extracted from the same titrated challenge virus [41]. Results were reported as EID₅₀/ml equivalents and the detection limit for calculated results was $10^{1.5}$ EID₅₀/ml per reaction.

2.5. HA gene sequencing and phylogenetic analysis

HA gene sequencing was performed as previously described [18] with modifications. The HA genes were amplified using a One-Step RT-PCR Kit (Qiagen, Valencia, CA) with specific HA primers described previously [18]. The RT-PCR products were purified using QIAquick gel extraction Kit (Qiagen) after agarose gel electrophoresis. Sequencing was performed with HA gene specific primers (available upon request) using the ABI BigDye

Table 1

Morbidity, mortality and mean death time of ducks vaccinated using Re-1 or Re-5 vaccines and challenged with different H5N1 HPAI viruses.

Experiment	Groups		Morbidity	Mortality	Mean death time (days)
	Vaccine	Challenge			
I	Non-vaccinated	VN/117/08 ^a	9/9	9/9	5.1 ^A
		VN/185/08 ^b	8/8	8/8	4.5 ^A
	Re-1 vaccinated	VN/117/08	0/10 ^c	0/10 ^c	– ^B
		VN/185/08	1/10 ^e	0/10 ^e	– ^B
II	Non-vaccinated	VN/398/10 ^c	8/8	8/8	5.4 ^A
		VN/421/10 ^c	8/8	8/8	4.0 ^A
	Re-1 vaccinated	VN/398/10	0/10 ^c	0/10 ^c	– ^B
		VN/421/10	0/10 ^c	0/10 ^c	– ^B
	Re-5 vaccinated	VN/398/10	3/10 ^c	0/10 ^c	– ^B
		VN/421/10	1/10 ^e	1/10 ^e	7.0 ^B
III	Non-vaccinated	VN/675/11 ^c	2/6	2/6	6.0 ^A
		VN/672/11 ^d	6/6	6/6	3.5 ^A
	Re-1 vaccinated	VN/675/11	1/10	0/10	– ^A
		VN/672/11	5/10	5/10	5.4 ^A
	Re-5 vaccinated	VN/675/11	0/10	0/10	– ^A
		VN/672/11	0/10 ^e	0/10 ^e	– ^B

^a HA clade 1.1.^b HA clade 2.3.4.3.^c HA clade 2.3.2.1.A.^d HA clade 2.3.2.1.B; – = not applicable.^e Significant difference in number of birds presenting morbidity and mortality compared to non-vaccinated group; Fisher's exact test, $P < 0.05$. Different superscript uppercase letters denote significant difference for survival when compared to the non-vaccinated group (Log rank) Mantel–Cox test. $P < 0.05$.

Terminator 1.1 reaction mix and the ABI 3730XL automated DNA sequencer (Applied Biosystems). The sequence of the vaccine seed viruses was obtained from GenBank and/or GISAID databases. The gene and protein sequence from each virus was compared with the vaccine virus sequence using DNASTAR program (Madison, WI) and BioEdit. Phylogenetic trees were constructed by neighbor-joining analysis of HA gene nucleotide alignments using MEGA5. The GISAID accession numbers for the HA sequences of the challenge viruses are: A/chicken/Vietnam/NCVD-185/2008: EPI284451; A/chicken/Vietnam/NCVD-117/2008: EPI284449; A/chicken/Vietnam/NCVD-398/2010: EPI284468; A/chicken/Vietnam/NCVD-675/2011: EPI405128 A/chicken/Vietnam/NCVD-421/2010: EPI284470; and A/duck/Vietnam/NCVD-672/2011: EPI330995.

2.6. Statistical analysis

Data was analyzed using Prism v.501 software (GraphPad Software Inc., La Jolla, CA, USA). The survival rate data was analyzed using the Mantel–Cox Log-Rank test. Morbidity, mortality, and number of birds shedding virus were tested for statistical significance using Fisher's exact test. Significant difference for mean viral titers between vaccinated groups and non-vaccinated controls was done using the unpaired t test. A P -value of <0.05 was considered to be significant.

3. Results

3.1. Protective efficacy of H5N1 vaccines: morbidity and mortality outcomes

Five of the six H5N1 HPAI viruses used to challenge vaccine immunity in ducks were lethal for non-vaccinated control ducks, except with the VN 675/11 virus (HA clade 2.3.2.1.A) in experiment III. This virus caused 33% mortality with a mean death time (MDT) of 6 days (Table 1). The most prominent clinical signs in non-vaccinated H5N1-challenged ducks were lethargy, anorexia, prostration, and neurological signs, similar to previous reports [16,17]. The survival of the vaccinated and non-vaccinated ducks is

shown in Supplementary Fig. 1. The mortality of ducks vaccinated with Re-1 and Re-5 and challenged with viruses from 2008 and 2010 was significantly reduced when compared to non-vaccinated ducks ($P < 0.05$). These ducks were significantly protected against H5N1 HPAI with minimal mortality and clinical signs (transient mild lethargy and anorexia). In contrast, the Re-1-vaccinated ducks were only partially protected from A/VN/672/11 (HA clade 2.3.2.1.B) virus challenge; these animals developed clinical signs similar to those of non-vaccinated controls, and 50% of the animals died with a MDT of 5.4 days. However, Re-5-vaccinated ducks challenged with VN/672/11 were fully protected against mortality and clinical signs. Re-1 or Re-5-vaccinated ducks challenged with VN/675/11 (HA clade 2.3.2.1.A) did not show clinical signs or mortality, but this was not significantly different than what seen with the non-vaccinated controls.

3.2. Serum antibody responses to vaccination and infection

Serological responses to vaccination and virus challenge were assessed by HI assays which detect antibodies specific to the HA glycoprotein (Fig. 1). The majority of vaccinated ducks developed pre-challenge HI titers ($\geq 4 \log_2$) when using the vaccine virus antigen in the test (surrogate homologous virus), indicating most vaccinated ducks seroconverted. However, some vaccinated ducks had HI titers under the limit considered positive ($\leq 3 \log_2$). The antibody response to the challenge viruses (heterologous virus relative to the vaccine) measured on samples collected before challenge, revealed differential HI responses; 100% of Re-1-vaccinated ducks showed cross HI responses to the VN 117/08 (HA clade 1) virus antigen with 5 mean $\log_2$ titer, in contrast, only one duck from the group challenge with the VN 185/08 (HA clade 2.3.4) was positive. Lower levels of HI cross reactivity was observed with samples from Re-1-vaccinated ducks where 0–50% were positive when using the 2010 and 2011 viruses in the test (HA clade 2.3.2.1) and ducks vaccinated with Re-5 were 0–70% positive. As expected, high post-challenge titers were observed in more than 80% of the vaccinated challenged ducks that survived in all three experiments when using the vaccine virus in the HI tests ($\geq 7 \log_2$). 80–100% of the vaccinated challenged ducks that survived had positive titers when using the challenge

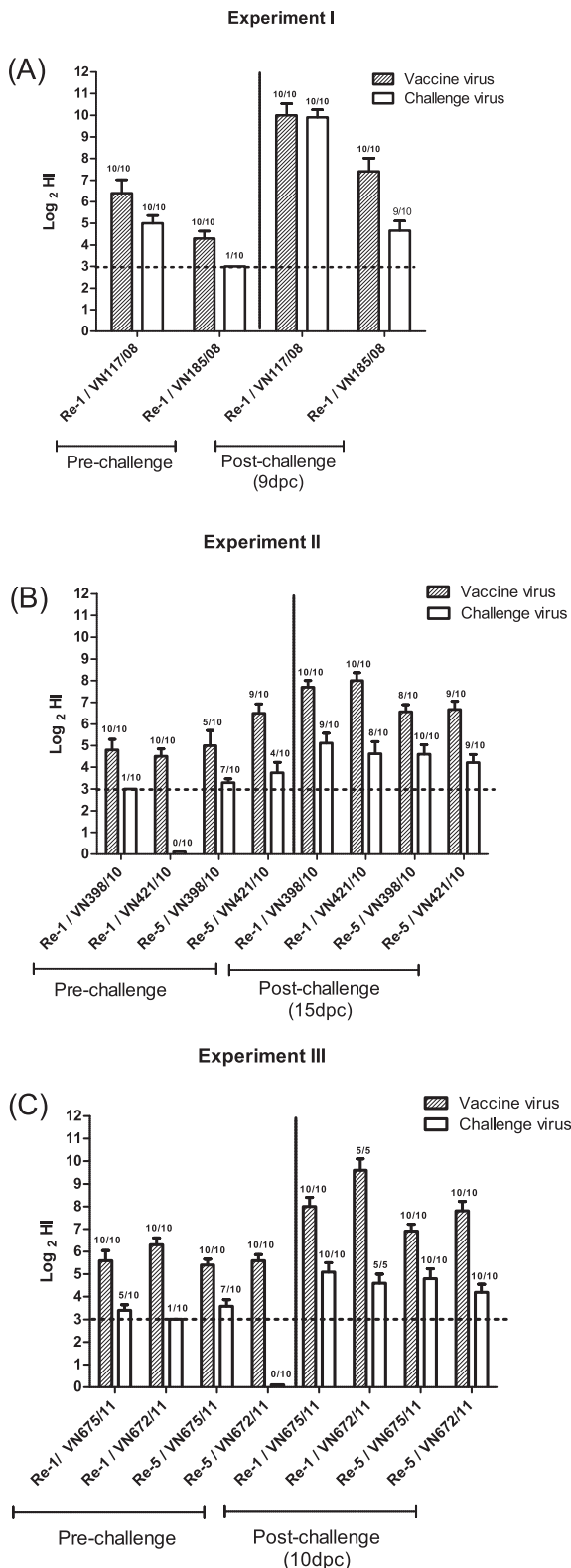


Fig. 1. Mean HI titer ($\log_2$) in Re-1 or Re-5 vaccinated-challenged ducks. Duck were vaccinated with the Re-1 or Re-5 vaccines two times, at the one week of age and 7 or 14 days later. Serum was collected at 30 days of age, before challenge, and 9–15 days post-challenge to examine the antibody responses. The number of ducks with positive HI titer is shown above each group [number of positive sera ($\geq 3 \log_2$)/total number of sera tested]. Bars represent standard deviation within groups.

virus in the HI tests ($\geq 4 \log_2$) indicating exposure and replication of the challenge viruses.

3.3. Protective efficacy of H5N1 vaccines: virus shedding outcomes

Virus shedding was monitored by quantification of viral RNA in oropharyngeal and cloacal samples collected at different time points after virus challenge. Limited virus shedding was noted at 3 and 5 dpc in ducks vaccinated with Re-1 and challenged with either of the two H5N1 HPAI viruses from 2008 (Table 2). Significantly less number of vaccinated ducks were shedding virus, and in less quantity, when compared to the non-vaccinated controls at 3 dpc. No virus shedding was detected at 7 dpc from any of the vaccinated ducks. In contrast, 40–60% of ducks vaccinated with Re-1 and challenged with either one of the 2010 viruses were still shedding at 7 dpc (Table 3). The Re-5 conferred more effective protection against 2010 viruses, with only 10–20% of ducks shedding at this time point. Of note, 40–90% of ducks vaccinated with Re-1 or Re-5 and challenged with the VN 675/11 virus were still shedding virus at 10 dpc (Table 4). 50–100% of ducks vaccinated with either vaccine and challenged with the VN/672/11 virus were still shedding virus at 10 dpc. Nonetheless, the vaccines significantly reduced the amount oropharyngeal virus shedding when compared to controls.

3.4. Amino acid changes in the HA sequences of challenge viruses

The HA gene sequences of the two vaccines were compared with those from the H5N1 HPAI challenge viruses to determine the similarity among them (Table 5; Supplemental Table 6). The challenge viruses were isolated in different years and belong to different HA clades or subclades (Supplemental Fig. 2). The viruses isolated in 2008, HA clades 1.1 and 2.3.4.3, showed 94.6% and 94.5% amino acid (aa) similarity with the Re-1 vaccine and had 22 aa changes in the mature HA1 proteins but only 21 or 3 substitutions, respectively, compared to Re-5. The viruses from 2010, both HA clade 2.3.2.1.A, showed 92.1% and 92.3% aa similarity with the Re-1 vaccine (31 and 32 aa in HA1) and 94.5% and 94.4% with the Re-5 vaccine (21 and 22 aa in HA1). The viruses from 2011, HA clade 2.3.2.1.A and 2.3.2.1.B, showed the least similarity with the vaccines, 92.4% and 91.6% with Re-1 (30 and 40 HA1 substitutions, respectively), and 94.9% and 93.1% with Re-5 (21 and 35 HA1 substitutions, respectively). The aa differences between the vaccines and challenge viruses HA were mostly found in the HA1 subunit protein region with a high proportion of these occurring in previously described antigenic sites. In addition to these substitutions, several changes between vaccine strains and challenge viruses were predicted to result in gain or loss of N-linked glycosylation at position 154–156. Relative to Re-1, clade 2.3.4 and 1.1 viruses possess a putative gain in glycosylation. Relative to Re-5, the clade 2.3.2.1 viruses demonstrate a loss of glycosylation. The lowest aa similarity (91.6%) and largest number of HA1 substitutions was found between the Re-1 vaccine and the VN 672/11 virus, this combination also showed the least protection in vaccinated ducks.

4. Discussion

In this study we examined the protective efficacy of two commercial inactivated vaccines in domestic ducks against infection with increasingly divergent H5N1 HPAI viruses isolated in Vietnam in 2008, 2010 and 2011. Despite control efforts, H5N1 HPAI viruses continue to circulate in Vietnam within various poultry populations, including domestic ducks. Introduction of new H5N1 genotypes as well as local evolution of viruses through mutation and reassortment continue to occur [6,46]. In the present study, we used viruses belonging to different HA clades and all, with the

Table 2

Experiment I, virus shedding. Ducks were vaccinated with the Re-1 vaccine and challenged with VN117/08 or VN185/08 H5N1 HPAI viruses.

Groups		Virus titers from swabs collected at different days post-challenge (dpc) # of positive ducks/total ducks ^a (Mean titer $\pm$ SD) ^b					
Vaccination	Challenge virus	3 dpc		5 dpc		7 dpc	
		OP ^c	C ^d	OP	C	OP	C
Non-vaccinated	VN 117/08	9/9 (4.7 $\pm$ 0.3) ^A	6/9 (2.0 $\pm$ 0.5) ^A	–	–	–	–
Re-1	VN 117/08	3/8 ^e (2.4 $\pm$ 1.2) ^B	0/8 ^e (–) ^B	4/10 (2.2 $\pm$ 1.4)	3/10 (1.5 $\pm$ 0.8)	0/10 (–)	0/10 (–)
Non-vaccinated	VN 185/08	8/8 (4.9 $\pm$ 1.0) ^A	7/8 (2.7 $\pm$ 0.8) ^A	–	–	–	–
Re-1	VN 185/08	2/10 ^e (2.5 $\pm$ 1.7) ^B	1/10 ^e (2.7) ^B	1/10 (1.6 $\pm$ 0.6)	0/10 (1.0 $\pm$ 0.1)	0/10 (–)	0/10 (–)

^a Swab samples were taken from all ducks remaining at each day post-challenge.^b Log₁₀ EID₅₀-equivalents were determined by qRT-PCR, numbers in parenthesis represent the mean of the viral titers $\pm$ standard deviation.^c Oropharyngeal swabs.^d Cloacal swabs; – = not applicable.^e Significant difference for number of positive ducks by qRT-PCR compared to non-vaccinated group, $P < 0.05$. Different superscript uppercase letters denote significant difference for mean viral titers between vaccinated and non-vaccinated groups, $P < 0.05$. For statistical purposes all swabs without viral RNA detection were given a numeric value of 1.5 Log₁₀ EID₅₀-equivalents.

exception of one of the 2011 viruses, caused severe clinical signs and mortality in non-vaccinated control ducks. The high virulence of H5N1 HPAI viruses in young domestic ducks has been previously reported and is not exclusive to a specific HA clade [16,47].

Many studies have shown that inactivated whole virion oil-adjuvanted vaccines are effective in protecting domestic ducks against H5N1 HPAI [41,42,48–52]. The efficacy of vaccines is a key factor in the success of control programs which rely on them to reduce virus spread. From 2005–2009, 90% of the national domestic duck and 50% of national chicken populations in Vietnam were vaccinated against H5N1 and the majority of outbreaks reported in 2005–2009 were in non-vaccinated ducks [37]. However, more recently, reports have emerged in H5N1 enzootic countries of vaccinated poultry flocks with outbreaks of HPAI [53]. Our studies demonstrate that the efficacy of two inactivated vaccines routinely used in Vietnam, Re-1 and Re-5 to protect ducks against H5N1 HPAI varied depending on the virus used for challenge. Ducks vaccinated with Re-1 were well protected when challenged with viruses from 2008. A decrease in protection, however, was observed in ducks vaccinated with Re-1 or Re-5 and challenged with the viruses from 2010 and 2011, with a substantial proportion of ducks having prolonged virus shedding, and in the case of A/VN/672, some mortality. Most ducks showed detectable HI titers after the prime-boost vaccine regimen, indicating that vaccination stimulated humoral antibody responses to the surrogate homologous virus. HI titers to the challenge virus antigens were also observed, albeit lower than the homologous viruses; however, these titers were not always associated with protection conferred by vaccination. Lack of antibodies detected by HI in vaccinated birds is not a predictor of

the complete lack of immunity, as previously reported [41,48,54]. Assays that better correlate the immune response to vaccination with the outcomes of infection with wild type viruses are urgently needed [55].

The HA of the 2008 H5N1 HPAI viruses had the highest aa similarity with the Re-1 vaccine HA sequence, consistent with previous reports indicating that HA similarity between vaccine and challenge virus is often associated with higher vaccine efficacy. Interestingly, VN 117/08 and VN 185/08 belong to clade 1.1 and 2.3.4.3, respectively, but the ducks were equally well protected after vaccination with the Re-1 vaccine (HA clade 0). These results recapitulate our previous findings showing that the Re-1 vaccine protected ducks against morbidity when challenged with H5N1 HPAI viruses isolated in Vietnam 2005 and 2007 with heterologous HA clades [41,42,48]. These studies underlined the importance of antigenic similarity between the vaccine and the virus challenge on the protective efficacy in ducks, but also highlighted the importance of other factors such as the vaccination regimen, the duck species and the virulence of the challenge virus used.

Despite protection against morbidity and mortality, a large proportion of vaccinated ducks challenged with the 2010 and 2011 H5N1 HPAI viruses were shedding virus and for longer periods, suggesting inadequate protection. Although the Re-5 is a more recently updated vaccine, it did not provide superior protection to one of the clade 2.3.2.1 (group B) viruses (VN/672/11). While the Re-5 vaccine had a greater similarity to the clade 2.3.4 viruses circulating in Vietnam until 2010, these viruses were suddenly replaced by the new clade 2.3.2.1 viruses. Consequently, the replacement of Re-1 with Re-5 vaccine (clade 2.3.4) in Vietnam did not meet the objective

Table 3

Experiment II, virus shedding. Ducks were vaccinated with Re-1 or Re-5 vaccine and challenged with VN395/10 or VN421/10 H5N1 HPAI viruses.

Groups		Virus titers from swabs collected at different days post-challenge (dpc) # of positive ducks/total ducks ^a (Mean titer $\pm$ SD) ^b					
Vaccination	Challenge virus	3 dpc		5 dpc		7 dpc	
		OP ^c	C ^d	OP	C	OP	C
Non-vaccinated	VN 398/10	8/8 (5.9 $\pm$ 0.4) ^A	8/8 (3.7 $\pm$ 0.5) ^A	5/5 (5.5 $\pm$ 0.5) ^A	5/5 (3.8 $\pm$ 0.4) ^A	3/3 (4.4 $\pm$ 0.8) ^A	3/3 (3.3 $\pm$ 0.5) ^A
Re-1	"	8/10 (3.8 $\pm$ 1.0) ^B	9/10 (3.5 $\pm$ 1.6) ^A	8/10 (3.0 $\pm$ 0.6) ^B	8/10 (3.0 $\pm$ 0.8) ^A	6/10 (2.5 $\pm$ 0.3) ^B	4/10 ^e (2.2 $\pm$ 0.5) ^B
Re-5	"	8/10 (3.2 $\pm$ 1.1) ^B	5/10 ^e (2.5 $\pm$ 0.2) ^B	4/10 ^e (2.3 $\pm$ 0.5) ^B	2/10 ^e (2.3 $\pm$ 0.3) ^B	2/10 ^e (1.8 $\pm$ 0.7) ^B	2/10 ^e (2.2 $\pm$ 0.1) ^B
Non-vaccinated	VN 421/10	5/5 (6.2 $\pm$ 0.5) ^A	5/5 (4.0 $\pm$ 0.7) ^A	3/3 (5.6 $\pm$ 0.3) ^A	3/3 (4.5 $\pm$ 1.3) ^A	1/1 (4.6) ^A	1/1 (3.9) ^A
Re-1	"	10/10 (3.7 $\pm$ 1.2) ^B	10/10 (3.1 $\pm$ 0.4) ^A	3/10 (2.4 $\pm$ 0.1) ^B	4/10 ^e (2.5 $\pm$ 0.3) ^B	2/10 (2.1 $\pm$ 0.4) ^B	4/10 ^e (2.3 $\pm$ 0.2) ^B
Re-5	"	9/10 (3.2 $\pm$ 1.8) ^B	6/10 (2.2 $\pm$ 0.4) ^B	3/10 (2.4 $\pm$ 0.1) ^B	5/10 (2.4 $\pm$ 0.3) ^B	1/10 (1.9) ^B	1/10 (2.0) ^B

^a Swab samples were taken from all ducks remaining at each day post-challenge.^b Log₁₀ EID₅₀-equivalents were determined by qRT-PCR, numbers in parenthesis represent the mean of the viral titers $\pm$ standard deviation.^c Oropharyngeal swabs.^d Cloacal swabs.^e Significant difference for number of positive ducks by qRT-PCR compared to non-vaccinated group, $P < 0.05$. Different superscript uppercase letters denote significant difference for mean viral titers between vaccinated and non-vaccinated groups, $P < 0.05$. For statistical purposes all swabs without viral RNA detection were given a numeric value of 1.5 Log₁₀ EID₅₀-equivalents.

Table 4
Experiment III, virus shedding. Ducks were vaccinated with Re-1 or Re-5 vaccine and challenged with VN675/11 or VN672/11 H5N1 HPAI viruses.

Groups	Challenge virus	Virus titers from swabs collected at different days post-challenge (dpc) # of positive ducks/total ducks ^a (Mean titer ± SD) ^b									
		3 dpc		6 dpc		8 dpc		10 dpc			
Vaccination		OP ^c	C ^d	OP	C	OP	C	OP	C		
Non-vaccinated	VN 675/11	6/6 (6.5 ± 0.7) ^A	6/6 (4.0 ± 0.4) ^A	4/4 (5.4 ± 0.4) ^A	4/4 (4.2 ± 0.3) ^A	4/4 (5.1 ± 1.0) ^A	3/4 (3.2 ± 1.6) ^A	4/4 (4.7 ± 0.3) ^A	4/4 (3.6 ± 0.3) ^A		
Re-1	"	9/10 (4.4 ± 1.0) ^B	4/10 ^e (2.6 ± 0.5) ^B	9/10 (2.6 ± 0.6) ^B	4/10 ^e (2.5 ± 0.2) ^B	6/10 (2.4 ± 0.1) ^B	4/10 (2.3 ± 0.2) ^A	4/10 (2.5 ± 0.2) ^B	1/10 ^e (3.2) ^B		
Re-5	"	7/10 (3.2 ± 0.8) ^B	5/10 ^e (2.4 ± 0.3) ^B	10/10 (3.4 ± 0.3) ^B	10/10 (3.0 ± 0.3) ^B	9/10 (2.9 ± 0.3) ^B	9/10 (2.8 ± 0.2) ^A	7/10 (3.4 ± 0.3) ^B	9/10 (3.1 ± 0.2) ^B		
Non-vaccinated	VN 672/11	5/5 (6.9 ± 0.3) ^A	5/5 (4.5 ± 0.4) ^A	–	–	–	–	–	–		
Re-1	"	10/10 (5.7 ± 0.6) ^A	10/10 (3.5 ± 0.5) ^B	6/6 (3.3 ± 0.9)	5/6 (2.3 ± 0.4)	5/5 (3.1 ± 0.5)	5/5 (2.3 ± 0.2)	5/5 (3.1 ± 0.4)	4/5 (2.3 ± 0.3)		
Re-5	"	10/10 (4.8 ± 1.2) ^B	10/10 (3.0 ± 0.4) ^B	5/10 (2.7 ± 1.2)	2/10 (1.8 ± 0.5)	4/10 (2.1 ± 0.3)	4/10 (2.1 ± 0.2)	5/10 (3.0 ± 1.1)	3/10 (2.0 ± 0.3)		

^a Swab samples were taken from all ducks remaining at each day post-challenge.
^b Log₁₀ EID₅₀-equivalents were determined by qRT-PCR, numbers in parenthesis represent the mean of the viral titers ± standard deviation.
^c Oropharyngeal swabs.
^d Cloacal swabs; – = Not applicable
^e Significant difference for number of positive ducks by qRT-PCR compared to non-vaccinated group, *P* < 0.05. Different superscript uppercase letters denote significant difference for mean viral titers between vaccinated and non-vaccinated groups, *P* < 0.05. For statistical purposes all swabs without viral RNA detection were given a numeric value of 1.5 Log₁₀ EID₅₀-equivalents.

Table 5
HA sequence similarity between commercial vaccines and challenge viruses.

Experiment	Viruses	Amino acid similarity (%)	
		Re-1 A/goose/ Guangdong/1/1996 (HA clade 0)	Re-5 A/duck/ Anhui/1/2006 (HA clade 2.3.4)
I	A/Chicken/Vietnam/ NCVD-117/2008 (HA clade 1.1)	94.6	95.2
	A/Chicken/Vietnam/ NCVD-185/2008 (HA clade 2.3.4.3)	94.5	98.9
II	A/Chicken/Vietnam/ NCVD-398/2010 (HA clade 2.3.2.A)	92.1	94.5
	A/Chicken/Vietnam/ NCVD-421/2010 (HA clade 2.3.2.A)	92.3	94.4
III	A/Chicken/Vietnam/ NCVD-675/2011 (HA clade 2.3.2.A)	92.4	94.5
	A/duck/Vietnam/ NCVD-672/2011 (HA clade 2.3.2.B)	91.6	93.1

of matching the vaccine antigenic characteristics to the predominant viruses circulating in 2011 (clade 2.3.2.1). In late 2012 a new H5N1 vaccine seed strain was licensed, Re-6, which is based on a 2.3.2.1 clade virus and closer genetically to the 2.3.2.1 H5N1 viruses circulating in Vietnam in 2011.

The effectiveness of vaccines in preventing infection, disease, and transmission of antigenically divergent viruses has not been extensively studied in domestic ducks. Previous reports in chickens with H5N2 avian influenza have shown that HA sequence similarity between the vaccine and challenge virus directly relates with the ability of the vaccine to reduce virus shedding [56–58]. Other studies showed that when the vaccine and the challenge virus belong to same H5N1 subtype, and expected to have high HA homology, vaccinated ducks were completely protected against challenge virus [49,51,54]. However, other studies revealed that genetically more distant vaccines can also protect ducks against infection with H5N1 viruses [35,48,52]. Although there is a correlation between genetic and antigenic distance, it is also possible that specific genetic differences of as little as a single aa can lead to a substantial difference in antigenic distance [59]. Other variables including host differences, vaccination schedules, and the use of different adjuvants, can also affect the protection conferred by vaccines.

5. Conclusion

In this study, we showed clear differences in vaccine efficacy in protecting domestic ducks against H5N1 HPAI viruses circulating in Vietnam. A suboptimal protection was observed in the vaccinated ducks challenged with the 2010 and 2011 viruses. HA sequence similarity between the vaccines and the challenge viruses correlated with the vaccine efficacy in this study. To ensure adequate protection of domestic ducks against constantly changing H5N1 HPAI field strains, routine vaccine efficacy testing is required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2013.08.046>.

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Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection

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Abstract

Protection of chickens against avian influenza (AI) is mostly attributed to production of antibodies against the viral glycoprotein hemagglutinin, whereas less is known about the protective role of antibodies to the other surface glycoprotein neuraminidase (NA). Therefore, vaccines encoding NA antigen (*e.g.*, DNA and alphavirus-based virus like replicon particles (VRP)) or baculovirus-expressed recombinant NA (rN2) were tested for their ability to protect against highly pathogenic AI (HPAI) in chickens. Vaccination with A/Pheasant/Maryland/4457/93 (Ph/MD) rN2 protein produced significantly higher levels of NA-inhibition (NI) activity and 88% protection from HPAI H5N2 challenge than vaccination with Ph/MD N2 DNA (25% protection). Vaccination with Ph/MD N2 VRP a minimum of two times also produced high levels of NI activity and protection against HPAI challenge (63% protection). Vaccination with VRP encoding an N2 gene that was genetically distant from the challenge virus N2 failed to protect chickens. Vaccines producing higher levels of NI activity conferred partial protection, but failed to affect viral shedding. Consideration of the homology between vaccine and challenge virus isolate NA genes may provide improved immunity if high levels of NI activity are obtained.

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Keywords: Avian influenza; Neuraminidase; Vaccine

1. Introduction

The humoral immune response is vital to protect chickens from highly pathogenic avian influenza (HPAI). The primary protective antibodies are produced against the surface glycoproteins, hemagglutinin (HA). The HA gene is extremely variable in sequence and 16 defined subtypes have been described where by definition antibody to one subtype will neutralize only virus in the same subtype. Although a strong antibody response is generated to some of the internal proteins, including the nucleoprotein and matrix 1, these antibodies are not protective, but are useful for diagnostic purposes. The other surface glycoprotein, neuraminidase (NA), also elicits antibody that can be protective. The amino acid

sequence of NA protein is also extremely variable, with nine defined subtypes. The major function of NA protein is to catalyze the release of terminal sialic acid, the receptor for the virus, from host and viral glycoproteins (*e.g.*, HA). This is vital to prevent aggregation of mature influenza virions during their release from infected cells [1]. Additional evidence suggests that NA may also be involved in viral adhesion to human respiratory epithelial cells [2]. Hemagglutinin-specific antibodies are known to prevent avian influenza (AI) infection by blocking attachment to host cell receptors. However, the role of NA antibodies in protection against AI infection is less clear. The balance of humoral immune response to influenza glycoproteins is also skewed towards HA because there is approximately 2.5 times more HA than NA antigen expressed on the surface of influenza virions, and HA immunologically out competes NA [3]. Attempts to balance the humoral immune response by vaccinating with equal

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amounts of HA and NA antigen produced approximate anti-HA and anti-NA responses [4]. Yet, when purified HA or NA were administered individually, protection against influenza challenge in mice was markedly different. For example, mice vaccinated with chromatographically purified HA were completely protected against influenza morbidity, mortality and pulmonary viral shedding, whereas mice vaccinated with purified NA were not protected against infection, but shed significantly less virus in their lungs [4]. Thus, NA-specific antibodies may produce “permissive immunity” [4], which function to limit mortality because they decrease viral release from the apical surface of infected cells, but fail to prevent viral infection [5].

Numerous studies have used different NA vaccines (*e.g.*, DNA vaccines, chromatographically purified protein, baculovirus expressed recombinant protein, reassorted killed virus and others) to examine the role of NA antibodies in protection against influenza in mice or ferrets, yet much less is known of their contribution in chickens. Rott et al. were first to demonstrate a protective role of NA antibodies in chickens infected with AI [6]. McNulty et al. demonstrated that administration of killed H7N1 virus protected against H5N1-associated mortality, but failed to completely protect against clinical disease because most vaccinated birds developed conjunctivitis post-challenge [7]. Webster et al. reported production of serum neuraminidase inhibition (NI) activity in chickens after vaccinating with inactivated virus, vaccinia virus encoding NA or purified NA. Vaccination with purified NA or killed virus completely protected against homologous challenge (H5N2), but did not protect against heterologous challenge (H7N7) [8]. Qiao et al. recently demonstrated production of NA-specific antibodies in chickens following administration of recombinant fowl-pox virus encoding H5 and N1, which were completely protected against H5N1 challenge [9]. However, an NA-only control was not included in this study, which makes it difficult to determine a role of NA-antibodies using this vaccine.

Vaccines engineered to express high levels of NA *in vivo* are needed to better understand the role of NI activity in protection against AI. Recent advances in genetic vaccines (*e.g.*, eukaryotic expression plasmid DNA and vectored replication-deficient viruses) have made it possible to specifically characterize the role of antibodies targeted to specific antigens. Although plasmid DNA vaccines are easy to produce in large quantities, major disadvantages include lack of ability to mass administer and the necessity to administer with transfection agents or other adjuvants to enhance transgene expression *in vivo*. Using a needle and syringe, intramuscular injection of large quantities of DNA are required (*e.g.*, 50–200 µg per vaccine) to induce a humoral immune response, yet it is estimated that less than 0.1% of plasmid DNA is transfected into cells. In contrast, replication-deficient viruses (*e.g.*, adenovirus, retrovirus, alphavirus and others) transduce a broad range of host cells for enhanced expression of vaccine genes, and have also been safely used for gene therapy and vaccine delivery in a

variety of species, including chickens [10,11]. The alphavirus genus consists of 24 different viruses, including Venezuelan equine encephalitis virus (VEE) [12]. Alphavirus-based virus like replicon particles (VRP) vaccines are engineered with the foreign target gene replacing structural genes, whereas four non-structural genes are left intact at the 5' end of the genome to allow for robust transcription and translation of the target gene [13,14]. Although production of VRP vaccines require complex transfections, they have the distinct advantage of higher rates of target gene expression *in vivo* [15]. Based on the fundamental differences between DNA, VRP and recombinant protein vaccines, we sought to determine whether different types of vaccines could produce protective levels of NI activity to protect against HPAI challenge.

2. Materials and methods

2.1. Animals

Two week old white leghorn chickens from the Southeast Poultry Research Laboratory (SEPR) specific pathogen free (SPF) flocks were vaccinated and housed for 6 weeks in a biosafety level 2 animal facility, and were moved to biosafety level 3 Ag facility for challenge with a highly pathogenic AI (HPAI) virus. Care was provided as required by the Institutional Animal Care and Use Committee.

2.2. Viruses

Stock cultures of HPAI A/chicken/Queretaro/14588-19/95 (Ck/Queretaro; H5N2), passaged in chickens before archiving [16], low pathogenic A/chicken/Pennsylvania/13609/93 (Ck/PA; H5N2), low pathogenic A/turkey/Ohio/313053/04 (Tk/OH; H3N2) and A/pheasant/Maryland/4457/93 (Ph/MD; H5N2) were maintained at –70 °C in the virus repository at SEPR. The egg infectious dose 50% (EID₅₀) of the challenge inocula was calculated using the method of Reed and Muench [17]. Briefly, day 10 embryonating chicken eggs (ECE) were infected with 100 µL of 10-fold dilutions in brain heart infusion medium (BHI; Difco, Detroit, MI) from stock virus. Eggs candled daily for 3 days to detect deaths, and were chilled overnight at 4 °C before harvesting amniotic fluid (AAF) and testing for hemagglutination. Incubation of chicken RBCs with control serum or PBS served as negative controls.

2.3. Alphavirus vaccination

Groups of 8 white leghorn chickens at 2 weeks of age were vaccinated subcutaneously in the neck every 2 weeks with 10⁷ VEE VRP (Alphavax, Inc., Research Triangle Park, NC) encoding the N2 gene from either Ph/MD or Tk/OH. Those chickens receiving three doses of VRP encoding Ph/MD or Tk/OH N2 were administered at 2, 4 and 6 weeks of age, and others vaccinated with two doses of VRP encoding Ph/MD

N2 were administered at 2 and 4 weeks of age and PBS at 6 weeks of age. To determine if priming the immune system with Ph/MD N2 expressed by VRP or DNA vaccine affected production of NI activity, chickens were vaccinated at 2 weeks of age in the pectoral muscle with 100 µg of low endotoxin (≤ 100 EU/mg DNA) plasmid (Aldevron; Fargo, ND) in pCI vector (Promega, Madison, WI) with lipofectin transfection reagent (Invitrogen, Carlsbad, CA) [18] or 10^7 VRP encoding Ph/MD N2 administered subcutaneously. Afterwards, chickens were boosted twice at 4 and 6 weeks of age with 10^7 VRP encoding Ph/MD N2 or 100 µg of plasmid DNA encoding Ph/MD N2, respectively. Control chickens received 100 µL of sterile PBS subcutaneously administered at 2, 4 and 6 weeks of age. Chickens were bled *via* the brachial vein 2 weeks after each vaccination. Serum was harvested and stored at -20°C prior to analysis. Chickens were challenged with HPAI at 8 weeks of age, as described below.

2.4. Baculovirus and DNA vaccination

Recombinant N2 protein (rN2) was prepared by infecting Sf9 insect cells with baculovirus encoding the Ph/MD N2 gene. Four days after infection the rN2 was harvested from Sf9 cells by DEAE column chromatography. Fractions with high levels of NA activity were pooled and prepared into an oil and water emulsion vaccine [19]. Groups of 6–8 white leghorn chickens at 2 weeks of age were vaccinated subcutaneously in the neck twice at 3-week intervals with 980 µg/0.5 mL of partially purified Ph/MD rN2. Other groups of 2-week old chickens were vaccinated twice at 3-week intervals in the pectoral muscle with 150 µg of low endotoxin (≤ 100 EU/mg DNA) plasmid encoding Ph/MD or Tk/OH N2 in pCI vector conjugated with lipofectin transfection reagent, as described above. Control chickens received 100 µL of sterile PBS subcutaneously administered at 2 and 5 weeks of age. Chickens were bled *via* the brachial vein 3 weeks after each vaccination. Serum was harvested and stored at -20°C prior to analysis. Chickens were challenged with HPAI at 8 weeks of age, as described below.

2.5. Challenge and determination of mortality and viral shedding

When the chickens were 8 weeks old, they were inoculated intranasally *via* the choanal slit with 10^6 EID₅₀/0.2 mL of highly pathogenic A/chicken/Queretaro/14588-19/95 (H5N2) [16]. Chickens were observed daily for morbidity and mortality for up to 15 days post-infection. Mortality data were analyzed and presented as Kaplan–Meier survival plots. Oropharyngeal, tracheal or cloacal swabs were sampled from infected birds at days 2–7 post-infection, suspended in 1 mL of BHI broth, and stored at -70°C . Total RNA was extracted by using an RNeasy mini kit (Qiagen), and quantitative real-time RT-PCR was performed with primers and probe specific for type A AI virus matrix RNA [20]. Qiagen one-step RT-PCR kit was used under the following conditions: 1 µL of

enzyme mixture including RT and Taq polymerase, 10 pmol of each primer, 0.12 µM probe, 320 µM (each) deoxynucleoside triphosphate, 3.75 mM MgCl₂, 13 U of RNase inhibitor, 1 × reaction buffer, 8 µL of total RNA per sample and nuclease free water for a total quantity of 25 µL. The RT reaction was performed for 30 min at 50°C , and 15 min at 94°C to activate the Taq polymerase. The following PCR cycling protocol was used; 45 cycles of denaturation at 94°C for 1 s and annealing at 60°C for 20 s with fluorescence data acquired at the end of each annealing step using a Smart Cycler II (Cepheid Sunnyvale, CA) real-time PCR machine. In some experiments, relative amounts of viral shedding were determined by cycle threshold (C_t) values. In other experiments, quantitative viral shedding (*e.g.*, number of copies of viral RNA recovered from swab/mL of BHI) was interpolated from the sample cycle thresholds by using a standard curve generated from known amounts of control Ck/Queretaro RNA (*e.g.*, titers were exactly 10^1 – 10^6 EID₅₀/mL).

2.6. Quantitative neuraminidase inhibition (NI) assay

A fluorescent substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt hydrate (MUN; Sigma Chemical Co., St. Louis, MO) was used to measure the NA enzymatic activity [21]. Briefly, 25 µL of Ck/PA or Tk/OH stock virus were diluted 2-fold in 25 µL of calcium saline buffer (CaS; 20 mM sodium borate, 7 mM calcium chloride, 154 mM sodium chloride, 12.5 mM sodium acetate; pH 7.2) in 96-well, clear, flat-bottom plates, and 15 µL of 0.5 mM MUN was added per well. Uninfected allantoic fluid or MUN alone served as negative controls. The kinetic production of fluorescent byproduct 4-methylumbelliferone was assayed every 3 min, for a total of 30 min using a Synergy HT Multi-Detection microplate reader (BioTek, Winooski, VT) with an excitation and emission wavelength of 360 and 440 nm, respectively. The linear range of NA activity was determined by plotting fluorescence data from 15 min incubation *versus* the inverse of viral dilution ($1/x$, where $x = \log_2$ dilution factor). Twenty-five microliters of virus at the lowest end of the linear range of fluorescence was added to each well, and 25 µL of chicken serum was diluted 2-fold to produce a range of 1:2 to 1:4096. Serum and virus were incubated for 30 min at room temperature. Fifteen microliters of 0.5 mM MUN was added to each well, and fluorescence was immediately analyzed, as described above. Samples with little to no fluorescence were determined to be positive for NI activity, as compared to control chicken serum. The NI titer was determined as one $\log_2$ dilution below which fluorescence began to achieve levels similar to that of SPF control serum.

2.7. Sequence analysis

Amino acid percent identity of different NA antigens was calculated using Megalign v5.08 (DNA Star, Madison, WI) with Clustal W algorithm. Accession numbers and sequence data for NA genes are available upon request.

2.8. Statistical analysis

Data were analyzed using Prism v4.03 Software package (GraphPad Software Inc., San Diego, CA), and are expressed as mean $\pm$ standard deviation from chickens per experimental group. The Log Rank test was used to analyze Kaplan–Meier survival rate data, and one way analysis of variance (ANOVA) with Tukey–Kramer post-test were used to analyze NI and viral shedding data. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Neuraminidase inhibition activity produced by rN2, DNA or VRP vaccination

To test the hypothesis that different types of vaccines affect production of NI activity, chickens were initially vaccinated twice with either 980 μ g of baculovirus expressed Ph/MD rN2 protein or 150 μ g of Ph/MD N2 DNA. Vaccination with rN2 or DNA produced significant levels of NI activity against Ck/PA virus after each vaccination compared to control sera ($P < 0.01$; Table 1). Because of the high degree of amino acid identity between Ck/PA and the challenge virus Ck/Queretaro N2 protein (97.7%), and Ck/PA is a low pathogenic AI isolate that can be handled in biosafety level 2 facilities, Ck/PA virus was selected as the antigen for NI assays to evaluate the

immune response towards Ck/Queretaro N2. Next, the abilities of two distant N2 antigens (*e.g.*, Ph/MD and Tk/OH) to produce NI activity against CK/PA virus were tested. Amino acid sequence similarity between Ph/MD and Tk/OH N2 demonstrated a distant relation (85% identical). Chickens were vaccinated intramuscular twice at 3-week intervals with 150 μ g of either Ph/MD or Tk/OH N2 DNA. Three weeks after the second DNA vaccine, NI activity was compared against Ck/PA and TK/OH viruses. Vaccination with Ph/MD DNA produced a significant increase ($P < 0.01$) in NI activity, using CK/PA virus as antigen, as compared to sera from control chickens or chickens vaccinated with Tk/OH DNA (Table 2). Comparison of amino acids between Ph/MD and Ck/PA N2 showed 95.9% identity, whereas Tk/OH was distantly related (84.3% identical) to Ck/PA N2. To exclude the possibility that NI activity may differ when using Ck/PA or Ph/MD virus as antigen (amino acid identity is 95.9%), serum NI was tested using Ph/MD virus, and no significant difference was detected (data not shown). The specificity of NI activity produced after vaccination was compared by using Tk/OH virus as antigen. Vaccination with Tk/OH DNA produced significantly enhanced levels of NI activity against Tk/OH virus ($P < 0.001$), as compared to sera from controls or chickens vaccinated with DNA encoding Ph/MD N2 (Table 2). Lastly, chickens were vaccinated with combinations of VRP or DNA vaccines encoding Ph/MD or Tk/OH N2. Chickens vaccinated two or three times with 1×10^7 VRP encoding Ph/MD N2 produced significantly elevated NI titers

Table 1

Summary of baculovirus expressed recombinant Ph/MD N2 protein and Ph/MD N2 DNA vaccination in chickens challenged with HPAI virus

	NI titer ($\log_2$) 3 weeks after 1st vaccine	NI titer ($\log_2$) 3 weeks after 2nd vaccine (pre-challenge)	Mortalities	Mean time to death (days)	Tracheal viral shedding C_t value (day 2 p.i.)
Control	1.5 $\pm$ 0.84 ^{a†}	1.83 $\pm$ 0.75 ^a	6/6 (100%)	2.8	32.04 $\pm$ 5.76
Ph/MD DNA	3.38 $\pm$ 1.30 ^b	4 $\pm$ 1.52 ^b	6/8 (75%)	4	29.86 $\pm$ 4.94
Ph/MD rN2	4.25 $\pm$ 0.71 ^b	8.5 $\pm$ 1.39 ^c	1/8 (13%)	5	33.87 $\pm$ 3.64

Sera from control or vaccinated birds were tested for NI activity against Ck/PA virus, as described in Section 2. Values represent mean $\pm$ standard deviation $\log_2$ NI activity. Tracheal swabs were tested for viral shedding, as described in Section 2. Values represent mean $\pm$ standard deviation C_t from surviving chickens 2 days post-infection (p.i.). No statistical significant changes in viral shedding were determined (Control: chickens vaccinated twice with PBS; Ph/MD DNA: chickens vaccinated twice with 150 μ g of Ph/MD N2 DNA vaccine; and Ph/MD rN2: chickens vaccinated twice with 980 μ g/0.5 mL Ph/MD baculovirus expressed recombinant Ph/MD N2 protein.)

[†] NI titer values in each column denoted by different letters (a–c) are statistically different from each other ($P < 0.05$) as determined by ANOVA.

Table 2

Summary of Ph/MD or Tk/OH DNA vaccination in chickens challenged with HPAI virus

	NI titer ($\log_2$) 3 weeks after 2nd vaccine (pre-challenge) Ck/PA antigen	NI titer ($\log_2$) 3 weeks after 2nd vaccine (pre-challenge) Tk/OH antigen	Mortalities	Mean time to death (days)	Tracheal viral shedding day 3 p.i. (C_t value)
Control	1.38 $\pm$ 0.74 ^{a†}	1.63 $\pm$ 0.74 ^a	8/8 (100%)	1.5	NA*
Ph/MD DNA	2.36 $\pm$ 0.76 ^b	4.72 $\pm$ 1.21 ^b	17/25 (68%)	6.4	32.62 $\pm$ 2.51
Tk/OH DNA	1.36 $\pm$ 0.56 ^a	6.92 $\pm$ 1.38 ^c	18/25 (72%)	6	32.33 $\pm$ 3.41

Sera from control or vaccinated birds were tested for NI activity against Ck/PA or Tk/OH virus, as described in Section 2. Values represent mean $\pm$ standard deviation $\log_2$ NI activity. Tracheal swabs were tested for viral shedding, as described in Section 2. Values represent mean $\pm$ standard deviation C_t from surviving chickens 3 days post-infection (p.i.). No statistically significant changes in viral shedding were detected (Control: chickens vaccinated twice with PBS; Ph/MD DNA: chickens vaccinated twice with 150 μ g of Ph/MD N2 DNA vaccine; and Tk/OH DNA: chickens vaccinated twice with 150 μ g of Tk/OH N2 DNA vaccine).

[†] NI titer values in each column denoted by different letters (a–c) are statistically different from each other ($P < 0.05$) as determined by ANOVA.

* Control chickens all died before sampling at 3 days p.i.

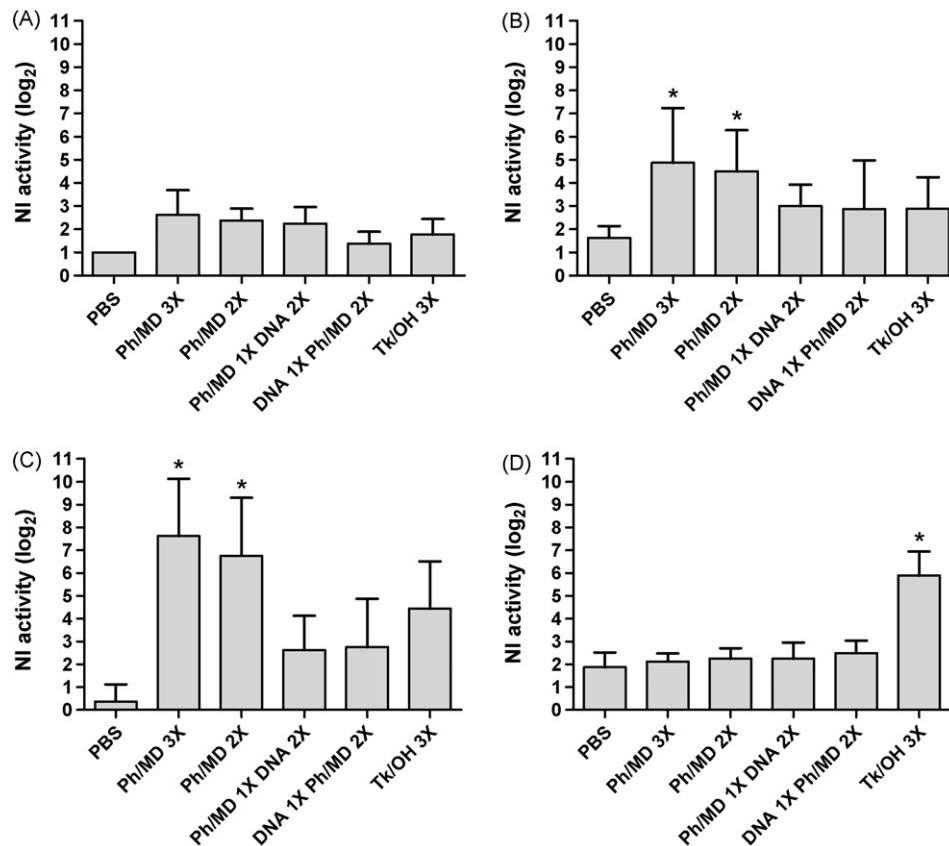


Fig. 1. Temporal response of serum neuraminidase inhibition (NI) activity after vaccination with VRP encoding Ph/MD or Tk/OH N2. Serum was collected 2 weeks after scheduled vaccinations with Ph/MD (panels A and B: NI after 1st and 2nd vaccination, and C and D after 3rd vaccination) (PBS: PBS mock vaccinated three times; Ph/MD 3×: Ph/MD N2 VRP three times; Ph/MD 2×: Ph/MD N2 VRP two times; Ph/MD 1×DNA 2×: Ph/MD N2 VRP one time and Ph/MD N2 DNA two times; DNA 1× Ph/MD 2×: Ph/MD N2 DNA one time and Ph/MD N2 VRP two times; and Tk/OH 3×: Tk/OH N2 VRP three times). Serum NI activity was determined using Ck/PA (panels A–C) and Tk/OH virus as antigen (panel D), as described in Section 2. Data represent mean $\pm$ standard deviation log₂ NI titer of eight chickens per vaccination group, performed in duplicate. Significant changes (* $P < 0.01$) in log₂ NI activity were detected, as compared to PBS vaccinated controls.

($P < 0.01$) against Ck/PA virus as antigen as early as 2 weeks after the 2nd vaccination, as compared to chickens receiving PBS control or VRP encoding Tk/OH N2 (Fig. 1A–C). There was no significant change in NI titer whether the chickens were primed with DNA or VRP encoding Ph/MD N2, and boosted with VRP or DNA, respectively. The specificity of NI activity produced by administration of Ph/MD or Tk/OH VRP was confirmed using Tk/OH virus as antigen. Only chickens vaccinated with VRP encoding Tk/OH N2 showed a significant elevation in Tk/OH virus NI activity ($P < 0.001$) (Fig. 1D). Thus, there is specificity to the immune response to VRP or DNA plasmid encoding different N2 antigens as is seen with vaccines with HA protein [18]. To determine whether production of NI was dependent on the number of VRP administered, chickens were vaccinated with 5×10^6 VRP encoding Ph/MD N2 every 2 weeks for a total of three vaccinations. Chickens receiving 50% fewer VRP produced significantly lower ($P < 0.01$) log₂ NI activity after the 3rd vaccination (4.00 ± 1.21), as compared to chickens vaccinated three times with 10^7 Ph/MD VRP (NI activity after 3rd vaccination = 7.63 ± 2.5). Overall, baculovirus expressed

rN2 and VRP encoding N2 produced the greatest increase in NI activity, whereas the DNA vaccines produced lower levels of antibody.

3.2. Morbidity and mortality after viral challenge

Comparison of amino acids between vaccine Ph/MD and challenge virus Ck/Queretaro N2 showed 97.7% identity, whereas Tk/OH N2 was 85% identical to Ck/Queretaro. Chickens vaccinated twice with 980 μ g of partially purified baculovirus expressed Ph/MD rN2 protein were significantly protected against HPAI challenge ($P < 0.01$; Table 1 and Fig. 2A), and survivors showed mild signs of clinical disease. When compared to baculovirus expressed rN2 protein, vaccination with Ph/MD N2 DNA failed to significantly protect against HPAI challenge or prevent clinical signs of disease (Fig. 2A), but did increase the mean time to death as compared to control chickens (Table 1). Similarly, there was no significant change in survival rate between chickens vaccinated with either Ph/MD or Tk/OH DNA, although their survival rates were significantly different in comparison to control chick-

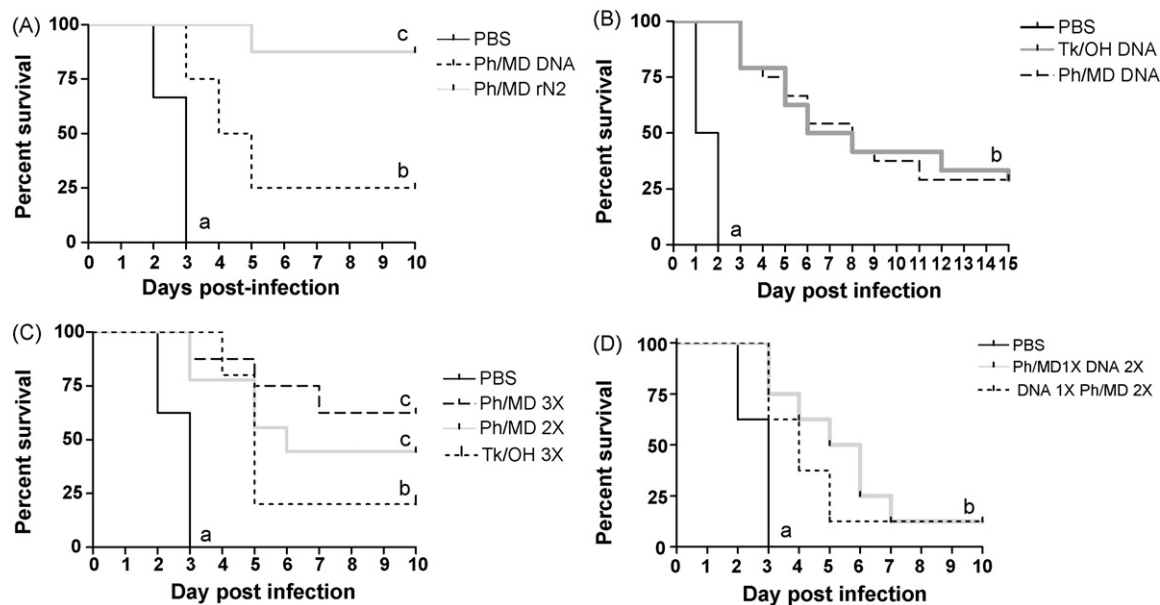


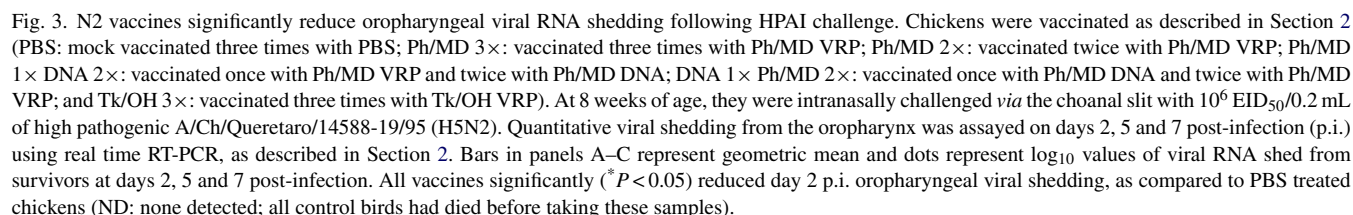
Fig. 2. Comparison of NA vaccines and survival against HPAI challenge. Chickens were vaccinated as described in Section 2. At 8 weeks of age, they were intranasally challenged *via* the choanal slit with 100 μ L of PBS containing 10^6 EID₅₀ of high pathogenic A/Ch/Queretaro/14588-19/95 (H5N2). Mortality was assessed daily for up to 15 days post-infection. Data in panel A (PBS: mock vaccinated twice with PBS; Ph/MD DNA: Ph/MD DNA vaccinated twice; and Ph/MD rN2: vaccinated twice with baculovirus expressed recombinant Ph/MD N2 protein), panel B (PBS: mock vaccinated twice with PBS; Ph/MD DNA: vaccinated twice with Ph/MD DNA; and Tk/OH DNA: vaccinated twice with TK/OH DNA), panels C and D (PBS: mock vaccinated three times with PBS; Ph/MD 3 $\times$: vaccinated three times with Ph/MD VRP; Ph/MD 2 $\times$: vaccinated twice with Ph/MD VRP; Ph/MD 1 $\times$ DNA 2 $\times$: vaccinated once with Ph/MD VRP and twice with Ph/MD DNA; DNA 1 $\times$ Ph/MD 2 $\times$: vaccinated once with Ph/MD DNA and twice with Ph/MD VRP; and Tk/OH 3 $\times$: vaccinated three times with Tk/OH VRP) represent percent of chickens surviving HP AI challenge. Values denoted by different letters are statistically different from each other ($P < 0.01$) as determined by Log Rank test.

ens ($P < 0.001$) (Fig. 2B). Similar to Fig. 2A, vaccination with either Ph/MD or Tk/OH N2 DNA increased the mean time to death in comparison to control chickens (Table 2). The survival rates for chickens vaccinated two or three times with VRP encoding Ph/MD N2 were significantly increased ($P < 0.001$) (Fig. 2C), as compared to chickens vaccinated with PBS or VRP encoding Tk/OH N2. Chickens vaccinated with VRP encoding Tk/OH N2 showed a significant change in survival rate ($P = 0.0013$) as compared to control chickens. None of the VRP vaccine strategies provided 100% protection, but the mean time to death for birds vaccinated two or three times with VRP encoding Ph/MD N2 was 4.8 and 5 days, respectively, compared to 2.9 days for control chickens. Likewise, chickens vaccinated with VRP encoding Ph/MD N2 which survived infection showed only mild clinical signs of disease such as conjunctivitis, and did not show the severe clinical signs seen in PBS or Tk/OH VRP vaccinated birds which included edematous and cyanotic wattles and comb, severe conjunctivitis, depression, lethargy and subcutaneous hemorrhage. The survival rate of chickens primed with Ph/MD DNA or VRP, and boosted with VRP or DNA, respectively, was significantly elevated ($P = 0.0043$ and 0.0017 , respectively) in comparison to control chickens (Fig. 2D), and an increase in the mean death time was observed (e.g., 4.4 and 4.9 days, respectively). However, survival rates from chickens vaccinated with combinations of VRP and DNA vaccines encoding Ph/MD N2 were signif-

icantly less ($P < 0.05$) than chickens vaccinated three times alone with VRP encoding Ph/MD N2. These data indicate that vaccines which produced the highest levels of NI activity closely related to the challenge virus significantly enhanced survival from HPAI.

3.3. Viral shedding

The effect of different NA vaccines on viral RNA shedding from the oropharynx or trachea of infected chickens was determined by real time RT-PCR for AI matrix RNA. Although different vaccines affected production of NI activity and survival from HPAI infection, there were no significant changes in tracheal viral RNA shedding C_t values from chickens vaccinated with Ph/MD rN2 protein or DNA encoding Ph/MD N2 (Table 1), or comparing Ph/MD *versus* Tk/OH N2 DNA (Table 2). Similarly, vaccination with Ph/MD rN2 protein or DNA encoding Ph/MD N2 had no effect on cloacal shedding of viral RNA; C_t values (mean $\pm$ standard deviation) 35.79 ± 4.58 and 36.14 ± 3.31 , respectively. Significant differences in viral RNA shedding from the oropharynx were seen in chickens vaccinated with combinations of VRP or DNA encoding Ph/MD N2 ($P < 0.05$) 2 days post-infection, compared to control chickens (Fig. 3A). However, there were no significant changes in oropharyngeal viral RNA shedding detected from survivors between different vaccine groups on days 5 and 7 post-infection (Fig. 3B and C).



The immune response to NA can provide protection in chickens against HPAI challenge when high levels of NI activity are produced. Vaccination with VRP encoding Ph/MD N2 or baculovirus expressed recombinant Ph/MD N2 protein produced the highest levels of NI activity against Ck/PA virus, whereas vaccination with N2 DNA produced less NI activity. The former were partially protected against HPAI challenge, and the opposite was true for the latter. Analysis of the N2 vaccine antigens used in the present study, Ph/MD and Tk/OH, demonstrated 85% amino acid identity. The inclusion of vaccines encoding TK/OH N2 was to determine whether a distantly related NA gene of the same

subtype could protect against heterologous HPAI challenge. A recent isolate from turkeys, the origin of this virus is likely the triple reassortant swine viruses commonly found in the United States [22]. The swine H3N2 viruses that have circulated in the United States since the late 1990s are thought to have obtained their HA and NA genes from a human influenza virus and the internal genes are of swine, human and avian influenza origin [23]. Vaccinating chickens with VRP encoding Tk/OH N2 produced modest increases in NI activity when using Ck/PA as antigen, but significantly higher levels of NI activity against the Tk/OH antigen. In spite of the high levels of NI antibody to Tk/OH, they were not protected from HPAI challenge. The poor protection from heterologous Tk/OH antigen was unexpected since it has been reported that

mice were protected using DNA vaccines encoding NA that shared 89% amino acid sequence similarity compared to the lethal influenza challenge [24]. In the same study, protection was not obtained with less stringently matched vaccine (N1) and challenge (N2) virus (*e.g.*, 42% amino acid identity) [3].

Mortality results from the present study are consistent with those obtained previously in chickens, where vaccination with NA similar to the challenge virus protected against mortality, but not morbidity [7,8]. Results indicate that vaccination with Tk/OH N2 does not induce high enough levels of protective NI activity because of the sequence difference to the challenge virus. It appears that NI activity produced by vaccines should be closely related to the challenge strain NA, even within the same subtype, to protect against HPAI infection. The level of sequence similarity needed for protection between vaccine and challenge strains was not determined in these studies, but levels of NI activity produced also need to be considered for prediction of protection.

This is the first report describing quantitation of post-vaccination NI activity using MUN as a substrate. The test provides rapid, sensitive and reproducible quantitative NI data to evaluate immune response against NA vaccine antigens, and may serve to differentiate infected from vaccinated animals. Vaccination with VRP encoding Ph/MD N2 or Ph/MD recombinant baculovirus expressed N2 protein were superior to DNA vaccines with respect to NI production, and levels of NI activity correlated with protection against HPAI challenge. This study highlights the need for high levels of NI activity to provide clinical protection. The most commonly used vaccine in the poultry industry, adjuvanted killed whole virus, produce NI activity, but NA is present in much lower quantities than HA. Thus, these vaccines likely result in little protection from the NA component of the vaccine to the challenge strain. This was previously reported using killed AI vaccines generated by reverse genetics that differ only in the NA subtype, which demonstrated there was no effect on virus shedding regardless of the vaccine NA subtype present in the vaccine [25]. Supplementation with exogenous NA may be a solution to a poor immune response towards vaccine NA, which has been reported in mice [26].

In our studies, baculovirus expressed recombinant N2 protein produced the greatest level of protection, but also required a large quantity of partially purified protein (*e.g.*, 980 μ g). Administration of 72% less (273 μ g/0.5 mL) of partially purified Ph/MD rN2 produced low levels of NI activity (2.13 ± 0.64) levels, albeit significantly elevated above control chickens (0.38 ± 0.74), and failed to significantly protect (2/8) against HPAI challenge. These results indicate that a large quantity of recombinant protein may be required to protect, but the minimum amount necessary to provide protection is not known.

This is the first investigation into the protective effect of VRP encoding NA on HPAI infection in chickens. The VRP vaccine transduces a diverse number of cells *in vitro*, which results in a single round of robust transgene transcription [15]. Although we did not determine which cells

were expressing VRP-transduced N2 protein after subcutaneous administration *in vivo*, NI activity produced after the 1st vaccination suggest that antigen presenting cells, in part, may have been transduced. Vaccination of chickens two or three times with VRP encoding Ph/MD N2 significantly elevated pre-challenge NI activity as early as after the 2nd vaccination, which further increased after the 3rd vaccine (Fig. 1C). Seventy-five and 80% of chickens that were vaccinated two or three times, respectively, with VRP encoding Ph/MD N2 that survived HPAI challenge had NI titers $\geq 7 \log_2$. Although a protective NI titer is not clearly identified in the present study, results indicate that NA vaccines should strive to produce NI levels to the challenge antigen of $\geq 7 \log_2$ to consistently achieve clinical protection. The duration of protective NI activity produced in response to VRP vaccination was not directly examined. A significant elevation in NI activity was detected 4 weeks after the last vaccination in chickens administered VRP encoding Ph/MD N2 two times (Fig. 1C). The production of NI was dependent on the number of VRP administered in the vaccine. A 50% reduction in VRP encoding Ph/MD N2 produced significantly decreased NI titers ($P < 0.01$). Similarly, serum HI activity was lower and AI mortality increased for chickens vaccinated *in ovo* and boosted with 90% fewer VRP encoding H5 [10].

The results of serum NI response and mortality after vaccination with DNA encoding Ph/MD N2 were unexpected. Vaccination every 2 or 3 weeks may not have been optimal for DNA vaccines used in the present studies, since monthly vaccinations are more commonly used for HA DNA vaccines [18]. It is not clear why pre-challenge NI titers from chickens vaccinated with Ph/MD N2 DNA with Ck/PA virus as antigen were not comparable to those obtained with Tk/OH N2 DNA with Tk/OH virus as antigen (*e.g.*, 2.36 ± 0.76 versus 6.92 ± 1.38 , respectively). In previous experiments, priming with DNA encoding various HAs produced modest increases in HI activity, but boosting twice produced the highest levels of HI activity [18]. Similarly, a single dose of VRP encoding H5 was partially protective against HPAI challenge, but complete protection and increased HI activity was produced after boosting [10]. Based on these findings, we hypothesized that priming and boosting with combinations of DNA or VRP would affect NI production, but we detected no significantly change in NI activity or mortality. Overall, these results indicate that plasmid DNA failed to produce high enough levels of NI activity to protect against HPAI challenge, and that mixing DNA and VRP were not useful to produce a protective immune response.

The use of the VEE vector in chickens has the advantage over other potential viral vectored vaccines like Newcastle disease virus because chickens are unlikely to be naturally exposed to this virus. A concern is that multiple vaccinations with this vector might generate an immune response to the vector, and result in immune-interference and poor expression of the transgene. Although we did not test serum for humoral response to VEE, serum NI levels continued to increase following each of three administrations. These

results suggest that immunity was not directly generated against VRP vaccine, and they can be safely administered to chicken multiple times. Additionally, there is little evidence of immune interference from preexisting immunity in humans to VRP, and have been shown to be effective for humans in the face of anti-vector responses (Personal communication with B. Hubby, Alphavax Inc.). Mass vaccination of chickens *in ovo* is a well established technique to protect chickens against Marek's disease virus. Likewise, *in ovo* vaccination with VRP encoding H5 resulted in partial protection against AI challenge [10]. Therefore, it may be possible to vaccinate *in ovo* with VRP encoding NA alone or in combination with recombinant NA protein to protect chickens against emerging AI isolates.

The effector mechanism of NI activity responsible for protecting chickens from AI is not clear, but it is clearly different from antibodies to the HA protein. One likely mechanism is the NI activity keeps AI trapped on the apical surface of infected cells [5], where they may be susceptible to cytotoxic T lymphocyte (CTL)-mediated killing. Although CTLs readily kill influenza infected cells in the respiratory tract of mice and humans [27,28], their contribution is unknown in chickens. This possibility is strengthened by reports that VRP vaccines encoding tumor antigens to produce specific CTL responses [29,30]. Serum NI activity may also activate classical complement pathway and cause disruption of viral envelope, and affect infectivity [31]. Finally, the NI activity may help aid viral clearance by an opsonin effect [32].

In summary, the immune response to NA can protect chickens against HPAI challenge when high levels of NI activity are produced. Killed whole virus vaccines are commonly used for chickens, but do not likely produce NI activity at high enough levels against NA protein [33–35] to enhance vaccine efficacy. This study, like earlier studies in mice, provides evidence that if vaccines can produce levels of NI activity over 7 log₂, protection against HPAI challenge can be achieved with just NA antigen. New technology like the VRP can produce effective levels of NI activity in chickens, and additional studies to provide practical recommendations for inclusion of NA in vaccines are warranted.

Acknowledgements

We thank Suzanne DeBlois, Joan Beck, Ronald Graham and Roger Brock at SEPRL for their technical assistance and Whitney Ellis at Alphavax Inc. for preparation of VRP. Mention of trade names or commercial products is solely for the purpose of providing specific information, and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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Señor Director

NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

Referencia: Expediente NC2017/0001614

Asunto: Solicitud de patente para: VACUNA EN VECTOR RECOMBINANTE DE
ADENOVIRUS AVIAR SEROTIPO 9

Solicitante: GRUPO INDUSTRIAL PECUARIO, S.A. DE C.V.

Fecha de solicitud: 20 de febrero de 2017

RESPUESTA A SEGUNDO EXAMEN DE PATENTABILIDAD

JOSE ANDRES RINCON USCATEGUI, identificado como aparece al pie de mi firma, en mi condición de apoderado de la sociedad solicitante de la patente de la referencia, doy respuesta al Oficio **11969**, notificado por fijación en lista el 5 de octubre de 2018.

I. MODIFICACIONES AL CAPÍTULO REIVINDICATORIO

En respuesta al examen de patentabilidad aportó un Capítulo Reivindicatorio modificado conformado por **14** reivindicaciones en las que se han incluido las siguientes modificaciones:

1. En la reivindicación 1 se ha reemplazado la palabra “interés” por “aves” y se ha incluido el fragmento “*y el vector de FAdV-9 comprende la secuencia SEQ ID NO:1*”;
2. Se han eliminado las reivindicaciones 3, 16 y 17.

Estas modificaciones se ajustan al Artículo 34 de la Decisión 486 de 2000, ya que no constituyen una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial.

II. EN CUANTO A LA SUPUESTA FALTA DE CLARIDAD

1. Según el Examinador, la reivindicación 1 no es concisa y carece de sustento porque la frase “*por lo menos una secuencia de nucleótidos exógena que codifica para por lo*

menos un antígeno de una enfermedad de interés” incluye una cantidad de secuencias exógenas que van más allá de la evidencia desarrollada por el solicitante. Por ello, el Examinador recomienda limitar esta reivindicación a una generalización razonable de lo que fue desarrollado y evaluado en la descripción.

En relación con esta objeción, el solicitante está procediendo a limitar la reivindicación 1 a una secuencia de nucleótidos exógena que codifica para por lo menos un antígeno de una enfermedad de aves, teniendo en cuenta que en los ejemplos de la presente solicitud se muestra información experimental del FAdV-9, un virus aviar, al cual le fueron insertadas en la región de interés secuencias de nucleótidos exógenas de diferente tamaño y provenientes de virus aviáres de diferente origen (familia).

2. Adicionalmente, el examinador indica que las reivindicaciones 16 y 17 carecen de sustento porque la descripción no muestra ninguna evidencia de la obtención de una vacuna que comprenda un vector FAdv-9 que tenga insertadas dos secuencias de nucleótidos exógenos en la región de interés, ni de vacunas con dos vectores FAdv-9 con dos secuencias exógenas insertadas cada una en la región de interés de cada vector. En este sentido, el Examinador sugiere también restringir la solicitud a la materia que esté debidamente sustentada en la descripción.

A fin de superar esta objeción, el solicitante está eliminando las reivindicaciones 16 y 17.

III. ESTADO DE LA TÉCNICA

El Examinador determinó dos documentos como relevantes para realizar el análisis de patentabilidad de la presente patente de invención:

1. D1, referido al documento titulado *“Antibody Response and Virus Shedding of Chickens Inoculated with Left End Deleted Fowl Adenovirus 9- Based Recombinant Viruses”* que se relaciona con la aplicabilidad de la región no esencial del extremo izquierdo del virus recombinante FAdV-9 como sitio de inserción de genes externos y su uso potencial como vacuna para aves.

2. D2, referido al documento titulado *“Development of fowl adenovirus 9 based vector vaccine expressing the hemagglutinin gene of an H5N1 influenza virus”* que se relaciona con el desarrollo de una vacuna basada en un vector FAdV-9 que comprende el

gen de hemaglutinina del virus de la influenza insertado en una región no esencial del genoma.

IV. EN CUANTO A LA SUPUESTA FALTA DE NIVEL INVENTIVO

Conforme a la opinión del Examinador, el documento D1 se considera el estado de la técnica más cercano a la invención de la reivindicación 1, al describir una vacuna recombinante de un vector de FAdV-9 que contiene un casete de expresión con el gen de la proteína verde fluorescente aumentada (EGFP) insertado entre los nucleótidos 491-2782. D1 menciona también que hubo una respuesta de anticuerpos anti EGFP detectable luego de la semana 2 con un incremento hacia el final del experimento, donde la inoculación con el vector FAdV-9Δ4-EGFP (que comprende el inserto en la región 491-2782) produjo una respuesta de anticuerpos mayor en comparación con el vector FAdV-9inEGFP (que contiene el casete de EGFP en el marco de lectura abierto 1B).

Así, señala el Examinador que la diferencia entre la materia reclamada en la reivindicación 1 y la vacuna divulgada en D1 consiste en que el vector reclamado en la solicitud comprende una secuencia de nucleótidos exógena que codifica para una enfermedad de interés y no para un gen reportero.

El efecto que se logra por el hecho de incluir una secuencia de nucleótidos exógena que codifica para una enfermedad de interés es que se consigue una vacuna estable contra una enfermedad de interés.

Por lo tanto, el Examinador considera que el problema técnico que se pretende resolver con la presente invención es “cómo modificar la vacuna divulgada en D1 para conseguir una vacuna estable contra una enfermedad de interés”.

Al respecto, señala el Examinador que la inserción de una secuencia exógena que codifica para una enfermedad de interés ya está divulgada en el documento D2, en el cual se describe una vacuna recombinante de vector de FAdV-9 que contiene la secuencia HA del virus de la influenza A insertada en una región no esencial del genoma del vector localizada en el extremo izquierdo. D2 divulga además que el vector es estable luego de cuatro pases, y que se logró la expresión de una proteína funcional de importancia como vacuna, sin comprometer la replicación del virus en cultivo celular.

Por ello, el examinador concluye que un técnico en la materia incluiría una secuencia exógena de nucleótidos según lo divulgado en D2 en la región correspondiente a los

nucleótidos 491- 2782 del vector divulgado en el documento D1, llegando así al objeto de la reivindicación 1 de la presente solicitud, por lo cual ésta se considera obvia o no inventiva, teniendo en cuenta además que D1 menciona el uso potencial de FAdV-9 como vector para desarrollar vacunas aviares basado en el remplazo de la región no esencial que se encuentra localizada en el extremo izquierdo del genoma viral, con un gen exógeno.

De manera respetuosa, el solicitante desea señalar que no está de acuerdo con la opinión del Examinador, y que la invención reclamada en la nueva reivindicación 1 de la presente solicitud es inventiva por lo menos debido a las siguientes razones:

En primer lugar, ninguno de los documentos citados describe ni sugiere las ventajas de la vacuna reclamada en la reivindicación 1, que comprende un vector de FAdV-9 que comprende la SEQ ID NO:1, ventajas tales como la estabilidad para la producción a escala industrial, efectividad y no interferencia al administrarse junto con una vacuna contra la enfermedad de Marek, entre otras.

El documento D1 únicamente se limita a mencionar que hubo una producción de anticuerpos anti EGFP en pollos inoculados con el vector FAdV9-9Δ4-EGFP, que comprende el inserto en la región 491-2782.

Al respecto, el solicitante está procediendo a presentar el documento del Anexo A, en el cual se realizó una prueba con diferentes vacunas contra influenza aviar, concluyéndose que no existe una correlación entre el título de anticuerpos y la protección contra mortalidad, ni entre el título de anticuerpos y la reducción de la eliminación viral. Asimismo, los documentos de los anexos B-D muestran que aun cuando hubo producción de anticuerpos después de la administración de diferentes vacunas, solo se logró una protección parcial o no óptima. La información contenida en estos documentos revela que, si bien puede haber producción de anticuerpos después de la administración de una vacuna, dichos anticuerpos no necesariamente producirán una protección óptima contra una enfermedad, lo cual refuerza el hecho de que la vacuna reclamada en la presente solicitud es inventiva a la luz de D1.

En cuanto a D2, este documento describe una vacuna de vector de FAdV-9 que contiene un inserto de HA del virus de influenza A, el cual está localizado en una región no esencial del genoma del vector localizada en el extremo izquierdo. Según D2, el virus recombinante resultante fue estable después de 4 pases, y el gen insertado se expresó como una proteína funcional.

No obstante, D2 no describe ni sugiere una vacuna como la de la presente invención, en la cual el virus recombinante es estable incluso después de 10 pases. Asimismo, D2 únicamente muestra la expresión del gen exógeno insertado. En este sentido, si un técnico en la materia insertara el gen de HA de D2 en el vector de D1, dicho técnico en la materia esperaría que dicho gen se expresara, y tal vez que se generaran anticuerpos contra la proteína expresada. Sin embargo, según lo señalado más arriba, esto no significaría que dichos anticuerpos producirían una protección óptima y completa contra una enfermedad, con lo cual la materia reclamada en la reivindicación 1 inventiva también a la luz de D2.

Según lo anterior la reivindicación 1, así como las reivindicaciones dependientes de la misma, son inventivas a la luz del arte previo citado.

V. PETICIÓN

1. Puesto que se ha dado respuesta a todas las objeciones formuladas por la Dirección de Nuevas Creaciones, solicito proceder al otorgamiento de la solicitud de patente.
2. Subsidiariamente, en caso de que su Despacho considere que la modificación introducida al Capítulo Reivindicatorio amerita revisión adicional, solicito un segundo examen de patentabilidad de conformidad con lo dispuesto en el Artículo 2 de la Resolución 3719 de 02 de febrero de 2016, para lo cual realizo el pago de tasa correspondiente.

VI. ANEXOS

1. Capítulo Reivindicatorio modificado conformado por 14 reivindicaciones;
2. Pago en línea por concepto de examen de patentabilidad adicional;
3. Anexos A, B, C y D;

Señor Director,

ANDRÉS RINCÓN USCÁTEGUI

C.C. 79.780.910 de Bogotá

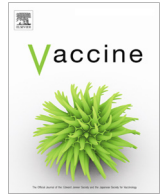
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Homologous and heterologous antigenic matched vaccines containing different H5 hemagglutinins provide variable protection of chickens from the 2014 U.S. H5N8 and H5N2 clade 2.3.4.4 highly pathogenic avian influenza viruses

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ABSTRACT

From December 2014 to June 2015, a novel H5 Eurasian A/goose/Guangdong (Gs/GD) lineage clade 2.3.4.4 high pathogenicity avian influenza (HPAI) virus caused the largest animal health emergency in US history resulting in mortality or culling of greater than 48 million poultry. The outbreak renewed interest in developing intervention strategies, including vaccines, for these newly emergent HPAI viruses. In these studies, several existing H5 vaccines or vaccine seed strains with varying genetic relatedness (85–100%) to the 2.3.4.4 HPAI viruses were evaluated for protection in poultry. Chickens received a single dose of either an inactivated whole H5 AI vaccine, or a recombinant fowl poxvirus or turkey herpesvirus-vectored vaccines with H5 AI hemagglutinin gene inserts followed by challenge with either a U.S. wild bird H5N8 (A/gyrfalcon/Washington/40188-6/2014) or H5N2 (A/northern pintail/Washington/40964/2014) clade 2.3.4.4 isolate. Results indicate that most inactivated H5 vaccines provided 100% protection from lethal effects of H5N8 or H5N2 challenge. In contrast, the recombinant live vectored vaccines only provided partial protection which ranged from 40 to 70%. Inactivated vaccine groups, in general, had lower number of birds shedding virus and at lower virus titers than the recombinant vaccine groups. Interestingly, prechallenge antibody titers using the HPAI challenge viruses as antigen in heterologous vaccine groups were typically low ($\leq 2 \log_2$), yet the majority of these birds survived challenge. Taken together, these studies suggest that existing vaccines when used in a single immunization strategy may not provide adequate protection in poultry against the 2.3.4.4 HPAI viruses. Updating the H5 hemagglutinin to be genetically closer to the outbreak virus and/or using a prime-boost strategy may be necessary for optimal protection.

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1. Introduction

The Eurasian-origin H5N8, and reassortant H5N2 and H5N1 HPAI viruses (clade 2.3.4.4) were isolated in the western U.S. in December 2014–January 2015 and caused the largest outbreak in poultry resulting in the death of approximately 48 million birds with greater than \$3.2B in economic losses [1–3]. The outbreak was the largest foreign animal disease emergency in the U.S., affecting the food supply and causing concern for public health [4]. During December 2014–June 2015, 21 states reported HPAI of H5N2, H5N8 and H5N1 subtypes in commercial premises, back-

yard flocks, captive wild birds and free-living wild birds [5]. The devastating impact to commercial poultry renewed interest in the development of vaccines against these viruses as an alternative to test and slaughter approach that was used to successfully control the outbreak.

Currently, the first line of defense against AI in poultry is focused on biosecurity, education, surveillance, rapid diagnosis, and slaughter of affected flocks to prevent spread to uninfected flocks [6]. These measures involve considerable veterinary infrastructure and financial resources to provide the control measures and provide the indemnity to flock owners affected by the disease. Because of the many hemagglutinin (HA) subtypes and antigenic variation within a subtype, routine preventative vaccination is not practical approach to prevent infection. Therefore AI

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vaccination as a preventative tool in poultry is currently considered as a secondary line of defense and depends on specific conditions including extent and impact of AI related disease, spread into areas with a high density of poultry populations, establishment of endemic infections, and increased risks of spill-over infections of zoonotic AI to humans. Successful AI vaccination will greatly reduce morbidity and mortality of exposed birds and prevent economic losses. Furthermore, vaccinated birds should shed much less virus, thus reducing risks of its further transmission and spread to susceptible birds. Reduction of the circulating virus is also important for preventing spill-over infections to humans when outbreaks are caused by zoonotic HPAI viruses.

Vaccines for AI include traditional egg-grown preparations, as well as novel recombinant approaches [7–11]. For use of egg-derived vaccines in poultry, typically low pathogenic AI virus seed stock from crude allantoic fluids is chemically inactivated and adjuvanted with an oil based emulsions. Vaccinations are done by the subcutaneous (s.c.) or intramuscular (i.m.) routes with the goal to elicit protective levels of systemic hemagglutination-inhibiting (HI) antibodies [12]. Recombinant vaccines for avian influenza include many experimental approaches including reverse genetics-derived, vectored vaccines, live-attenuated vaccines, subunit, DNA and “universal” vaccines. Live viral vector vaccines, such as herpes virus of turkeys (HVT) and fowlpox virus (FPV), continue to rise in popularity in the field due to the capacity to persistently replicate in the host, induce both humoral and cell-mediated immunity, and they are relatively easy to produce [11,13–17]. DNA vaccines have demonstrated protection against HPAI in limited studies [18,19]. In addition, many experimental vectored vaccines expressing the AI HA, including Newcastle disease virus, duck enteritis virus, infectious laryngotracheitis virus, and vesicular stomatitis virus, have demonstrated complete protection of chickens from HPAI challenge [20–23]. Subunit vaccines, including virus-like particles, have demonstrated complete protection against homologous HPAI challenges [24–26]. When properly applied, vaccination has been shown to protect poultry against morbidity and mortality, and markedly reduce virus shedding in vaccinated birds, thereby reducing transmission potential. However, the available vaccine strategies provide no universal solution for poultry vaccination so the efficacy of vaccines against novel isolates must be tested empirically.

In this study, we evaluated several currently available vaccines expressing different HA proteins derived from different H5 AIV and demonstrated that vaccination had variable protection in chickens from experimental challenges with the two 2014 U.S. H5N8 and H5N2 HPAI viruses.

2. Materials and methods

2.1. Viruses

A total of six different H5 AIVs were used in these studies as inactivated vaccine seed strains which included two wild-type low pathogenic avian influenza (LPAI) vaccine seed strains and two HPAI isolates homologous to the challenge strains from the U.S., and two recombinantly derived LPAI vaccine seed strains from China. The two U.S. low pathogenic H5 isolates were USDA approved vaccine master seed strains for H5 vaccines for poultry: A/turkey/Wisconsin/1968 H5N9 (TW68) and A/turkey/California/2002 H5N2 (TC02). The two highly pathogenic H5 U.S. viruses were A/gyrfalcon/Washington/40188-6/2014 H5N8 clade 2.3.4.4 (GW14) and A/northern pintail/Washington/40964/2014 H5N2 clade 2.3.4.4 (NW14) which were used as both homologous vaccines as well as the challenge viruses. Two reverse genetics engineered (RE) inactivated vaccine seed strains, commercially

available in China, were tested including RE-5 containing the HA gene from A/duck/Anhui/1/2006 H5N1 clade 2.3.4 (DA06) and RE-6 containing the HA gene from A/duck/Guangdong/1332/2010 H5N1 clade 2.3.2 (DG10). Both of the Chinese viruses were altered to be a LPAI virus by changing the HA cleavage site from a HPAI cleavage site to a low pathogenic cleavage site and they were assembled in a PR8 influenza A vaccine seed strain backbone. These seed strains were rescued at SEPRL from plasmids provided by Harbin Veterinary Research Institute (Courtesy of Hualan Chen and Yanbing Li).

Two additional H5 isolates, A/Whopper Swan/Mongolia/3/2005 (H5N1) clade 2.2 (WM05) and A/turkey/Ireland/1378/1983 (H5N8) (TI83), based on commercially available vaccines, were used as antigen for serology studies described below. All AIVs were propagated in specific pathogen free (SPF) embryonating chicken eggs according to standard procedures [27]. Allantoic fluid containing virus was harvested as antigen for vaccine formulation or titration of HPAI challenge virus. All experiments using HPAI viruses, including work with animals, were reviewed by the institutional biosecurity committee and were performed in biosecurity level-3 enhanced (BSL3E) facilities at SEPRL.

2.2. Vaccines

A total of eight different avian influenza virus vaccines were tested in these studies: two as live recombinant vectored vaccines, and six as inactivated vaccines (Supplemental Table 1). For the recombinants, a HVT vector AI vaccine (Vectormune® AI, CEVA Animal Health, Lenexa, KS) was constructed by inserting the HA gene of the HPAI A/swan/Hungary/4999/2006 H5N1 clade 2.2 strain into the genome of HVT FC-126 strain (rHVT-AI), and a FPV vectored vaccine (TROVAC®-AIV H5, Merial Select, Inc., Gainesville, GA) contained the FP recombinant vFP89, which expresses the HA gene of the TI83 H5N8 (rFPV-AI). The cleavage site of the HA gene used in the rHVT-AI and rFPV-AI was altered to a typical cleavage site sequence of LPAI virus strains. Both vaccines are licensed for use in the U.S., but currently neither have been used commercially in the U.S., but they have been used internationally. The live recombinant vaccines, HVT-AI and FPV-AI, were used per manufacturer's recommendations.

Individual inactivated H5 vaccines were produced with the six AIV isolates described above following growth in SPF eggs and harvesting of allantoic fluid. Following BPL-inactivation of allantoic fluid, each vaccine virus was diluted to provide a concentration of 512 HA units per 0.5 ml when mixed (70/30) with Montanide ISA VG70 oil emulsion (SEPPIC Inc., Fairfield, NJ) according to the manufacturers' recommendations [28].

2.3. Chickens

For all experiments, mixed-sex SPF chickens (White Leghorn (egg-layer type)) were obtained from and housed at the Southeast Poultry Research Laboratory in a BSL2 facility for vaccination, and transferred to a BSL3E facility for challenge. Birds were maintained in HEPA filtered isolation cabinets with access to feed and water *ad libitum*. All bird experimental procedures were approved and performed under the SEPRL Institutional Animal Care and Use Committee.

2.4. Vaccination and challenge protocol

One-day of age SPF birds (n = 10) received a recombinant AI vaccine (either rHVT-AI or rFPV-AI) delivered in a single dose subcutaneously in 0.1 ml volume per the manufacturers recommendations. At four weeks of age birds were challenged intranasally with 10^{6.5} EID₅₀ per bird of GW14 or NW14 HPAI.

At three-weeks of age SPF birds ($n = 10$ per vaccine) received a single dose delivered subcutaneously of the six inactivated vaccines. Sham vaccinated birds received PBS in 0.5 ml Montanide adjuvant as above. At six-weeks of age birds were challenged intranasally with $10^{6.5}$ EID₅₀ per bird of GW14 or NW14 HPAI. After challenges, birds were monitored daily for clinical signs, serum was taken at day 0 and day 14 post challenge (pc), with oral and cloacal swabs taken at day 2 and 4 pc to measure virus shedding.

2.5. Hemagglutination assay

Viruses were serially diluted in PBS at twofold increments in 50 μ l volume in a 96-well plate. Chicken red blood cells (RBC) were utilized as recommended by the WHO as a reagent for animal influenza diagnosis and surveillance [29]. To each virus dilution, 50 μ l of 1% chicken RBC working solution was added, mixtures of virus and RBCs were gently mixed and the plate was incubated at room temperature for 30–60 min. Negative hemagglutination results appeared as dots in the centers of the wells. The titer was calculated as the highest dilution factor that produced a positive reading.

2.6. Serology and antibody assays

Hemagglutination inhibition (HI) assay was performed using standard methods [30]. Homologous vaccine virus as well as inactivated HPAI challenge virus were used as antigens to test pre-challenge serum. The WM05 was used as a close surrogate for the rHVT-AI insert HA. Post-challenge serum was tested for antibody titers to the challenge virus. HI titers are reported as log₂ values, with 3 log₂ being the minimum titer considered as positive.

2.7. Determination of virus shedding

Oropharyngeal and cloacal swabs were collected in sterile brain heart infusion medium and kept frozen at -70°C . Viral RNA was extracted using Trizol LS reagent (Invitrogen, Carlsbad, CA) and the MagMAX AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX). Quantitative real time RT-PCR (qRRT-PCR) was performed as previously described [9]. Briefly, qRRT-PCR targeting the influenza M gene was conducted using AgPath-ID one-step RT-PCR Kit (Ambion) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). For viral quantification, a standard curve was established with viral RNA extracted from the titrated challenge virus, GW14 H5N8, or NW14 H5N2. Results were reported as EID₅₀/ml equivalents and the lower limit of detection being $10^{0.9}$ EID₅₀/ml for samples from chickens.

2.8. Phylogenetic comparison of HA from vaccine and challenge isolates

Avian influenza HA sequences were obtained from GenBank and Influenza Research Database (<http://www.fludb.org/brc/home.do?decorator=influenza>). Sequences were aligned with Clustal V (Lasergene 10.0, DNASTar, Madison, WI), and phylogenetic analysis for the H5 HA gene was performed with MEGA version 6 [31].

2.9. Statistical analysis

Kaplan-Meier survival curves were generated with Prism 5 (GraphPad Co., San Diego, CA). The Mantel-Cox log-rank test was used to compare survival curves between the experimental groups (Prism 5). Statistical differences in mean and standard error between HI titers were analyzed using ANOVA (Prism 5). ANOVA was also used for comparison of virus titers from oral and cloacal

swabs between sham and vaccinated groups (Prizm 5). All statistical tests were performed using $P < 0.05$.

3. Results

3.1. Phylogenetic comparison of HA from HPAI and vaccine isolates

In this study a wide range of H5 vaccines or vaccine seed strains were selected for use to determine their ability to protect birds from lethal challenge with two related U.S. 2014 HPAI clade 2.3.4.4 isolates. Phylogenetic analysis of the HA protein from the H5 challenge viruses demonstrated that both GW14 and NW14 isolates were 99.8% similar, and further analysis show high sequence similarity to Asian clade 2.3.4.4 viruses which indicates a common lineage within the Gs/GD H5 lineage (Fig. 1). Vaccine relatedness to the HPAI challenge viruses were closest with the RE-5 (DA06) clade 2.3.4 HA and the RE-6 (DG10) clade 2.3.2, demonstrating 93% and 91% sequence similarity, respectively. The HA insert genes used for the rHVT-AI (SM05) and rFPV-AI (TI83) were 91% and 88% similar, respectively, to the U.S. HPAI isolates tested here. Finally, the older U.S. H5 isolates, TW68 (H5N9) and TC02 (H5N2), were approximately 85% and 86% related, respectively, to the challenge viruses.

3.2. Protective efficacy following HPAI challenge

Efficacy of the vaccines was evaluated based on protection from mortality following challenge and reduction in viral shedding. The sham vaccinated birds challenged with GW14 H5N8 had mortality beginning on day 2 and ending on day 8 pc (Fig. 2A). The mean time to death in this group was 5 days. Groups of birds receiving heterologous or homologous inactivated vaccine, including RE-5, RE-6, TC02, TW68, NW14 and GW14, demonstrated 100% protection from mortality with GW14. In addition, no morbidity was observed in these groups (data not shown). Birds that received the rHVT-AI demonstrated mortality on days 3 and 4 pc, with 60% of birds surviving challenge. Birds receiving the rFPV-AI demonstrated 40% protection, with mortality observed between days 2 and 8 pc. Surviving birds in the rHVT-AI and rFPV-AI groups did not demonstrate signs of morbidity throughout the challenge period.

Vaccine efficacy following challenge with the second U.S. HPAI isolate, NW14 H5N2, is presented in Fig. 2B. Sham-vaccinated birds exhibited bi-phasic mortality with 8 birds dying between day 2 and 4, and two birds dying on days 8 and 9 pc. The mean time to death following challenge with this virus was 4 days. Groups of birds receiving heterologous clade 2.3 vaccine (RE-5 and RE-6) or homologous (GW14 and NW14) were 100% protected from mortality. In contrast, vaccines based on older U.S. H5 vaccines, TW68 and TC02, were 60% and 90% protected, respectively. Birds vaccinated with rHVT-AI demonstrated 60% protection, whereas rFPV vaccinated birds demonstrated 70% protection.

3.3. Humoral response to vaccination and challenge

Antibody responses following application of inactivated or recombinant vaccines was tested prior to challenge against both the vaccine antigen and HPAI challenge antigen, and at 14 days pc against the HPAI challenge virus (Fig. 3). In the first trial, results demonstrate that vaccines homologous to the 2.3.4.4 clade isolate, GW14 and NW14, provided the highest prechallenge HI titers (6 log₂) to the GW14 challenge virus (Fig. 3A). Following challenge, HI titers increased significantly to 7.2 and 6.6 (log₂) in the GW14 and NW14 groups, respectively. Groups of birds vaccinated with the RE-5 or RE-6 vaccines demonstrated high HI titers (appx 7.3)

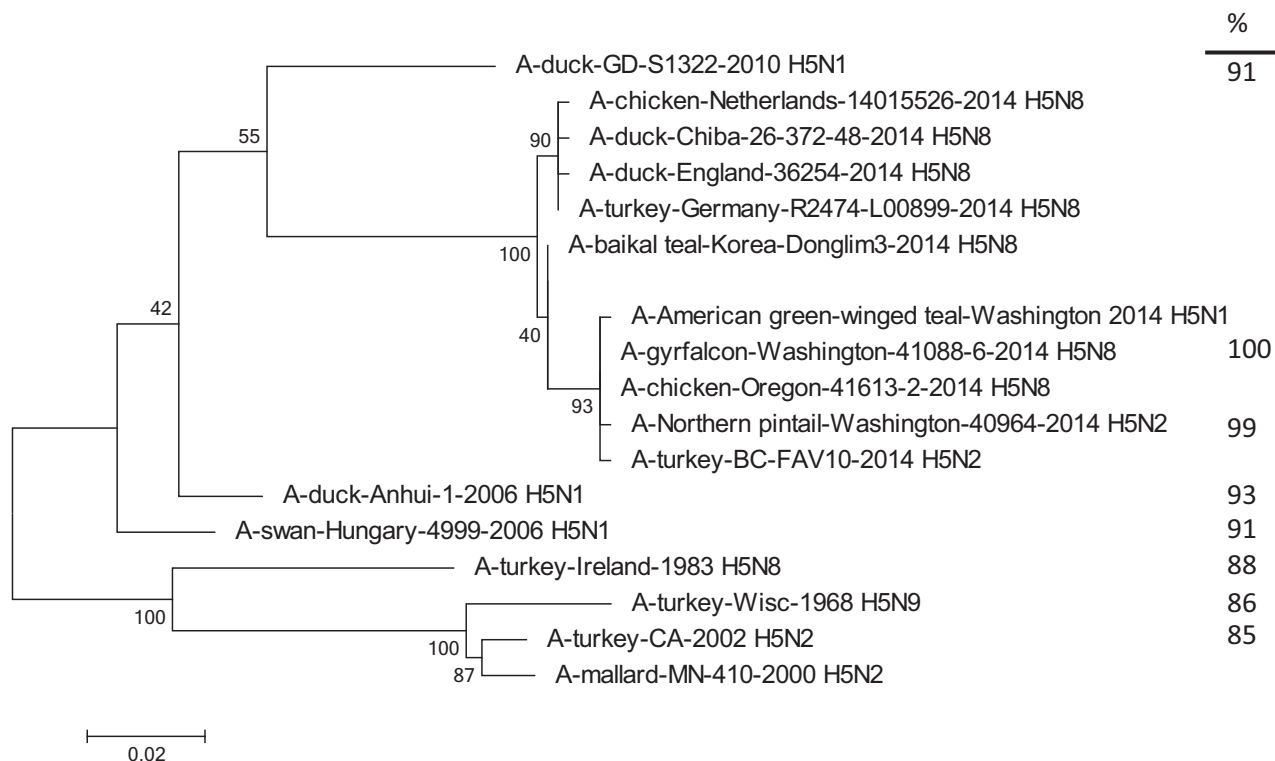


Fig. 1. Phylogenetic tree illustrating the similarity between the amino acid sequences of the H5 hemagglutinin (HA) proteins from the isolates used in these studies. Sequences were aligned with Clustal V (Lasergene 10.0, DNASTar, Madison, WI). Phylogenetic analysis for the H5 gene was performed with MEGA 6 using the maximum likelihood tree building method, with heuristic search and 500 bootstrap replicates. Percent relatedness of HA from vaccine isolates to A/gyrfalcon/Washington/2014 H5N8 is shown.

to their homologous vaccine antigens prior to challenge (Fig. 3B). However, when tested against the GW14 challenge antigen, a significant reduction in mean titer (≤ 2) was observed. Following challenge, HI titers were not significantly changed in either vaccine group. Groups of birds vaccinated with the TW68 or TC02 vaccine also demonstrated high prechallenge HI titers to the vaccine antigen of approximately 7.2 and 7.4, respectively (Fig. 3C). However, prechallenge titers to the GW14 antigen were significantly reduced to less than 2. Following challenge, HI titers in both vaccine groups increased significantly to the GW14 antigen. Finally, HI titers following application and challenge in birds receiving recombinant vaccines, rHVT-AI and rFPV-AI, is demonstrated in Fig. 3D. Groups of birds receiving the rHVT-AI demonstrated prechallenge HI titers of 4.7 and 0.6 to vaccine and GW14 antigen, respectively. Following challenge, mean HI titers increased to 3.5 to the GW14 antigen. Similar results were observed with rFPV-AI vaccinated birds. Prior to challenge, HI titers to vaccine antigen (TI83) and GW14 were demonstrated to be 3.8 and 0.1 respectively. However a significant increase in antibody titer was observed to the GW14 antigen after challenge. Antibody titers following challenge with H5N2 HPAI (NW14) were similar to those observed with H5N8 (GW14) (Supplemental Fig. 1).

3.4. Virus shedding after challenge with H5N8 and H5N2 clade 2.3.4.4 viruses

Virus shedding was significantly reduced following using the challenge strains as autogenous vaccines against the H5N8 HPAI challenge virus (Fig. 4). Following virus challenge, virus replicated and was shed efficiently in the upper respiratory (Fig. 4A and B) and intestinal tract (Fig. 4C and D) of sham immunized control chickens with mean titers of approximately $10^{6.5}$ and $10^{4.1}$ EID₅₀

per ml, respectively, on day 2 pc. Virus shedding continued at high levels at day 4 pc. Interestingly, the recombinant vaccines failed to significantly reduce shedding compared to the sham vaccinated birds by either route on day 2 pc, with mean oral titers of $10^{5.3}$ and $10^{6.1}$ detected in the rHVT-AI and rFPV-AI groups, respectively. The rHVT-AI vaccine group demonstrated reduced levels (titers $< 10^1$) by day 4 pc. In contrast, birds immunized with any of the inactivated vaccines (TW68, TC02, RE-5, RE-6, GW14 and NW14) displayed significant reductions in H5N8 viral shedding titers compared to the sham birds at each site and on each day post challenge. Shedding was typically lower from cloacal swabs compared to oral swabs in all groups.

Virus shedding following H5N2 HPAI challenge demonstrated some differences compared to the H5N8 challenge (Fig. 5). Sham vaccinated birds shed high levels of H5N2 virus from both oral and cloacal swabs on day 2 and 4 pc (Fig. 5A–D). The highest titers ($10^{7.1}$ and $10^{6.9}$) were observed from oral swabs on day 2 and 4 respectively in the sham group. No statistical difference in titer was observed between sham and rHVT-AI vaccinated groups on day 2 pc, in either oral or cloacal swabs. However, rFPV-AI vaccinated birds did show reduced shedding compared to sham vaccinated birds on day 2 pc, but not day 4 pc. No differences in shedding levels were observed in oral swabs from TW68, RE-5, and RE-6 on day 2 and 4 pc. Significant reductions in shedding compared to sham birds were observed in H5N8 and H5N2 groups on both days tested and in both oral and cloacal swabs.

4. Discussion

Worldwide, AI remains an important zoonotic diseases as evidenced by the spread of H5 HPAI viruses that have evolved in Southeast Asia from the Gs/GD lineage, and outbreaks of H7N9 in

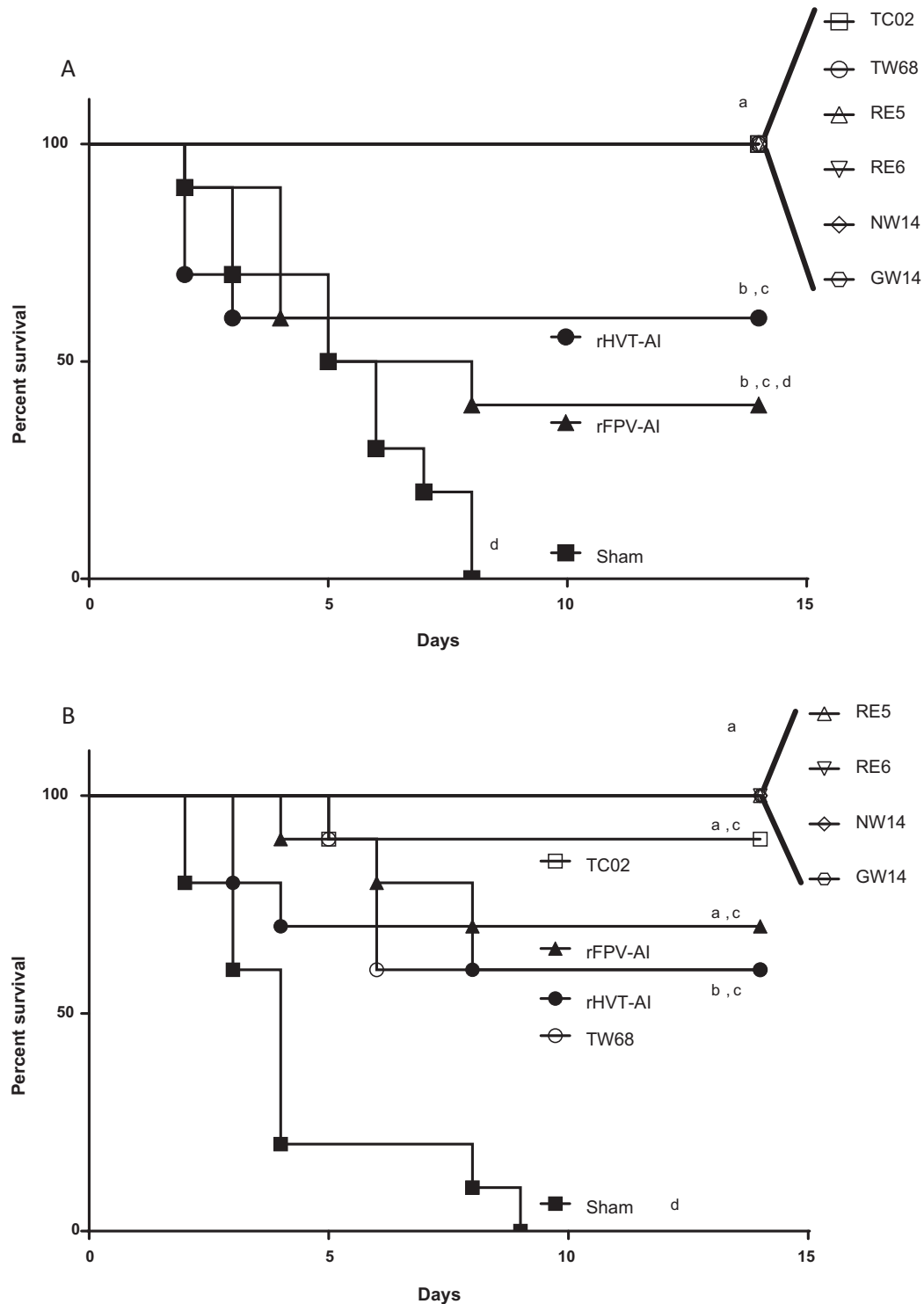


Fig. 2. Kaplan-Meier survival plots following challenge with 2014 U.S. H5N8 (A) and H5N2 (B) highly pathogenic avian influenza (HPAI) viruses. The percent survival versus day post challenge against A/gyrfalcon/Washington/2014 (GW14) H5N8 clade 2.3.4.4 or A/northern pintail/Washington/2014 (NW14) H5N2 clade 2.3.4.4 is shown. Vaccine groups included USDA H5 vaccine isolates, A/turkey/Wisconsin/1968 H5N9 (TW68) and A/turkey/California/2002 H5N2 (TC02), the two homologous HPAI H5 U.S. viruses GW14 and NW14, recombinant live virus vaccines expressing the hemagglutinin (HA) from A/Whopper Swan/Mongolia/3/2005 (rHVT-AI) H5N1 clade 2.2 and A/turkey/Ireland/1378/1983 (rFPV-AI) H5N8, and inactivated reverse engineered (RE) viruses expressing HA from A/duck/Anhui/1/2006 (RE-5) H5N1 clade 2.3.4 and A/duck/Guangdong/S1322/2010 H5N1 clade 2.3.2 (RE6). Statistical significance in survival curves between groups determined by Log-rank (Mantel-Cox) Test ($P < 0.05$) indicated by different letters.

China, H7N7 in Italy, H5N2 in Canada, and H7N3 in Mexico [9,32–35]. The recent outbreak of H5N8, H5N1 and H5N2 clade 2.3.4.4 HPAI resulted in the largest foreign animal disease outbreak in the U.S, and renewed interest in vaccine development for potential

inclusion in a control strategy [2]. In these studies, the two most commonly used types of vaccines for AI in poultry, inactivated and recombinant, with different HA antigen configurations, were compared. It has been demonstrated previously that matching

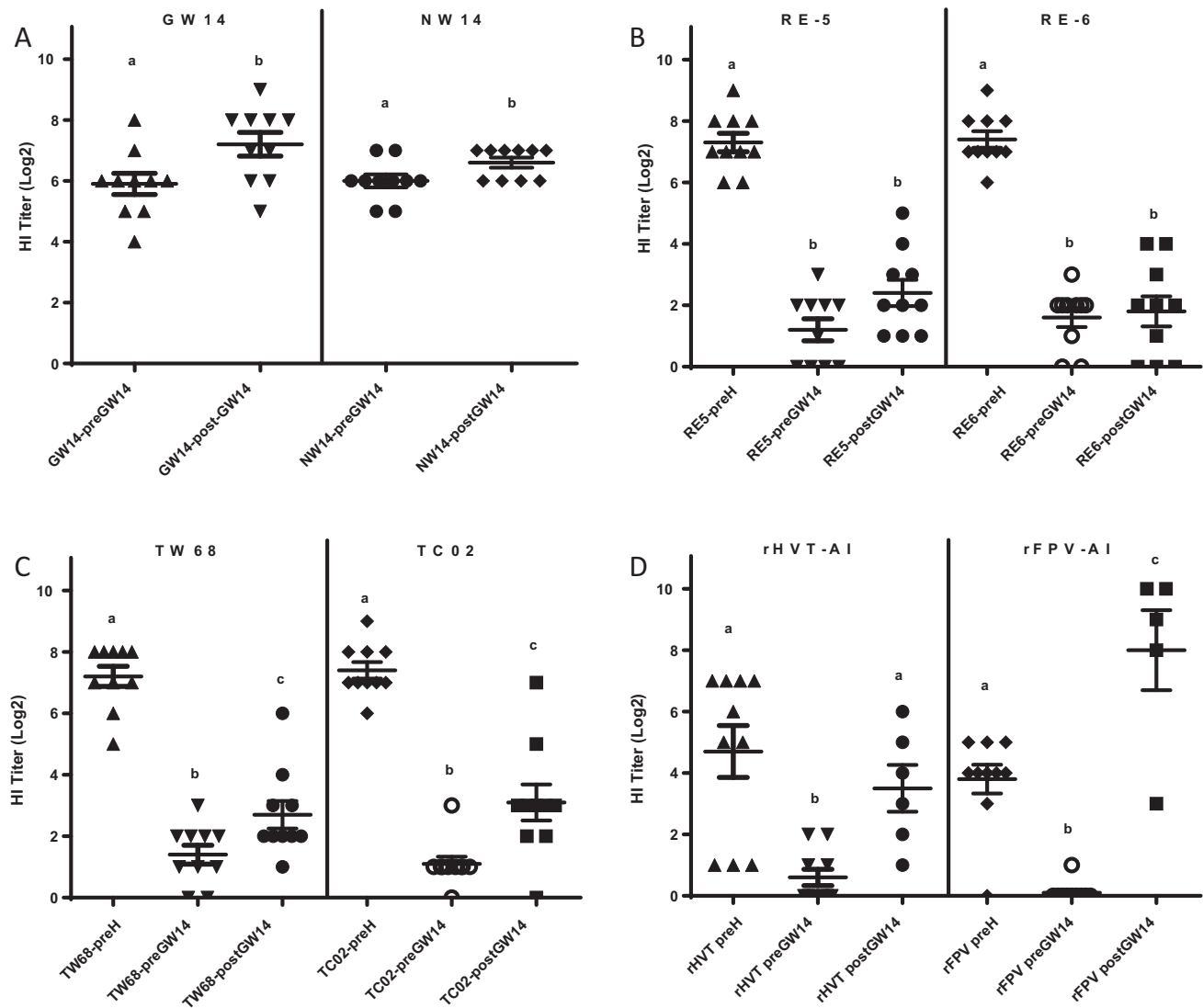


Fig. 3. Individual HI titers (log₂) and standard error for vaccine bird groups tested pre-challenge against the homologous vaccine antigen (preH), and the HPAI challenge virus (preGW14), and two weeks post-challenge against HPAI challenge virus (postGW14).

antigenic similarity of the HA between the vaccine and challenge virus provides the best protection against mortality and virus shedding assuming a comparable host immune response [12,36–40]. In the current study we demonstrate that several AI vaccines protected against the H5N8 and H5N2 HPAI viruses. Overall, the inactivated vaccines provided superior protection against the H5N8 and H5N2 viruses in a single vaccination strategy, even those with less than 90% HA similarity, compared with the recombinant vaccines. As expected, the homologous vaccines based on the challenge viruses provided the best protection against mortality, highest antibody titers and lowest oral and cloacal shedding titers. However, because the homologous strains are HPAI viruses, it isn't possible use these as seed strains in the U.S. because there are no BSL-3 production facilities that can safely work with these viruses on a commercial scale. Future vaccines can be antigenically matched using genetic technology containing the 2.3.4.4 HA insert in a number of different recombinant vaccine platforms.

Minor differences were observed between the two different challenge isolates used. For example, the two U.S. H5 vaccine isolates, TW68 and TC02, provided less protection when tested against the H5N2 HPAI virus. Although both viruses are closely similar in the H5 genes, the viruses because of reassortment have

3 unrelated genes and differences in pathogenicity with these viruses in poultry species has been reported [41].

The two RE vaccines demonstrated complete protection from mortality with little to no shedding of challenge virus from oral or cloacal swabs. This is not unexpected since both were based on H5N1 2.3 clade origin viruses, and also demonstrated the highest HA gene similarity to the HPAI challenge viruses tested here. However, at least one report determined the RE-5 vaccine only induce 66% protection from a clade 2.3.4 HPAI challenge virus in chickens [42]. While both of these vaccines provided good clinical protection, neither are licensed for use in the United States and are unlikely to become commercially available in their current configurations. The two commercially available viral vectored recombinant H5 AI vaccines demonstrated lower degrees of protection in a single vaccination strategy. The rHVT-AI vaccine has previously been shown to protect against a wide spectrum of H5 HPAI Gs/GD lineage viruses [10,11,43], but only provided partial protection from clinical disease and only a limited reduction in viral shedding and likely reflects the high level of antigenic drift as reflected in the clade system. The rFPV-AI vaccine has a Eurasian virus hemagglutinin insert that is not in the Gs/Gd lineage, and at the amino acid level was even more distant at only 88% sequence homology. Both

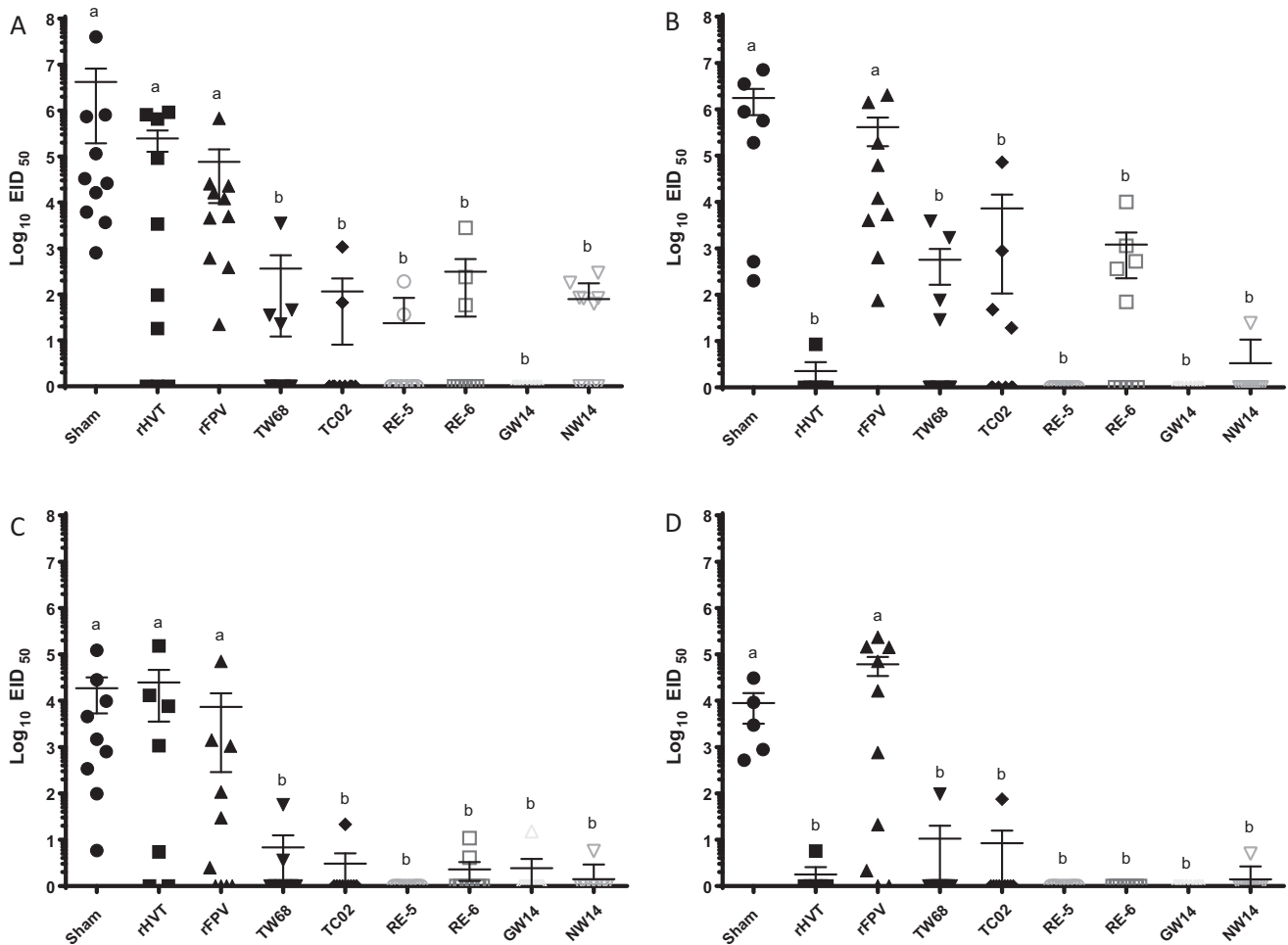


Fig. 4. Viral titers from oral and cloacal swabs on day 2 (A, C) and day 4 (B, D) post-challenge following challenge against H5N8 HPAI. Birds were vaccinated at 1 (rHVT-AI or rFPV-AI) or 21 days of age (inactivated vaccines), and challenged at day 28 or 35, respectively, with A/gyr/falcon/Washington/2014 H5N8 HPAI. Viral titers are expressed as log₁₀ EID₅₀ per ml. The lower limit of detection is 0.9 log₁₀ EID₅₀ per ml. (A, B) Oral titers; (C, D) Cloacal titers.

viral vectored vaccines, as has previously been reported, do not produce high levels of antibody as measured by HI, and clinical protection is considered to be derived from both the antibody response as well as a cell mediated immune response [11,16]. It should be recognized that the differences in the vaccine regimen may have contributed to the decreased protection, as birds were vaccinated at a younger age with the viral-vectored recombinant vaccines, whereas the inactivated vaccines were applied when the birds were more immunologically mature. It was apparent that the mismatched HAs contained within the inactivated vaccines provided better protection than the mismatched HAs in the recombinant vaccines. Our previous results with HP H7 vaccine challenge models also demonstrate good protection from mismatched HAs with inactivated vaccines in poultry [38]. It is possible that antigens other than those found on the HA contributed to the improved protection of the inactivated vaccines. Likewise, since the challenge viruses in this study were not poultry-adapted the threshold for antibody protection may be lower than what normally is required [12,44]. Finally, the earlier vaccination schedule with the rHVT and rFPV was conducted because both are licensed for application in day old chicks and would not be applied at three weeks of age due to potential immunological interference against the vector itself.

Although the HI antibody titers induced to homologous vaccine-antigen were high, the heterologous vaccines did not induce high levels of cross reactive HI antibodies to the challenge

virus prior to challenge. The presence of HI antibodies against challenge virus has been predictive of protection from mortality [11,42,44]; i.e. high positive predictive value. However, the lack of HI antibodies to challenge virus does not predict lack of protection from mortality; i.e. low negative predictive value [44]. Because the HI test measures antibodies involved with receptor binding, it is likely that virus-neutralizing antibodies localizing to the stalk region, which interfere with pH-induced conformational changes associated with membrane fusion, or that interfere with neuraminidase activity of progeny release, would go undetected, allowing protection in vaccinated poultry that lack HI antibody. Indeed, recent studies have identified protective epitopes in the stalk and other regions of the HA protein that induce neutralizing antibodies in mammals in the absence of HI activity [45–47]. A more complete evaluation of the epitopes involved with avian influenza neutralization in poultry may demonstrate why the inactivated vaccines provided better protection compared to the recombinant vaccines in these studies. Present studies are underway to evaluate the contribution of antibodies not involved with receptor binding to protection from challenge.

5. Conclusion

Taken together, these data demonstrate that immunization of poultry offered significant protection against lethal disease

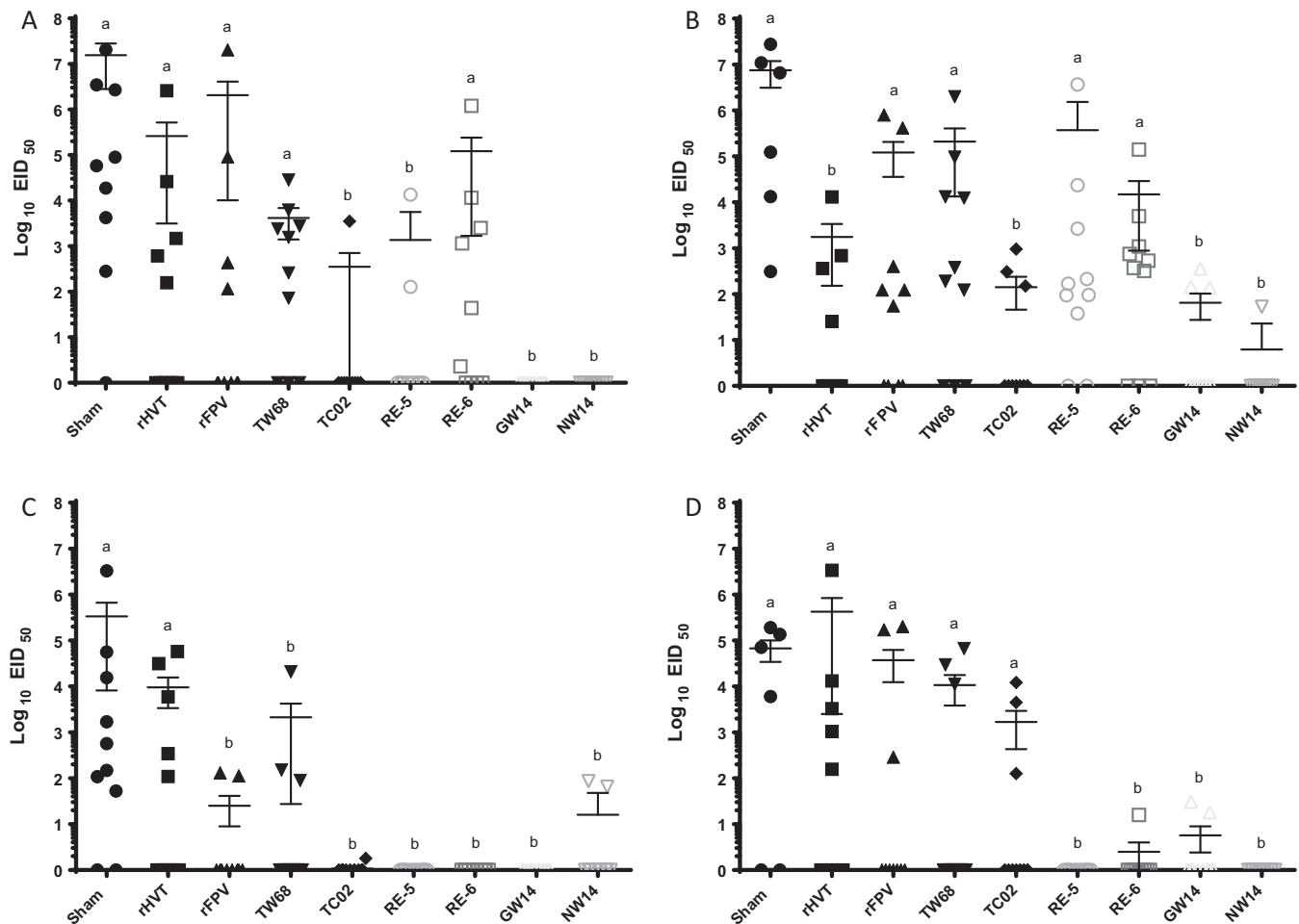


Fig. 5. Viral titers from oral and cloacal swabs on day 2 (A, C) and 4 (B, D) post-challenge following challenge against H5N2 HPAI. Birds were vaccinated at 1 (rHVT-AI or rFPV-AI) or 21 days of age (inactivated vaccines), and challenged at day 28 or 35, respectively, with A/northern pintail/Washington/2014 H5N2 HPAI. Viral titers are expressed as $\log_{10}$ EID₅₀ per ml. The lower limit of detection is 0.9 $\log_{10}$ EID₅₀ per ml. (A, B) Oral titers; (C, D) cloacal titers.

following challenge with two different but related H5 HPAI clade 2.3.4.4 isolates. In addition, shedding levels were best repressed when using antigenically matched vaccines, which underscore the absolute need to develop future vaccines using genetic technology to match currently circulating viruses. Finally, prechallenge antibody titers based on HI testing did not reflect protective indices as previous studies have shown, however they may be indicative of a need to better match vaccine antigen to field virus.

Conflict of interest statement

The authors declare that they have no competing financial interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2017.04.042>.

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Variation in protection of four divergent avian influenza virus vaccine seed strains against eight clade 2.2.1 and 2.2.1.1. Egyptian H5N1 high pathogenicity variants in poultry

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Background Highly pathogenic (HP) H5N1 avian influenza virus (AIV) was introduced to Egyptian poultry in 2006 and has since become enzootic. Vaccination has been utilized as a control tool combined with other control methods, but for a variety of reasons, the disease has not been eradicated. In 2007, an antigenically divergent hemagglutinin subclade, 2.2.1.1, emerged from the original clade 2.2.1 viruses.

Objectives The objective was to evaluate four diverse AIV isolates for use as vaccines in chickens, including two commercial vaccines and two additional contemporary isolates, against challenge with numerous clade 2.2.1 and clade 2.2.1.1 H5N1 HPAIV Egyptian isolates to assess the variation in protection among different vaccine and challenge virus combinations.

Methods Vaccination-challenge studies with four vaccines and up to eight challenge strains with each vaccine for a total of 25

vaccination-challenge groups were conducted with chickens. An additional eight groups served as sham-vaccinated controls. Mortality, mean death time, morbidity, virus, and pre-challenge antibodies were evaluated as metrics of protection. Hemagglutination inhibition data were used to visualize the antigenic relatedness of the isolates.

Results and conclusions Although all but one vaccine-challenge virus combination significantly reduced shed and mortality as compared to sham vaccinates, there were differences in protection among the vaccines relative to one another based on challenge virus. This emphasizes the difficulty in vaccinating against diverse, evolving virus populations, and the importance of selecting optimal vaccine seed strains for successful HPAIV control.

Keywords H5N1, highly pathogenic avian influenza virus, poultry vaccination.

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Introduction

Use of vaccination for the control of avian influenza virus (AIV) has become routine, combined with other control measures such as improved biosecurity in areas where H5N1 highly pathogenic (HP) AIV is endemic in poultry. Historically, only a few H5 AIV seed strains have been used in commercial vaccines. Two of the most widely used have been A/goose/Guangdong/1/1996 which is the source of hemagglutinin (HA) and neuraminidase (NA) genes in the

inactivated Re-1 vaccine¹ (use discontinued in China in mid-2008² although its use continued in Egypt and Vietnam) and A/Mexico/232/1994 H5N2 (Mex/94) low pathogenic (LP) AIV which is used in several inactivated commercial products. Currently, vaccines with the Mex/94 lineage virus and a reverse genetics produced vaccine with an Egyptian 2010 strain antigen are among the vaccines being used. Because of the ability of influenza A viruses to mutate and drift antigenically, there are reports of H5 field viruses which escaped immunity induced by these common vaccines

in areas where they have been used widely,^{3–5} including Egypt.

H5N1 HPAIV was first detected in poultry in Egypt in 2006.⁶ As then vaccination has been one of the control measures utilized to help control the virus and reduce the impact of infection on poultry. However, H5N1 HPAIV outbreaks have continued to occur and vaccination alone has not been as effective as expected. One of the critical factors known to affect vaccine efficacy is antigenic matching between the challenge virus and vaccine. In countries that have used the same vaccines for an extended period of time, the field viruses have evolved to evade the immune response to the vaccine.^{4,5,7} Improper vaccination⁸ and inadequate coverage of poultry populations⁹ make control of virus spread difficult which may contribute to the emergence of antigenic variants. In Egypt, a new variant, 2.2.1.1, (previously referred to as 'variant clade 2.2.1', 'sublineage B' or 'sublineage E') first described in 2007 is possibly an example of this.^{6,10} The emergence of this clade could complicate vaccination strategies if the currently available vaccines have reduced efficacy against viruses in this clade.

Goals of this work include evaluating the efficacy of the current vaccines against numerous Egyptian isolates including isolates clade 2.2.1.1 and to determine if local, contemporary isolates from Egypt would provide any improvement in protection. Also, as experimental evaluations of AIV vaccines are often published later than is practical, a broader goal was to assess the consistency of protection against multiple challenge viruses, some of which were relatively closely related genetically and antigenically. To accomplish this, we have conducted vaccination-challenge studies with four vaccines and up to eight H5N1 HPAIV challenge viruses per vaccine.

Materials and methods

Virus source and propagation

Virus isolates used for vaccine-challenge studies and antigenic cartography were obtained from either the repository at Southeast Poultry Research Laboratory, USDA-ARS (SEPR; Athens, GA, USA), or the National Laboratory for Quality Control in Poultry Production (NLQP; Cairo, Egypt). All influenza isolates were propagated in specific pathogen-free (SPF) embryonating chicken eggs (ECE) by standard procedures.¹¹ Allantoic fluid containing infectious virus was used as challenge virus, and as the antigen source for vaccines and hemagglutination inhibition (HI) assays. The sequence data for the HA genes of all viruses used here have been published with analysis (isolates from 2006 to 2008)⁶ or have been submitted to GenBank (isolates from 2009 to 2010).

Vaccines

Four viruses were selected for use as vaccine antigens. Two were selected because of their widespread use as commercial

vaccines: reverse genetic produced A/goose/Guangdong/1996 H5N1 (GS/GD/96) HPAIV and wild-type A/chicken/Mexico/232/1994 H5N2 (Mex/94) LPAIV. The other two, A/chicken/Egypt/13-NLQP/2008 (Egy/13/08) HPAIV, and A/chicken/Egypt/202-NLQP/2007 (Egy/202/07) HPAIV were selected to represent clades 2.2.1 and 2.2.1.1, respectively.

All vaccines were inactivated, whole virus, adjuvanted in an oil emulsion. The vaccine prepared with GS/GD/96 was the commercial product, Re-1 (Harbin Veterinary Research Institute, Harbin, China),² all others were experimental vaccines prepared in-house as described by Stone *et al.*¹² using 0.1% beta-propiolactone inactivated allantoic fluid from ECE and adjusting the pH to 7.0 with sodium bicarbonate. Sham vaccine was prepared with allantoic fluid from uninfected ECE. Viruses used to produce vaccines in-house were evaluated for HA titer by standard methods¹¹ and by titration in ECE as described previously.¹¹ The HA and 50% egg infectious dose (EID₅₀) titers for the viruses are given in Table 1.

Challenge viruses

Eight challenge viruses were selected from among H5N1 HPAIV viruses from poultry in Egypt, aiming to represent a diverse sampling of HA1 protein sequences. The clade 2.2.1 isolates were: A/chicken/Egypt/959-NLQP/2006 (Egy/959/06), Egy/13/08) A/goose/Egypt/20-NLQP/2009 (Egy/20/09), A/duck/Egypt/44-NLQP/2009 (Egy/44/09), A/chicken/Egypt/102d-NLQP/2010 (Egy/2d/10). The clade 2.2.1.1 challenge viruses were as follows: Egy/202/07, A/chicken/Egypt/65-NLQP/2008 (Egy/65/08), and A/chicken/Egypt/1063-NLQP/2010 (Egy/63/10). All viruses were propagated in ECE as described above.

Vaccination-challenge studies in chickens

Each vaccine-challenge group contained 10 individually tagged, SPF white leghorn chickens from SEPR in-house

Table 1. Titers of viruses used to prepare vaccines and the geometric mean titer (GMT) of antibody for all birds receiving each vaccine at the time of challenge (3 weeks post-vaccination) based on HI assay

Virus isolate in vaccine	HA titer	EID50 titer (log ₁₀ /ml)	GMT of antibody prior to challenge
A/chicken/Mexico/232/94	512	8.9	49.2 ^a
A/chicken/Egypt/202-NLQP/2007	256	8.1	13.6 ^b
A/chicken/Egypt/13-NLQP/2008	128	8.7	19.5 ^b
A/goose/Guangdong/1996 (Re-1)	Unknown:		60.6 ^a
	commercial vaccine		

Superscripts denote statistical groups within the same column.

Table 2. Mortality, mean death time, and number of chickens shedding virus at detectable levels in oropharyngeal swabs 2 days post-challenge (DPC) by challenge group

Vaccine	Vaccine HA clade	Challenge virus HA clade	Challenge virus	Percent mortality	Mean death time	Number of chickens orally shedding 2DPC*
Sham	Not applicable	2.2.1	A/chicken/Egypt/959-NLQP/2006	100 ^c	2.3 ^{ab}	10/10 (100) ^a
			A/chicken/Egypt/13-NLQP/2008	100 ^c	2.4 ^{ab}	4/4 (100)
			A/goose/Egypt/20-NLQP/2009	100 ^c	3.2 ^a	10/10 (100) ^a
			A/duck/Egypt/44-NLQP/2009	100 ^c	2.1 ^b	2/2 (100)
			A/chicken/Egypt/2d -NLQP/2010	100 ^c	2.7 ^{ab}	10/10 (100) ^a
			A/chicken/Egypt/202-NLQP/2007	100 ^c	2.1 ^b	1/1 (100)
			A/chicken/Egypt/65-NLQP/2008	100 ^c	2.6 ^{ab}	10/10 (100) ^a
A/chicken/Mexico/232/1994 H5N2	Classical H5	2.2.1	A/chicken/Egypt/63-NLQP/2010	100 ^c	2.3 ^{ab}	10/10 (100) ^a
			A/chicken/Egypt/959-NLQP/2006	0 ^a	—	8/10 (80) ^a
			A/goose/Egypt/20-NLQP/2009	0 ^a	—	6/10 (60) ^a
			A/chicken/Egypt/2d-NLQP/2010	0 ^a	—	7/10 (70) ^a
			A/chicken/Egypt/65-NLQP/2008	10 ^a	9.0	10/10 (100) ^a
			A/chicken/Egypt/63-NLQP/2010	10 ^a	8.0	10/10 (100) ^a
			A/chicken/Egypt/959-NLQP/2006	0 ^a	—	8/10 (80) ^{ab}
Re-1 Goose/Guangdong/1996 H5N1	0	2.2.1	A/chicken/Egypt/13-NLQP/2008	10 ^a	3.0	10/10 (100) ^a
			A/goose/Egypt/20-NLQP/2009	0 ^a	—	4/10 (40) ^b
			A/chicken/Egypt/2d-NLQP/2010	0 ^a	—	7/10 (70) ^{ab}
			A/duck/Egypt/44-NLQP/2009	0 ^a	—	8/10 (80) ^{ab}
			A/chicken/Egypt/202-NLQP/2007	20 ^{ab}	6.0	9/10 (100) ^a
			A/chicken/Egypt/65-NLQP/2008	50 ^b	7.0	9/10 (90) ^a
			A/chicken/Egypt/63-NLQP/2010	40 ^{ab}	7.0	10/10 (100) ^a
A/chicken/Egypt/13-NLQP/2008 H5N1	2.2.1	2.2.1	A/chicken/Egypt/959-NLQP/2006	0 ^a	—	9/9 (100) ^a
			A/chicken/Egypt/13-NLQP/2008	0 ^a	—	9/10 (90) ^a
			A/goose/Egypt/20-NLQP/2009	0 ^a	—	2/10 (20) ^b
			A/duck/Egypt/44-NLQP/2009	10 ^a	6.0	10/10 (100) ^a
			A/chicken/Egypt/202-NLQP/2007	20 ^{ab}	4.0	9/9 (100) ^a
			A/chicken/Egypt/65-NLQP/2008	0 ^a	—	7/8 (87.5) ^a
			A/chicken/Egypt/959-NLQP/2006	0 ^a	—	8/8 (100) ^a
A/chicken/Egypt/202-NLQP/2007 H5N1	2.2.1.1	2.2.1	A/chicken/Egypt/13-NLQP/2008	30 ^{ab}	2.7	9/10 (90) ^a
			A/goose/Egypt/20-NLQP/2009	0 ^a	—	1/9 (11.1) ^b
			A/duck/Egypt/44-NLQP/2009	70 ^{bc}	6.1	10/10 (100) ^a
			A/chicken/Egypt/202-NLQP/2007	20 ^{ab}	3.5	10/10 (100) ^a
			A/chicken/Egypt/65-NLQP/2008	0 ^a	—	7/8 (87.5) ^a
			A/chicken/Egypt/959-NLQP/2006	0 ^a	—	8/8 (100) ^a
			A/chicken/Egypt/13-NLQP/2008	30 ^{ab}	2.7	9/10 (90) ^a

Superscript letters denote the statistical group within the same column (groups without superscripts were not included in statistical calculations due to inadequate sample numbers).

*Number positive/total (% positive).

flocks. Vaccine-challenge virus groups are listed in Table 2. Chickens were vaccinated between 3 and 4 weeks of age with 0.5 ml of vaccine by the subcutaneous route. Three weeks after vaccination, the birds were bled for serum to evaluate antibody to both the vaccine and challenge virus by HI assay. The birds were also challenged 3 weeks post-vaccination with 10⁶ EID₅₀ of HPAIV per bird administered in 0.1 ml by the intratracheal route.

Oropharyngeal swabs were collected 2 days post-challenge (DPC) to evaluate virus shed titers by quantitative real-time RT-PCR (qrRT-PCR).

Quantitative real-time RT-PCR

Oropharyngeal swabs collected at 2 DPC were processed for qrRT-PCR to determine virus shed titers. RNA was extracted as described by Das *et al.*¹³ using a combination of Trizol LS (Invitrogen, Inc., Carlsbad, CA, USA) and the MagMAX 96 AI/ND Viral RNA isolation kit (Ambion, Inc., Austin, TX, USA) with the KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA, USA).

Quantitative rRT-PCR which targets the influenza M gene¹⁴ was performed using the 7500 FAST Real-time PCR System (Applied Biosystems, Foster City, CA, USA) and the

AgPath-ID OneStep RT-PCR kit (Ambion, Inc.) in accordance with the U.S. National Veterinary Services Laboratories protocol AVSOP1521.01. The standard curve for virus quantification was established with RNA extracted from dilutions of the same titrated stock of each virus used to challenge the chickens and was run in duplicate.

Hemagglutination inhibition assay

Sera were tested by the HI assay to evaluate antibody levels to both the vaccine and challenge virus in vaccinated birds pre-challenge. Because GS/GD/96, the source of the HA in the Re-1 vaccine was not available, a closely related isolate which was available was used: A/HongKong/156/1997 H5N1 (HK/156) (98.1% amino acid identity in the HA1).

The assay antigens were prepared by inactivating infectious allantoic fluid with 0.1% beta-propiolactone and adjusting the pH to 7.0 with sodium bicarbonate. The HI assays were performed in accordance with standard procedures.¹⁵ Titers were calculated as the reciprocal of the last HI positive serum dilution.

Antigenic cartography

A total of 23 H5N1 HPAIVs from Egypt were selected for characterization by antigenic cartography. Each virus used as a vaccine and/or challenge strain was included. Additional isolates were selected to represent divergent H5 HA1 protein sequences. In addition to the isolates from Egypt, five H5N1 isolates from different HA clades were included (the HK/156 antigen was used to approximate GS/GD/96). Two classical H5 clade viruses were also used, one of which was the Mex/94 isolate included as one of the vaccines in this study.

Reference sera were produced with an inactivated, oil emulsion vaccine prepared with the Montanide ISA50V adjuvant (Seppic, Inc., Fairfield, NJ, USA) using allantoic fluid prepared as described under HI assay. Three-week-old SPF white leghorn chickens from SEPRL in-house flocks were inoculated with 0.5 ml of vaccine by the subcutaneous route, and serum was collected 3 weeks post-vaccination. Sera were treated with 5% chicken red blood cells for 30 minutes at ambient temperature to adsorb non-specific agglutinins. The HI assay was performed by standard procedures.¹⁵

The antigenic map for the test antigens was constructed using matrix completion-multiple dimensional scaling (MC-MDS).¹⁶ Given the HI table h with n antigens ($n = 30$ in this study) and m antisera ($m = 28$ in this study), each observed value h_{ij} was transformed into $h_{ij} = [H_{ij}] - \log_2(H_i/H_{ij})$, where H_{ij} is the maximum HI titer in the HI table h and H_j is the maximum HI titer for serum j , $1 \leq i \leq n$, and $1 \leq j \leq m$. The value of 2 was defined as a low reactor, and a value of 4096 was used for those entries with '>4096'. A low rank matrix completion algorithm was applied to identify those entries without experimental data and to reduce the noise in

HI table. The antigenic distance was then calculated and mapped to a two dimensional antigenic map.

Statistical methods

Shed titers by vaccine and challenge virus, mean death times, and antibody titers to the vaccine and challenge virus prior to challenge were tested for statistical significance using the one-way ANOVA, all pairwise (Tukey test). If normality failed then the Student–Newman–Keuls method was used (pairwise). Linear regression was used to evaluate the correlation between antibody levels to the vaccine or challenge virus and virus shed titers. All statistical analyses were conducted with SIGMAPLOT 11.2 (Systat Software, Richmond, CA, USA). A P value of <0.05 was considered to be significant. Statistical groups are denoted in tables as superscript and on graphs by lowercase letters. Groups with different letters are significantly different at a P value of <0.05 . Groups apply to values in the same column. Samples which were negative by rRT-PCR were counted as below the limit of detection (titer = 10 EID 50/ml).

Results

Serology

Antibody levels to each vaccine 3 weeks post-vaccination (immediately prior to challenge) were evaluated by HI assay (Figure 1). The Re-1 vaccine (commercial product) and the in-house vaccine produced with the Mex/94 isolate produced significantly higher levels of antibody (geometric mean titers) than either of the experimental vaccines produced with the Egyptian H5N1 isolates: Egy/202/07 or Egy/13/08. Antibody levels using the challenge viruses as test antigen in the HI assay for each group varied widely by vaccine-challenge virus combination, but were consistently lower than antibody levels to the vaccine (in all non-homologous groups) (Figure 1).

Amount of virus shed was evaluated against antibody titer to either the vaccine or challenge virus immediately prior to challenge. There was no correlation between the level of virus shed and the antibody titer to either the vaccine or challenge virus for any of the treatment groups. There was also no association between antibody titers and mortality.

Vaccination challenge

Four vaccines were evaluated with up to eight H5N1 challenge viruses from Egypt for a total of 25 vaccine-challenge virus combinations and eight additional sham vaccinated groups (Table 2). Protection was measured as prevention of mortality and decrease in virus shed. There was 100% mortality in all sham vaccinated groups and mortality varied by vaccine-challenge virus combination (Table 2). There was not a clear trend for prevention of mortality in groups vaccinated with Egy/202/07 or Egy/13/08 based on

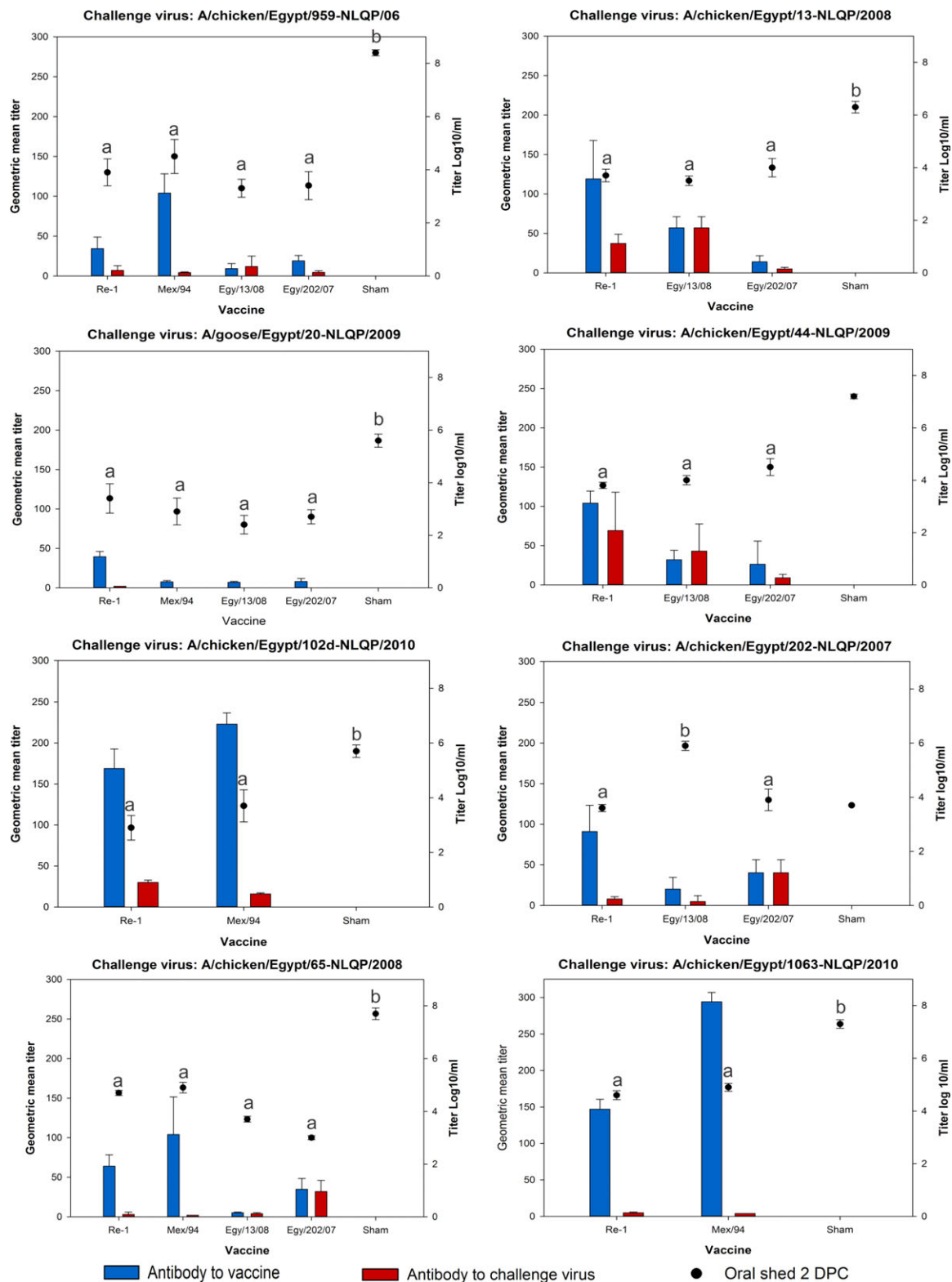


Figure 1. Pre-challenge geometric mean antibody (Ab) titers (determined by HI assay) to both the vaccine virus and challenge virus versus oral shed titers ($\log_{10}/\text{ml}$) 2 days post-challenge. Statistical groups ($P < 0.05$) for mean shed titers are denoted by a letter where different letters indicate statistical difference (mean shed titers without a letter indicate insufficient surviving birds to compare shed titers). Error bars represent standard error.

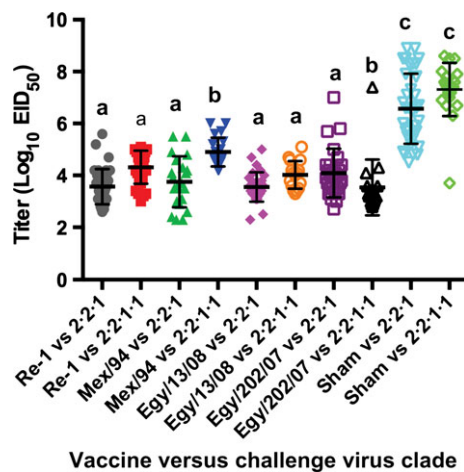


Figure 2. Oral virus shed 2 day post-challenge collectively by HA clade of challenge virus and vaccine. Letter denote statistical groups ($P < 0.05$) where different letters indicate statistical difference. Error bars represent standard deviation.

challenge virus clade; however, in groups vaccinated with Re-1 or Mex/94 mortalities were nearly exclusive to the groups challenged with viruses from clade 2.2.1.1. This trend was more pronounced with the Re-1 vaccinated group. When collectively evaluated by vaccine and clade (mortality from all challenge viruses of the same clade combined) mortality was significantly higher for the chickens challenged with clade 2.2.1.1 isolates versus clade 2.2.1 isolates with Re-1, Mex/94 and Egy/13/08 vaccinated groups and significantly lower in the Egy/202/07 vaccinated groups.

Oral virus shed at 2 DPC varied by vaccine and challenge virus combination (Figure 1); all vaccines significantly reduced shed compared to sham vaccinated chickens except for the group vaccinated with Egy/202/07 and challenged with Egy/13/08 (note that the groups challenged with Egy/202/07, Egy/44/09 could not be statistically evaluated versus sham vaccinates, because there was only one or two sham vaccinates, respectively, which survived long enough to be sampled for shed). All vaccines reduced shed similarly for seven of the eight challenge viruses; that is, the titers of virus shed was not significantly different among vaccinated birds for any virus, regardless of vaccine (Figure 1).

When virus shed was compared between the two clades for each vaccine, the differences in mean titers were significantly lower with challenge viruses from clade 2.2.1 versus challenge viruses from clade 2.2.1.1 for Re-1, Mex/94, and Egy/13/08 (Figure 2). In contrast, shed titers were significantly higher with challenge viruses from clade 2.2.1 than clade 2.2.1.1 from chickens vaccinated with Egy/202/07.

Antigenic profiling by antigenic cartography

Antigenic mapping showed that the Egyptian H5N1 viruses in clade 2.2.1 do predominantly cluster apart from the

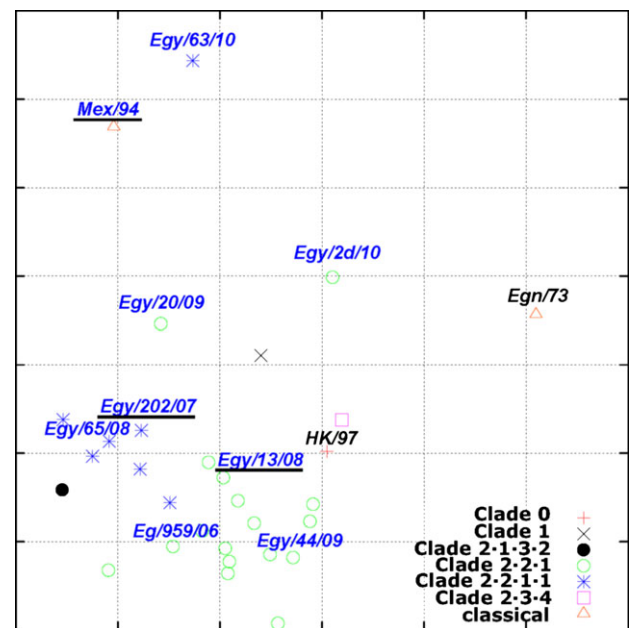


Figure 3. Influenza antigenic cartography for the vaccine strains and Egyptian H5N1 HPAIVs. Both the vaccine strain and challenges are labeled in blue text, and the vaccine strains are underlined. The antigenic cartography is constructed using AntigenMap (<http://sysbio.cvm.msstate.edu/AntigenMap>).

Egyptian H5N1 viruses in clade 2.2.1.1 (Figure 3). The antigenic distances within clade 2.2.1 is 1.4609 ± 0.7470 (mean $\pm$ standard deviation) antigenic units (an antigenic unit is equal to a twofold difference in HI assay titer), and within clade 2.2.1.1 is 1.6291 ± 1.3042 units. However, the intercluster distance is 2.1875 ± 0.9904 units. The antigenic distances within each clade (2.2.1 or 2.2.1.1) are statistically smaller than those between these two clades, $P < 0.001$ (this result is consistent to those reported previously¹⁷). The antigenic distances between the vaccine strains and challenge viruses are shown in Table 3.

Discussion

As expected, minor differences in protection (reduction of mortality and reduction of virus shed) based on vaccine and challenge virus combination were observed. Also there were higher mortality and shed titers in the Re-1 vaccinated groups that were challenged with clade 2.2.1.1 isolates versus clade 2.2.1 isolates. This data support the hypothesis that antigenic drift of H5N1 HPAIV in Egypt may be a result of immune pressure, and highlights the need for updated vaccines. In mid-2008, Re-1 was discontinued in China² where was replaced by a vaccine with a more recent H5N1HA gene, although, use of Re-1 continued for at least another year in Egypt. Continued use of Mex/94 based vaccines should also be critically reviewed; collective analysis of the

Table 3. Antigenic distances in antigenic units of the vaccine strains and challenge viruses used in this study

Virus	Virus									
	Clade	Abbreviation	Classical	0	2.2.1		2.2.1.1			
					Egy/95/06	Egy/13/08	Egy/20/09	Egy/44/09	Egy/2d/10	Egy/202/07
A/chicken/Hidalgo/232/1994*	Classical	Mex/94	0.0000	3.6026**	3.9055	3.1541	2.5579	4.1733	2.7647	3.0629
A/Hong Kong/156xPR8/1997***	0	HK/97	0.0000	0.0000	1.9033	1.4016	1.9132	1.4025	2.5845	2.2506
A/chicken/Egypt/959-NLQP/2006	2.2.1	Egy/95/06			0.0000	1.0912	2.3717	1.4155	3.0723	1.4924
A/chicken/Egypt/13-NLQP/2008	2.2.1	Egy/13/08				0.0000	1.8489	1.4559	2.1018	1.0159
A/goose/Egypt/20-NLQP/2009	2.2.1	Egy/20/09					0.0000	2.3698	2.6067	2.0364
A/duck/Egypt/44-NLQP/2009	2.2.1	Egy/44/09						0.0000	2.7450	2.1748
A/chicken/Egypt/102d-NLQP/2010	2.2.1	Egy/2d/10							0.0000	2.1433
A/chicken/Egypt/202-NLQP/2007	2.2.1.1	Egy/202/07								0.0000
A/chicken/Egypt/65-NLQP/2008	2.2.1.1	Egy/65/08								2.2167
A/chicken/Egypt/1063-NLQP/2010	2.2.1.1	Egy/63/10								0.3058
										0.0000
										0.0000

*The viruses in bold were used as vaccines.

**One antigenic unit is equal to a twofold difference in HI.

***HK/97 strain is used as an antigenically similar strain to GS/GD/96 from clade 0.

two clade 2.2.1.1 challenge isolates tested here showed significantly more virus shed versus the clade 2.2.1 challenge isolates, although mortality was not significantly different. The protection provided by Mex/94 will likely continue to deteriorate from its current levels similar to what has been noted with long term use of AIV vaccines in poultry in the past.³⁻⁵

Our results are similar to what was reported by Grund *et al.*¹⁸ based on protection by clade, although they used different challenge viruses from Egypt (one from each clade) and three of their four vaccines were different (Mex/94 was used commonly). Grund *et al.*'s study also showed that the best protection against mortality and reduction of shed against clade 2.2.1.1 challenge was by vaccination with a clade 2.2.1.1 virus. While improved protection was noted with the clade 2.2.1.1 based vaccine in this study, it was still poorer than the protection Re-1 and Mex/94 provided against the clade 2.2.1 viruses.

Although there are differences among the vaccines in overall reduction of virus shed based on challenge virus clade, individually 17 of 18 vaccinated groups (the seven groups challenged with either Egy/202/07 or Egy/44/09 were not included in the statistical analysis because too few sham vaccinates survived long enough to be tested to determine the mean shed titer with adequate power) did reduce shed significantly versus sham vaccinates. Individually, 24 of 25 of the vaccine challenge groups also had significantly lower mortality when compared to sham vaccinates challenged with the same isolate. This has important implications for evaluating vaccines because most vaccines will provide measurable protection against mortality when compared to non-vaccinated or sham vaccinated birds in an experimental setting, but optimal protection may only be recognized in comparison to less protective vaccines. Furthermore, protection is always better in the lab than in the field; small differences in an experimental setting may be amplified in the field.

Somewhat interestingly, there was no significant difference in the number of chickens shedding detectable levels of virus based on vaccine or challenge virus clade. There was one challenge virus, Egy/20/09, for which significantly fewer chickens shed virus with three of the four vaccines (the exception was Mex/94). This virus also had the longest MDT with the shams, although this was only significant as compared to two other isolates. This suggests that vaccine efficacy may differ even among relatively closely related challenge viruses due to minor biological variations; if this isolate had been the only one used most of the vaccines would have appeared to have been more efficacious at reducing shed than they were. Because halting the transmission is a primary goal of vaccination, the optimal vaccines will substantially reduce both the shed titers and number of animals shedding virus. Interestingly, through 2012 the 2.2.1

clade remained predominant; the 2.2.1.1 clade did not replace it,¹⁹ reinforcing that the selection pressures on influenza are numerous (e.g., immune pressure, biological, epidemiological), and complex.

The serological results were also interesting in that antibody titers to neither the vaccine nor the challenge virus were predictive of either survival or shed. This is in contrast to the somewhat loosely held principle that HI titers over 40 indicate that a bird will be protected from mortality²⁰ and greater than 120 prevent replication.²¹ However, others have reported good protection against mortality in groups when geometric mean titers were below an HI titer of 40^{22–24} and it has been recognized that this cut-off is less predictive with vaccine viruses which are not well matched to the challenge virus among other factors.²⁵ This may be because HI assay is not measuring antibody to all protective epitopes, such as antibody to the fusion domain of the HA protein²⁶ or some other immunological component induced by inactivated adjuvanted vaccines.

Serology also revealed that Re-1 and Mex/94 induced higher antibody levels than either of the Egyptian isolates. Importantly, the differences in antibody levels did not translate into differences in protection. The lower immunogenicity of the Egyptian isolates is probably not due to vaccine formulation since the Mex/94 vaccine was prepared in-house identically to the Egyptian virus based vaccines and antigenic masses were similar based on HA titer. Therefore, there may be an inherent difference in the immunogenicity of the HA proteins of these isolates for chickens. Poor immunogenicity may be under selection to evade the immune response and may help explain why there was mortality with Egy/202/07 homologous vaccination challenge. Poorly immunogenic HA protein is thought to be why vaccines produced with A/chicken/Pakistan/NARC-1/1995 H7N3 produced poor immunity in the field and protected poorly against homologous challenge in experimental studies.^{27,28} Conversely, good immunogenicity may explain why some isolates can provide excellent protection against relatively unrelated challenge viruses (e.g., Re-1 and Mex/94 vaccines with clade 2.2.1 challenge viruses).²⁹ As antigenic proximity did not correlate with protection based on the HI assay data, these data suggest that immunogenicity may be at least as critical as antigenic matching when selecting vaccine seed strains. Importantly, the observed segregation based on clade has been previously reported.¹⁷ With this data HI based antigenic profiling alone is not sufficient to draw more general conclusions and nor is it clear what the effect of adjuvant may be on antibody reactivity. Finally, when updating vaccines, protection needs to be evaluated carefully, as simply using a recent local virus of the same genetic lineage may not always provide optimal protection.

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