Cross-protection by inactivated H5 pre-pandemic vaccine seed strains against diverse Goose/Guangdong lineage H5N1 highly pathogenic avian influenza viruses

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Abbreviations

Amino acid (aa); Animal biosafety level 2 (ABSL-2); Animal biosafety level 3 enhanced (ABSL-3E); Avian influenza virus (AIV); Broth heart infusion medium (BHI); Centers for Disease Control and Prevention (CDC); Days post-challenge (dpc); Defective interfering (DI); Embryo infectious dose (EID50); Embryonated chicken eggs (ECE); General time reversible (GTR); Geometric mean titers (GMT-log2); Goose/Guangdong lineage (Gs/GD); Hemagglutinin (HA); Hemagglutination inhibiting (HI); High efficiency particulate air (HEPA); Highly pathogenic avian influenza virus (HPAIV); Institutional Animal Care and Use Committee (IACUC); Low pathogenic avian influenza virus (LPAIV); Maximum likelihood (ML); Mean death time (MDT); Multiplicity of infection (MOI); Negative predictive value (NPV); Oropharyngeal (OP); Positive predictive value (PPV); Quantile-Quantile (Q-Q); Quantitative real time PCR (qRRT-PCR); Receptor binding site (RBS); Southeast Poultry Research Laboratory (SEPRL); Specific pathogen free (SPF).
Abstract

The highly pathogenic avian influenza virus (HPAIV) H5N1 A/goose/Guangdong/1996 lineage (Gs/GD) is endemic in poultry across several countries in the world, and has caused lethal, sporadic infections in humans. Vaccines are important in HPAI control for both poultry and in pre-pandemic preparedness in humans. This study assessed inactivated pre-pandemic vaccine strains in a One Health framework, focusing on the genetic and antigenic diversity of field H5N1 Gs/GD viruses from the agricultural sector and assessing cross protection in a chicken challenge model. Nearly half (47.92%) of the forty-eight combinations of vaccine/challenge viruses examined had bird protection of 80% or above. Most vaccinated groups had prolonged mean death time (MDT) and the virus shedding titers were significantly lower compared to the sham group (p ≤ 0.05). The antibody titers in the pre-challenge sera were not predictive of protection. Although vaccinated birds had higher titers of hemagglutination inhibiting (HI) antibodies against homologous vaccine antigen, most of them also had lower or no antibody titer against the challenge antigen. The comparison of all parameters, homologous or closely related vaccine and challenge viruses, gave the best prediction protection. Through additional analysis, we identified a pattern of epitopes substitutions in the hemagglutinin (HA) of each challenge virus that impacted protection, regardless of the vaccine used. These changes were situated in the antigenic sites and/or reported epitopes associated with virus escape from antibody neutralization. As a result, this study highlights virus diversity, immune response complexity, and the importance of strain selection for vaccine development to control H5N1 HPAIV in the agricultural sector and for human pre-pandemic preparedness. We suggest that the engineering of specific antigenic sites can improve the immunogenicity of H5 vaccines.
Importance

The sustained circulation of highly pathogenic avian influenza virus (HPAIV) H5N1 A/goose/Guangdong/1996 lineage (Gs/GD) in the agricultural sector and some wild birds has led to the evolution and selection of distinct viral lineages involved in the escape from vaccine protection. Our results using inactivated vaccine candidates from the human pandemic preparedness program in a chicken challenge model identified critical antigenic conformational epitopes on the H5 hemagglutinin (HA) from different clades that were associated with antibody recognition and escape. Even though other investigators have reported epitope mapping in the H5 HA, much of this information pertains to epitopes reactive towards mouse antibodies. Our findings validate changes in antigenic epitopes of HA associated with virus escape from antibody neutralization in chickens, which has direct relevance to field protection and virus evolution. Therefore, the knowledge of these immunodominant regions is essential to proactively develop diagnostic tests, improve surveillance platforms to monitor AIV outbreaks, and design more efficient and broad-spectrum agricultural and human prepandemic vaccines.

Keywords: Highly pathogenic avian influenza; H5N1; Goose/Guangdong lineage; Immunity; Chicken; Inactivated vaccine; Vaccine.

Introduction

The highly pathogenic avian influenza virus (HPAIV) H5N1 A/goose/Guangdong/1996 lineage (Gs/GD) is widespread, producing infections in poultry, wild birds, and humans in 84 different countries through Asia, Africa, Europe and North America (1, 2, 3). Since the first report of H5N1 in China during 1996, the H5N1 Gs/GD lineage HPAIV has spread across...
continents, with successful eradication in many countries (4). But the geographic isolation and maintenance of some H5N1 strains through endemic infections in the agricultural sector with spillover into natural ecosystems of migratory aquatic birds, genetic and antigenic drift has resulted in viral diversity, creating sub-lineages grouped into 10 genetically distinct virus clades (0-9) and multiple subclades (2). Continued circulation further resulted in some clades becoming extinct, while new antigenically distinct variants emerge. In total, four waves of intercontinental transmission of Gs/GD lineage H5Nx virus have been identified: (1) the 2005-2006 wave caused by clade 2.2 H5N1 HPAIV; (2) the 2009-2010 wave caused by clade 2.3.2.1c H5N1 HPAIV; (3) the 2014-2015 wave involving two separate lineages 2.3.4.4a H5Nx and clade 2.3.2.1c H5N1 that differed antigenically from the 2009–2010 viruses; and (4) the 2016-2017 wave caused by clade 2.3.4.4b H5Nx (5).

Ever since the AI viruses were identified as cause of fowl plague in 1955, more than forty-four documented outbreaks of HPAIV have occurred (5). Only the Gs/GD lineage epizootic has affected more poultry in countries than the other forty-three HPAI outbreaks combined (5). The circulation of H5N1 HPAIV Gs/GD lineages continue to threaten agricultural systems, natural ecosystems and human health; ie One Health. Since June 2016, countries in both Europe and Asia have detected infections in migratory aquatic birds of the natural ecosystems and/or domestic poultry of the agricultural sector with HPAIV H5N1 clade 2.3.4.4, showing mortality in wild birds (6). Further spread into other countries can still occur due to the possible spread along the migratory route of aquatic birds. Although the likelihood of human infection with the clade 2.3.4.4 H5Nx virus is low, it should be noted that human infection with H5N6 HPAIV of clade 2.3.4.4 has already occurred (7). In addition, zoonotic infections of HPAI H5N1 clades 2.1 and 2.2 have been reported, predominantly in Egypt and Indonesia since 2014 (5, 8).
Thus, although the risk of human infection is low, we cannot ignore its possibility – H5N1 continues to evolve and potentially acquiring mutations that promote transmission to humans (7).

In countries where Gs/GD lineage is endemic, the control of H5N1 HPAI in poultry by limited stamping-out programs is supplemented by vaccination, primarily through inactivated oil emulsified adjuvanted whole virus vaccines with more limited use of live-vectored vaccines (9). The genetic diversification and rapid antigenic drifting of field Gs/GD viruses in the agricultural sector have frequently led to inadequate protection. In addition, the prolonged vaccination programs adopted in endemic countries, such as China, Egypt, and Vietnam, have subsequently seen variant strains resistant to inactivated vaccine seed strains emerge (8, 10-13). Thus, to maximize protection of both poultry and human, the antigenic characteristics of vaccine seed strains should be periodically re-evaluated by *in vitro* and *in vivo* assays against the current circulating field viruses (14) to determine whether the available vaccine strains should be changed.

Currently, there is no vaccine seed strain capable of eliciting protective immunity across the various clades and subclades of H5 Gs/GD lineage of HPAIV in circulation (15). There is a need for a protective H5 HPAIV vaccine that provides broad coverage of co-circulating antigenically distinct variants in poultry and as a pre-pandemic vaccine for humans.

Within this study, we evaluated inactivated pre-pandemic vaccine strains in a One Health framework, focusing on genetic and antigenic matched and mismatched clade challenge viruses from the agricultural sector and assessing protection in a chicken challenge model in a variety of protection parameters, including a) survival, b) mean death time (MDT), c) decrease in virus shedding titers and number of birds shedding, d) HI antibody levels measured against both the
vaccine and challenge viruses HA protein pre- and post-challenge, in the context of genetic and antigenic differences between HA of vaccine and challenge viruses.

Results

Protection of vaccinated birds

We examined the vaccination efficacy of six inactivated vaccine strains against challenge with eight H5N1 Gs/GD lineage HPAI viruses in chickens (Table 1). Vaccine protection based on the percentage of bird survival varied greatly among the 48 homologous and heterologous combinations of vaccine strains and challenge viruses tested. We observed a survival efficacy rate of 90 percent or above for 16 out of the 48 possible combinations of vaccines and challenge viruses (Fig. 1 and Table S1). In contrast, survival rates of ≥80 and ≥70 percent were observed for a total of 23 and 28 combinations (Fig. 1 and Table S1), respectively. The homologous combination of vaccine 6 (7.1/Vn016/08) and challenge virus 7 (7.1/Vn016/08) had 100% protection. Further, most of vaccines and challenge strains from homologous clade had protection rates between 89% and 100% (Fig. 1 and Table S1), except for vaccine 2 (2.1.3.2/Indo/05) and challenge virus 2 (2.1.3/WJ29/07), vaccine 4 (2.3.2.1a/Hub/10) and challenge virus 6 (2.3.2.1b/Vn672/11), and vaccine 5 (2.3.4/Anh/05) and challenge virus 7 (2.3.4/HK8825/08) which had 20%, 60%, and 70% of protection, accordingly (Fig. 1 and Table S1).

To analyze changes in protection of multiple vaccines on the same set of challenge viruses we used the Wilcoxon matched-pairs signed rank test, a non-parametric version of the dependent t-test. Results showed statistically significant changes in protection between vaccines 2 (2.1.3.2/Indo/05) and 3 (2.2.1.1/E3300/08) (P=0.0078), vaccines 2 (2.1.3.2/Indo/05) and 4
(2.3.2.1a/Hub/10) (P=0.0078), and vaccines 2 (2.1.3.2/Indo/05) and 6 (7.1/Vn016/08) (P = 0.0234) (Fig. 2 and Table S2). Clearly, these analyses (Fig. 2 and Table S2) illustrated that vaccine 2 (2.1.3.2/Indo/05) (“white” boxplot) had the lowest protection out of all six vaccines tested. Since there were no statistical differences between the other five vaccines, and vaccines 1 (1.1/Camb/07), 3 (2.2.1.1/E3300/08), 4 (2.3.2.1a/Hub/10), 5 (2.3.4/Anh/05), and 6 (7.1/Vn016/08) all had protection levels of ≥ 70% (“blue” dotted line in Fig. 2), our results suggest, for this study, a minimum protective level of 70% as the standard value for measuring the effectiveness of vaccines. However, if we consider a higher minimum protective value (≥ 80%, see “red” dotted line in Fig. 2), which is usually the minimum acceptable level used in license of poultry AIV vaccines (16), our results show that none of the six individual vaccines we tested provide sufficient protection against all eight challenge HPAI viruses. Despite that, we found that vaccine 3 (2.2.1.1/E3300/08) offered the broadest protection against different clades of Gs/GD viruses, providing protections against 6 out of the 8 challenge viruses (Fig. 1, 2, and Table S1). Moreover, all six vaccines provided acceptable survival to meet the licensure requirement (≥ 80% protection level) against challenge viruses 3 (2.2.1/Egy102d/10) and 8 (7.1/V016/08) (Fig. 1, 2, and Table S1).

All sham vaccinated bird groups, positive control for the lethality of challenge viruses, had 100% mortality (Fig. 1), with MDT ranging from 2 to 3.5 days (Table S1). In all combinations of vaccines and challenge viruses that mortality was observed, MDT varied from 2 to 8.5 days (Table S1). Further, for all vaccine strains with low to intermediate survival rates (< 70%), the MDT average was longer (majority ranged from 4 to 8.8 days, except for one group that was 3 days) compared to sham groups (MDT 2.6±0.4). In other words, in these birds the vaccine delayed progression to death.
Evaluation of virus shedding

Virus shedding was detected at 2 days post challenge (dpc) in the oropharyngeal (OP) swabs from all birds in the sham groups, independent of the challenge virus, with mean titer ranging between 4.6 to 7.0 log_{10} EID_{50}/ml (Fig. 3, Table S3). Birds that received one of the six vaccines had either no or very low levels of viral shedding, with mean virus titers ranging from 1.8 to 4.6 log_{10} EID_{50}/ml (Table S3). Moreover, our results showed that 44 of 48 combinations of vaccinated birds had statically significant lower OP titers compared to sham birds (p<0.05) (Fig. 3). The four combinations that failed to decrease the level of shedding were only related to vaccine 2 (2.1.3.2/Indo/05) and 4 (2.3.2.1a/Hub/10) against challenge virus 6 (2.3.2.1b/V672/11) and 7 (2.3.4/HK8825/08) (Fig. 3, Table S3). Also, in 15 of 48 combinations, not only the titer but also the number of the birds shedding virus were significantly reduced compared to shams (* in Table S3).

The evaluation of virus shedding in the vaccinated groups also showed differences between each challenge virus used (Fig. S1). In general, comparison only between the vaccinated group showed that the majority of the birds challenge against virus 7 (2.3.4/HK8825/08) had significantly higher virus shedding titers than the other challenge viruses used in this study (Fig. S1). On the opposite, vaccinated birds challenged against virus 8 (7.1/Vn016/08) usually had the lowest levels of virus shedding in almost all vaccines with a few exceptions, such as challenge virus 4 (2.2.1.1a/Egy1063/10) for vaccines 1 (1.1/Camb/07) and 4 (2.3.2.1a/Hub/10), and challenge virus 3 (2.2.1/Egy102d/10) for vaccine 5(2.3.4/Anh/05) (Fig. S1).

Antibody levels in immunized birds pre- and post-challenge with HPAIV
Sera from samples collected from all birds before challenge (0 dpc) and the surviving birds (14 dpc) after challenge were analyzed by HI assay to measure humoral immune response against homologous and heterologous antigens (Fig. S2).

All vaccinated birds had high titers of HI antibodies against homologous vaccine antigen in the pre-challenge (mean GMT ranging 5.7 to 10 log₂) with the quantity maintained for the surviving birds after 14 dpc. The three exceptions were one bird in the group vaccinated with vaccine 1 (1.1/Camb/07) and challenge virus 1 (1.1/Vn118/08), one bird with vaccine 1 (1.1/Camb/07) and challenge virus 6 (2.3.2.1b/Vn672/11), and one bird vaccinated with vaccine 2 (2.1.3.2/Indo/05) and challenged virus 6 (2.3.2.1b/Vn672/11). These three birds were excluded in further analyses (Table S4).

To observe differences in the antibody response, we tested sera from vaccinated birds with the specific challenge virus antigen (Fig. 4 and Table S4). A serum sample with a HI titer 3 log₂ GMT or more were considered seropositive. None of the sham birds had detectable HI antibody titers before challenge (data not shown). Most vaccinated birds had low or no antibody titer detected in the pre-challenge sera, with mean GMT ranging from 2.0 to 3.2 log₂; except for the homologous combination of vaccine 6 (7.1/Vn016/08) and challenge virus 8 (7.1/Vn016/08) (GMT 6.9 log₂). Thus, the antibody titer in the pre-challenge sera was not associated with protection against mortality. However, the number of antibody positive birds which survived per total was higher when at least 50% of vaccinated birds in a group had detectable antibodies to the challenge virus in the pre-challenge sera (Table S4). All vaccinated birds had statistically significant higher HI titer in the post-challenge sera (mean GMT ranging 3.4 to 8.5 log₂) compared to pre-challenge sera (p <0.05) (Fig. 4); except for a few groups in which all birds died, vaccine 2 (2.1.3.2/Indo/05) against challenge 7 (2.3.4/HK8825/08) (Figure 4B), or the
number of live birds were insufficient for statistical analysis, such as with vaccine 2 (2.1.3.2/Indo/05) against challenge 2 (2.1.3/WJ29/07) (Fig. 4B) and vaccine 5 (2.3.4/Anh/05) against challenge 6 (2.3.2.1b/Vn672/11) (Fig. 4E).

Molecular analysis of vaccines and challenge H5 avian influenza virus strains

The genetic divergence for the HA1 protein between vaccines and challenge viruses was evaluated (Fig. 5, and S3, and Table 2), as well as specific amino acid changes in the antigenic sites (Table S5) and predicted potential N-glycosylation sites (Table S6).

The paired HA1 amino acid (aa) identities between vaccine strains and challenge viruses varied from 84.7 to 100%, except for the homologous combination (vaccine 6 and challenge virus 8) and 3 heterologous combinations (i.e., vaccine 1 and challenge virus 1; vaccine 3 and challenge virus 4, and vaccine 4 and challenge virus 5) with ≥ 97.2% homology (Table 2). Thus, a high HA1 aa identity between vaccine strain and challenge virus alone did not provide a predictive value for protection.

In phylogenetic analysis, the vaccine and challenge strain of all clades, except 7.1, were not clustered together (Fig. 5 and Fig. S3 A-F). The analysis of the antigenic sites in HA protein showed aa differences across these sites between strains even from the same clade (Table S5). Most of the aa changes were observed in the globular head of HA1 within or close to the antigenic site regions (data not shown). Moreover, some of these changes in the antigenic sites correspond to the acquisition of potential N-linked glycosylation (Table S6) that can potentially mask these regions from antibodies access. Specifically, potential N-glycans were observed in the antigenic sites B (aa 154-157) for vaccine 2 (2.1.3.2/Indo/05), D (aa 236-239) for both vaccine 3 (2.2.1.1/E3300/08) and challenge 4 (2.2.1.1a/Egy1063/10), E (aa 72-75) for vaccines 3
Antigenic relationships were analyzed with a cartography map (Fig. S4) generated from the HI data and no clear association was observed between antigenic distance, percentage of aa identity, and clinical protection (Fig. S5).

To identify mutations in the HA that influence vaccine protection, we compared the position of the amino acid changes in the vaccine/challenge strains that occurred only when the bird survivability was below 80%. Our results showed a pattern of a few amino acid mutations in the HA of each challenge virus strain regardless of the vaccine clade/virus used (Fig. 6). These amino acid substitutions were localized in the globular head of HA protein, mostly within antigenic sites (Fig. 6A-B). The decrease in vaccine protection associated with challenge viruses from clade 1.1 and 2.1.3 was mostly associated with changes in epitopes of antigenic sites A or E, or adjacent to this region while challenge viruses from clade 2.2.1.1a and 2.3.2.1 had mutations within antigenic sites A, B, and close to these sites. In contrast, the most recent H5N1 challenge virus clades, 2.3.2.1b and 2.3.4, had epitopes changes in all five immune dominant antigenic sites (A to E), affecting the level of vaccine protection. Similar results were observed when the cut off value for vaccine protection was ≥70% (yellow circles in the aa position Fig. 6B), excluding a few amino acid positions. Among all amino acid changes, those at the position 140 (H5 HA numbering) were observed in nearly all the challenge viruses, with a few exceptions (Fig. 6 A and B) regardless of vaccine.

Discussion
The use of vaccines to control H5N1 Gs/GD lineage HPAIV infection in poultry where the virus is endemic is one of the main measures to assure livelihoods, food security and decrease the negative economic impact of AIV (1,14). Due to the rapid antigenic evolution of HA, virologic surveillance and in vivo experiment data are important to assure that the antigenic content of vaccines is updated in a timely fashion to protect individual bird and control AIV. Yet, vaccine protection for AIV is a complex multifactorial issue.

Results from this study showed ≥80% efficacy against mortality in nearly half (47.92%) of the combinations of vaccine strains and challenge viruses in this study (Table 1 and S1). The protection of these vaccines against challenge viruses was even higher (58.33%) with a survival rate value of ≥70%. In general, vaccine 2 (2.1.3.2/Indo/05) was less protective against H5N1 Gs/GD lineage challenges. Although regulatory requirements vary from country to country, considering a ≥80% efficacy threshold of protection, as the minimum acceptable level for licensing of poultry AIV vaccines (16), all H5N1 Gs/GD vaccines tested failed to protect against challenges with 2.3.2.1b and 2.3.4 HPAI viruses. Even in the 25 combinations with lower levels of survival, the MDT was prolonged for most vaccinated groups compared to the sham groups (Table S1), suggesting partial but inadequate protection to prevent death. For example, vaccine 2 (2.1.3.2/Indo/05) only provided 20% survival rate against challenge 2 (2.1.3/WJ29/07) and zero against challenge 7 (2.3.4/HK8825/08), and was accompanied by prolonged MDT of 5.5 and 6 days, respectively, as compared to a MDT of 3 and 2.5 days for the respective sham vaccinate groups. While none of the individual vaccines protected against all HPAI virus challenges, the vaccine strain from clade 2.2.1.1 (Vaccine 3- 2.2.1.1/E3300/08) was able to protect against six virus clades, but not 2.3.2.1b (challenge 6) and 2.3.4 (challenge 7). According to previous studies, serum and antigens of clade 2.2 avian influenza viruses appeared to be optimal for
detecting most of the HPAIV H5N1 virus and sera, respectively (17). Although new distinct
clusters had emerged, as H5N1 viruses from clade 2.2.1.2 in Egypt, no marked difference in
antigenicity and receptor preference was identified in this group of viruses before 2014 (18),
probably explaining why some vaccines were able to recognize this clade before this period (19).
Several studies have suggested a strong link between genetic and antigenic distances, even
though other factors could be involved.

Humoral responses to influenza vaccines are usually assessed by measuring antibodies
against the circulating field virus HA protein by HI assay, and such results are interpreted as
predictors of protection. Some studies suggested that presence of pre-determined levels of HI
antibodies would predict protection against the challenge virus used in HI assay (20, 21),
however other studies also report that birds with low or no detectable levels of HI antibodies can
survive some challenge viruses (12, 22-24). In our study, high HI antibody levels to the vaccine
were predictive of survival for genetically identical or closely related vaccine/challenge virus
combination, but not predictive for survival when the challenge virus was antigenically divergent
from the vaccine seed strain. Furthermore, in a previous study (16), the detection of HI
antibodies using challenge virus as HI antigen was associated with protection; i.e. positive
predictive value (PPV). Similar PPV results against mortality using challenge virus were
observed in the present study when the HI titers were ≥ 32 (GMT 5 log$_2$) (data not shown),
which is in agreement with the OIE’s efficacy requirement for new vaccines in terms of
achieving mortality reduction (25). In contrast, some divergent vaccine/challenge virus
combinations lacked detectable HI antibodies, but birds were still clinically protected [i.e. poor
negative predictive value (NPV) of lack HI titers and mortality], suggesting possible protection
by humoral non-HI antibodies, or cellular immunity, or a combination of them. Although,
inactivated vaccines are poor inducers of cellular immunity (14), some minor induction of cellular immunity may have contributed to protection. A previous study found that an inactivated H5N2 North American low pathogenic avian influenza virus (LPAIV) vaccine formulation, provided efficient vaccine protection and reduced virus shedding against challenge with Gs/GD lineage clade 2.2.1 virus from Egypt (23) despite low level of antigenic cross-reactivity by HI. In contrast, a comprehensive study with H5 vaccines against several genetic and antigenic dissimilar Indonesian H5 Gs/GD lineage 2.1.3 clade field strains concluded that the HI antibodies against the vaccine antigen was a poor positive predictor of survival after lethal challenge (16). Also, several studies noted lack of association between vaccine induced HI titers and protection in chickens for H5 (12, 26) and H7 (22, 27), and mice for H6 influenza A viruses (28). Therefore, even though detection of HI antibodies against a challenge virus are usually reported as a proxy of protection (i.e. PPV), HI antibodies are not the only active immune effectors against infection, and other parameters such as humoral Fc-mediated functions and non-humoral immunity of other conserved influenza proteins may play some role in protection (14, 29, 30).

In this study, we also tested 78 serum samples by virus neutralization assay (data not shown) because the HI assay alone does not detect all protective antibodies such as those to the fusion domain of the HA or other proteins such as neuraminidase. However, no clear association of the presence of neutralizing antibodies and survival efficacy could be made as several birds with neutralizing antibodies (even with titers as high as 256) died after challenge. Based on our limited sample set, the results of the neutralization assays by themselves were not enough to explain why some groups were protected when lacking neutralizing antibodies and others were not protected despite the presence of neutralizing antibodies. Thus, it reinforces the complexity...
of the immune response required to provide full protection after challenge with antigenic and genetically diverse, rapid and systemic-replicating HPAI viruses. Similar results with a lack of correlation of the HI or neutralizing antibody data with protection have been reported in humans against H5N1 infection (31, 32).

In general, homologous or closely related vaccine/challenge virus pairs in the same genetic clade had the best protection across multiple metrics (Fig. 5). The homologous vaccine and challenge performed with 7.1/V016/08 had 100% survival rate and only two birds shed low levels of virus. Vaccine 3 (2.2.1.1/E3300/08) protected against mortality and significantly decreased the virus shedding and the number of birds shedding with the two most closely related challenge viruses, 3 (2.2.1/Egy102d/10) and 4 (2.2.1.1a/Egy1063/10). Birds vaccinated with 2.3.2.1a/Hub/10 (Vaccine 4) were protected against mortality and presented a significant decrease in virus shedding when challenged with viruses 6 (2.3.2.1b/Vn672/11) and 5 (2.3.2.1/Vn398/10). Vaccine 1 (1.1/Camb/07) protected against mortality and decreased virus shedding after challenge with the same clade (1.1/Vn118/08) but did not significantly decrease the number of birds shedding. Despite these results, not all the closest related vaccine/challenge virus combinations provided acceptable protection. Birds vaccinated with 2.1.3.2/Indo/05 (Vaccine 2) and challenged with 2.1.3/WJ29/07 (challenge 2) (96.3%) had low survivability (20%) and although the titer of shedding was significantly decreased at 2 dpi, the number of birds shedding was equivalent to the sham controls. Although our study demonstrated most vaccine seed strains reduced challenge virus shedding at 2 dpi, the peak of virus replication and shedding, similar trends in reduction of oropharyngeal shedding has been reported from samples collected at later dpi (14, 16, 21, 33-35).
While the level of HA1 aa identity between vaccine and challenge for all the combinations ranged from 84.7% to 100%, the level of HA aa sequence identity was not consistently a good predictor of survival (except for combinations with ≥97.2% identity). Ten combinations that had low survival rates (0-40%) had an identity between vaccine and challenge ranging from 87.2% to 96.3%. This finding is not unexpected since previous studies had similar results (26). For example, chickens vaccinated with vaccine 2 (2.1.3.2/Indo/05) and challenged with closely related HPAI virus 2 (2.1.3/WJ29/07, 96.3% HA1 homology), only had a 20% of survival rate. By contrast, 90% survival rate was obtained with vaccine 4 (2.3.2.1a/Hub/10), followed by a challenge with virus strains 8 (7.1/Vn016/08) (85.9% homology), and 100% survival rate with vaccine 5 (2.3.4/Anh/05) when challenged with strain 8 (7.1/Vn016/08) (86.6% homology). This suggests that specific changes in critical antigenic sites might be a better predictor of protection than overall sequence identity of HA protein. The results of in silico analyses associated with in vivo vaccine/challenge combinations highlighted the importance of specific polymorphisms observed during H5N1 AIV evolution. Some important positions for antigenic drift mutations that allow the 2002-H5N1 virus to escape antibody neutralization, such as positions 86, 94, 124, 140, and 189 (36), were detected in our study as important epitopes alone or in a combination that affects vaccine protection regardless of the clade of the H5N1 challenge viruses. In general, we found that mutations in position 140 (H5 HA numbering) were predominant and strongly associated with the loss of vaccine protection in almost all combinations between vaccine and challenge strains. The amino acid at position 140, located in the antigenic site A, was previously characterized by different H5N1 strains and panel of chimeric and mouse monoclonal antibodies which target the receptor binding site (RBS) in the HA (37-41). This position is important since it impacts the H5 HA structure involving specific
monoclonal antibodies recognition and mediates neutralization escape (37-41). Similarly, changes in position 162 were also detected and negatively associated with vaccination protection. Other studies showed amino acid 162 in the HA as part of a neutralization epitope outside or the RBS and in proximity to site B (42, 43).

Sequence alignments of representative viruses from each clade and sequence variations mapped onto the H5N1 HA structure have demonstrated that the most variable inter-clade positions are largely located on the membrane distal globular head close to the receptor binding site (RBS) (44). The identification of these critical antigenic epitopes or “hot spots” highlighted the complexity of the conformational epitopes on the H5 HA structure involved in antibody recognition and escape neutralization. Even though several groups reported epitope mapping in the H5 HA, much of this information pertains to antigenic epitopes reactive towards mouse antibodies instead of human or chicken (44). Therefore, knowledge of human and chicken immunodominant regions is important to proactively develop diagnostic tests and improve surveillance platforms to monitor AIV outbreaks and design more efficient and broad-spectrum pre-outbreak vaccines.

To characterize the antigenic relationships among vaccines and challenge viruses, we used antigenic cartography based on HI assay reactivity (Fig. S4). Viruses from within each clade, cluster close to each other, although variation was still observed with each clade and subclade, as previously observed in other studies (45, 46). However, it was not possible to establish a clear association between antigenic distance or location on the map with the prediction of clinical protection or high HA1 aa homology of these in vivo studies (Fig. S5), with the specific panel of chicken antisera used. Previously, with a few exceptions, a strong association between antigenic distances for vaccine and challenge viruses and clinical protection
against challenge for distances lower than 4 antigenic units were reported for HPAI in chickens (47). Other studies also suggested that antigenic cartography can be an important tool to characterize and map virus diversity (48), but the lack of association between clinical protection and specific antigenic differences, visualized by antigenic cartography alone, has been previously reported for H5, H7 and H9 AIV in chickens (22, 33, 49), and ducks (29), especially the absence of HI reactivity being a predictor for lack of protection.

Prior research has shown that amino acid changes in the antigenic sites and glycosylation composition in the HA can alter the antigenicity of AIV (48, 50-52). The evaluation of the antigenic sites displayed specific amino acid changes across sites A through E (37, 38, 53) in the vaccine and challenge strains used in this study. However, no direct association was observed between complete antigenic site sequence and survivability, not even a decrease in oropharyngeal virus shedding. But some amino acid changes were associated with additional N-glycosylation sites, which also included positions 72-75, 154-157, 195-198, and 236-239 (H5 HA numbering) within the antigenic sites (Figure 6). To our surprise, vaccine 2 (2.1.3.2/Indo/05) was the only tested vaccine with N-linked glycosylation on the in silico evaluated antigenic site B (154-157) (Table S6). This vaccine demonstrated the lowest protection rate against the tested challenge viruses, regardless of the high protein sequence identity. It has been recognized that the presence of N-glycosylation on the globular head of the HA can mask or modify antigenic sites recognized by neutralizing antibodies (51). In a previous study with ferrets, the masking of the antigenic epitopes at 154-157 in the HA globular head of A/Vietnam/1203/2004(H5N1) vaccine virus by N-linked glycosylation affected virus antigenicity, resulting in lower antibody response (54). The presence of N-linked glycosylation on the tested antigenic site 154-157 observed in the 2.1.3.2/Indo/05 vaccine could be a factor involved in the poor protection
observed against some challenge viruses with high protein sequence homology with the vaccine strain. The addition of three N-glycosylation sites on a 2015 emergent H7N3 HPAI field viruses was associated with a decrease in protection from a recombinant fowl poxvirus vaccine containing a 2002 H7 HA gene insert (34). This vaccine gave good protection against the 2012 H7N3 HPAI virus, which had four glycosylation sites as do the 2002 vaccine strain (34). Therefore, we should consider the potential N-glycosylation sites to select AIV vaccine candidates and vaccination effectiveness against AIV field strains. Future studies are necessary to understand how the predicted N-glycosylation sites identified in this study can influence the efficacy of protection.

Before the widespread and long-term usage of AI vaccines in poultry, researchers had suggested that an HA similarity of >85% identity could be a good predictor of protection (55). However, virus evolution and the massive use of vaccination could have contributed to virus evasion mechanisms. This would have been possible with only a few critical genetic and antigenic changes in specific sites of the HA that would need to have occurred in order to overcome immunity. Our findings suggested that a combination of aa mutations (in contrast to a single substitution) are usually involved in the vaccine immunity overcome. Most of the clade-specific sequence variations in the HA of H5 Gs/GD identified were localized in the antigenic sites and reported epitopes associated with virus escape from antibody neutralization (44). Our data show differences in the pattern of amino acid substitutions between each Gs/GD virus clade, which highlighted important regions of immune pressure in these H5 HPAIV field viruses. Studies have been suggesting that antigenic evolution in AIV involves transitions from one antigenic cluster to another (46, 56) as we observed in these results. Further, the sustained circulation of H5N1 strains has led to the evolution and selection of distinct viral lineages with a
significant variation in the HA sequence (5, 17, 36, 46), which also explained the increase of epitopes involved in the escape of vaccine protection observed in clades 2.3.2.1b and 2.3.4 AIV.

Our goal with this study was to test vaccine candidates originally included in the human pandemic preparedness program and to evaluate whether they can protect a chicken model against challenge viruses relevant for poultry in endemic regions. We conclude that the effectiveness of the vaccine will be dependent on the challenge virus, especially specific epitopes in the antigenic sites. Although most vaccines could reduce oropharyngeal virus shedding, only half of the vaccine/challenge virus combinations had survivability of ≥ 80% (Table 1). The lack of consistent association between HI titers and protection for all vaccine/challenge virus combinations, as well as homology of HA1 and antigenic sites with clinical protection, reinforce that killed vaccine-induced protection against H5N1 HPAIV Gs/Gd lineage in chickens is a complex process. Several variables are involved, and one single criterion such as specific genetic and/or antigenic differences or HI results cannot by themselves predict clinical protection. On the other hand, we were able to identify HA epitopes in the challenge viruses that correlated with vaccinal protection. Most of the mapped high mutation sites observed were previously associated with virus escape from antibody neutralization, which supports the direct correlation of our findings connecting the in vivo and silico results. Our data also reinforces the need for further studies to better understand the antigenic drift mutations in the immune-dominant regions in the HA and other AIV proteins involved in virus escape against vaccine protection.

Materials and methods

Vaccines and challenge viruses
All vaccine seed strains used in this study (Table 1) are H5N1 vaccines produced by reverse genetics with PR8 internal gene segments. The vaccines were obtained from the US Centers for Disease Control and Prevention (CDC, Influenza Division, Atlanta, Georgia, USA) and the National Institute for Biological Standards and Controls (Hertfordshire, UK) as part of the WHO pre-pandemic vaccine preparedness program. The isolates were propagated in specific pathogen-free (SPF) 9-11-day-old embryonated chicken eggs (ECE) by following standard procedures (57). Allantoic fluid harvested from infected eggs was inactivated with 0.1% beta-propiolactone and used as antigen for vaccine production and serological assays. All the vaccines were adjuvanted by oil-in-water emulsion formulation using mineral oil as previously described (58). The antigens were adjusted to have all the vaccine with high antigen content as measured by hemagglutinating (HA) units (512 HAU per 0.5ml dose). Usage of between 256-512 HAU units per dose in poultry vaccine is standard across experimental studies and in veterinary biological manufacturing. Sham group vaccines were produced using sterile allantoic fluid from uninfected embryonated chicken eggs and the same formulation.

Eight H5N1 HPAIV isolates (Table 1) were selected as challenge viruses based upon distinct clades of Gs/GD from poultry found in some endemic areas, such as Asia, Africa, and the Middle East. The isolates were propagated using the same procedures described for the vaccines.

Animals and housing

Vaccinations were performed in animal biosafety level 2 (ABSL-2) facilities at Southeast Poultry Research Laboratory (SEPRL), United States Department of Agriculture (ARS/USDA). The birds were transferred before the challenge to the animal biosafety level 3 enhanced (ABSL-
facilities at SEPRL where they were housed in negative pressure high efficiency particle air (HEPA) ventilated isolation cabinets and had *ad libitum* access to feed and water throughout the experiment. All studies were reviewed and approved by the U.S. National Poultry Research Center (USNPRC) Institutional Animal Care and Use Committee (IACUC) and conducted with appropriate measures to maintain biosecurity and biosafety.

*Experimental design*

Experiment design and sampling were done using animals, vaccination, and challenge protocols following identical conditions as previously published from our group. In summary, a total of five experiments were performed using six different vaccine strains against eight Gs/Gd H5N1 HPAI challenge viruses (total of 48 combinations with an initial 10 birds per group) (Table 1). A sham-vaccinated (sham) group was included with each experiment as an unvaccinated challenge control, and all data comparisons were performed using each specific sham group for the appropriate challenge virus. All birds were 3-week-old SPF white leghorn chickens from SEPRL *in-house* flocks. The vaccines were administered via the subcutaneous route in the nape of the neck, in 0.5 ml dose per bird. Three weeks post-vaccination, birds were bled to evaluate the serological response and challenged by the intranasal route via the choanal cleft with target $10^6$ 50 percent embryo infectious dose (EID$_{50}$) in 0.1 ml per bird. The inoculum titer was verified by back titration in ECE that was for challenge virus (different studies were performed, so back titration titers are provided as a range for the challenge virus that had different back-titers in different studies): 1) 1.1/Vn118/08: $10^{5.0}$-$10^{6.1}$ EID$_{50}$/0.1ml, 2) 2.1.3/WJ29/07: $10^{5.9}$-$10^{6.1}$ EID$_{50}$/0.1ml, 3) 2.2.1/Egy102d/10: $10^{5.9}$ EID$_{50}$/0.1ml, 4) 2.2.1.1a/Egy1063/10: $10^{5.9}$-$10^{6.1}$ EID$_{50}$/0.1ml, 5) 2.3.2.1/Vn398/10: $10^{5.9}$-$10^{6.1}$ EID$_{50}$/0.1ml, 6)
2.3.2.1b/Vn672/11: $10^{5.9}$-10$^{6.1}$ EID$_{50}$/0.1ml, 7) 2.3.4/HK8825/08: $10^{5.7}$-10$^{5.9}$ EID$_{50}$/0.1ml, 8) 7.1/Vn016/08: $10^{6.1}$ EID$_{50}$/0.1ml. Two days post-challenge (dpc), OP swabs were collected and placed in Becton-Dickinson BBL brain heart infusion (BHI) medium with 2X antibiotics (Penicillin/Streptomycin/Fungiezone; Hyclone, Logan, UT, USA), and stored at −80°C until tested to determine virus shed titers. All birds were observed daily, and clinical signs and mortality were recorded from 0 to 14 dpc. Birds presenting severe clinical signs, e.g. unresponsive or unable to reach feed and water, or other HPAI infection signs were euthanized for humane reasons due to severe disease and were counted as death on the following day for MDT calculations. Fourteen days after challenge all the surviving birds were bled to evaluate antibody titers post-challenge and euthanized according to the approved IACUC protocol.

Virus shedding

OP swabs collected at 2 dpc were processed for quantitative real-time PCR (qRRT-PCR) to determine the viral shed from the oropharynx after challenge. We used a standard protocol that demonstrated the high correlation between qRRT-PCR and infectious titer qRRT-PCR because: a) used only influenza A challenge viruses chicken adapted and propagated in ECE, b) used low multiplicity of infection (MOI) to inoculate ECE in propagating the viruses to generate the challenge inoculum which minimizes defective interfering (DI) RNAs, and c) used the same specific challenge virus stock to generate the standard curve with each dilution point directly comparing qRRT-PCR CT values and infectious titer (59). This methodology has been used in several published veterinary influenza vaccine studies (59-61). Briefly, the RNA was extracted using MagMAXTM-96 AI/ND Viral RNA Isolation Kit® (ThermoFisher Scientific, Carlsbad, CA, USA) following the manufacturer’s instruction. Further, qRRT-PCR that targets the matrix
gene of avian influenza was performed with the AgPath-ID OneStep RT-PCR kit (ThermoFisher Scientific) using 7500 FAST Real-time PCR System (Applied Biosystems, Foster City, CA), as previously described (62). Virus quantity was established with a standard curve from RNA extracted from 10-fold dilutions of the challenge virus in duplicate. The limit of detection obtained for each challenge virus was 1) 1.1/Vn118/08: 2.5 log_{10} EID_{50}/ml, 2) 2.1.3/WJ29/07: 1.7 log_{10} EID_{50}/ml, 3) 2.2.1/Egy102d/10: 2.3 log_{10} EID_{50}/ml, 4) 2.2.1.1a/Egy1063/10: 2.5 log_{10} EID_{50}/ml, 5) 2.3.2.1/Vn398/10: 2.5 log_{10} EID_{50}/ml, 6) 2.3.2.1b/Vn672/11: 2.5 log_{10} EID_{50}/ml, 7) 2.3.4/HK8825/08: 2.3 log_{10} EID_{50}/ml, and 8) 7.1/Vn016/08: 1.9 log_{10} EID_{50}/ml. For statistical analysis, negative samples were considered lower than the limit of detection mentioned above for each specific challenge viruses tested.

Hemagglutination inhibition assay

Sera collected pre- and post-challenge were evaluated for specific antibodies using the appropriate vaccine or challenge virus antigens in the hemagglutination inhibition (HI) assay. The antigens were prepared as previously described (63) and the HI assay performed following standard procedures (64). Titers were calculated as the reciprocal of the last HI positive serum dilution and were converted to log2. Titers were expressed as geometric mean titers (GMT-log2). Samples were considered positive for the presence of AI antibodies at the limit of detection (1:8 dilution; or 3 log2). Negative samples were assigned as 2 log2 GMT for statistical purposes.

Antigenic cartography

All the vaccine strains and challenge viruses plus additional reference strains were antigenically characterized and mapped with statistical robustness tests as previously described.
The antigenic maps were produced from HI assay data. The HI assays were performed as previously described (64) using isolate specific polyclonal chicken sera produced in-house to serve as mono-specific reference antibodies. The antiserum was produced by vaccinating chickens with an oil emulsion vaccine prepared with Montanide ISA 50V adjuvant (SEPPIC, Inc., Paris, France) using infectious allantoic fluid inactivated with 0.1% beta-propiolactone. Five-week-old SPF chickens were vaccinated with 0.5 ml of the vaccine by the subcutaneous route, and serum was collected 3 weeks after vaccination. The serum was treated with 5% chicken red blood cells for 30 min at room temperature to decrease non-specific reactions.

Molecular analysis of HA

The HA segments of vaccine and challenge viruses used in this study were aligned using MUSCLE (66) and amino acid sequences at the previously reported antigenic sites of HA1 were evaluated (33, 48). All available HPAI clade 1.1; 2.1.3; 2.2.1; 2.3.2.1; 2.3.4; and 7.1 HA gene sequences were downloaded from the Influenza Virus Resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) on February 22, 2017. Complete HA sequences of clade 2 viruses were further pruned by using the software cd-hit (67) to remove redundant sequences at 99.3-99.7% similarity level. The final dataset consisted of 65 taxa for clade 1.1, 119 for clade 2.1.3, 112 for 2.2.1, 115 for 2.3.2.1, 106 for 2.3.4, and 82 for 7.1.

The maximum likelihood (ML) tree of each clade (Figure 1S) was generated by using RAxML (68) using the general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites (with four rate categories). Statistical analysis of phylogenetic tree was determined by bootstrap analysis with 1000 replicates.

Potential N-glycosylation sites were predicted using NetNGlyc server 1.0 (69).
We created a series of visualizations in the R software (Version 3.5.0) using the ggplot2 package (70) to help identify the amino acid changes that were correlated with a loss of effectiveness in vaccine protection using a threshold of 80% and 70% of survival. Each challenge virus strain was compared to the vaccine strains to identify the amino acid substitutions. Only amino acid changes that occurred in the combination of vaccine and challenge strains with bird survival below 80% or 70% were considered. The aa mismatch was excluded from the final analyses (Table 6A) if it was observed simultaneously in the effective and ineffective vaccination. The H5 HA subtype numbering conversion was obtained in the Influenza Research Database using the closest reference sequence (A/Vietnam/1203/04; HPAI) suggested by the website.

The HA structure was modeled using the H5 HA template (PDB accession number, 2FK0) in the SWISS-MODEL server (71, 72). The 3D molecular HA structures were visualized using the PyMOL Molecular Graphics System (Version 2.0 Schrödinger, LLC).

Statistical analysis

Statistical analyses were performed using Prism 7 (GraphPad Software, San Diego, CA, USA). The survival rate data was analyzed using the Mantel-Cox Log-Rank test. Fisher’s exact test was used to analyze the statistical significance of virus shedding compared among vaccinated and sham groups within experiments. Also, one-way-ANOVA using Kruskal-Wallis and Dunn’s multiple comparisons tests were performed to evaluate virus shedding between vaccinated groups. Statistical differences in mean viral titers and antibody levels between groups were analyzed using Mann–Whitney test and Tukey one-way ANOVA. To analyze changes in the protection of multiple vaccines on the same set of challenge viruses, we performed the
Wilcoxon matched-pairs signed-rank test, a non-parametric version of the dependent t-test. A P-value of <0.05 was considered significant.

Data availability

All the hemagglutinin gene sequences used in this study were downloaded from the Influenza Virus Resource Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). The avian influenza vaccine strains and challenge viruses tested are available in the GenBank under accession numbers listed in Table 1. The H5 HA structure template is available in Protein Data Bank (PDB) under accession number 2FK0.

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specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

The authors declare that they have no conflict of interest.
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Table captions

Table 1. Avian influenza vaccine strains and challenge viruses tested, clade and accession number of HA genes.

Table 2. HA1 amino acid identity between vaccine strains and challenge viruses.
Figure legends

Figure 1. Survival curve of vaccinated and not vaccinated (Sham) chickens challenged at 6 weeks of age with different clades of H5N1 HPAIV at a dose of $\sim10^{6.0}$ EID$_{50}$/0.1 ml. The inactivated vaccines used are: a) 1 (1.1/Camb/07); b) 2 (2.1.3.2/Indo/05); c) 3 (2.2.1.1/E3300/08); d) 4 (2.3.2.1a/Hub/10); e) 5 (2.3.4/Anh/05); f) 6 (7.1/Vn016/08). Each challenge virus used in the vaccinated and not vaccinated (sham, positive control) groups are identified by the same color but different symbols as shown in the figure legend.

Figure 2. Differences in vaccine protection against challenge with eight H5N1 HPAI viruses from Gs/Gd lineage. To analyze changes in the protection of multiple vaccines on the same set of challenge viruses, we performed the Wilcoxon matched-pairs signed-rank test. Each vaccine is represented by boxplot and challenge viruses by numbers and symbols (square). The white boxplot represents the vaccine that was less efficient in birds protection out of the six vaccines tested. The threshold of 80 % (red line) and 70% (blue line) of vaccine protection were also represented. The p < 0.05 are indicated by “*”.

Figure 3. Scatter plot of oropharyngeal virus shedding for vaccinated and sham group represented in Log$_{10}$EID$_{50}$/ml. The vaccinated ( ) versus sham ( ) groups are listed for each challenge virus in the: a) Vaccine 1 (1.1/Camb/07); b) Vaccine 2 (2.1.3.2/Indo/05); c) Vaccine 3 (2.2.1.1/E3300/08); d) Vaccine 4 (2.3.2.1a/Hub/10); e) Vaccine 5 (2.3.4/Anh/05); f) Vaccine 6
Each challenge virus is represented by numbers as labeled in the figure legend. The p < 0.05 are indicated by “*”.

**Figure 4.** Scatter plot of HI titers from vaccinated individual chickens pre- (filled circles “●”) and post-challenge (open circles “○”) sera. The HI titers (vertical axis) using challenge virus as antigen are shown for each vaccine (horizontal axis): a) Vaccine 1 (1.1/Camb/07); b) Vaccine 2 (2.1.3.2/Indo/05); c) Vaccine 3 (2.2.1.1/E3300/08); d) Vaccine 4 (2.3.2.1a/Hub/10); e) Vaccine 5 (2.3.4/Anh/05); f) Vaccine 6 (7.1/Vn016/08). Titers were expressed as geometric mean titer (GMT-log2), and samples with titer below 3 log2 GMT were considered negative (dotted horizontal line). ns: birds did not survive. The p < 0.05 are indicated by “**”.

**Figure 5.** Maximum-likelihood phylogeny of hemagglutinin gene for each vaccine and challenge virus used in this study. Numbers along branches indicate bootstrap values >70%. Brackets indicate the genetic subclades. Scale bar indicates nucleotide substitutions per site.

**Figure 6.** Molecular analyses of H5N1 avian influenza virus hemagglutinin for vaccines and challenge viruses. A) The table shows in silico analyses of the amino acid changes associated with loss of vaccine protection (80% or above) in each challenge virus compared to the vaccines used in this study. Each position is listed as “vaccine strain amino acid”-position (H5 HA numbering)-“challenge strain amino acid”. The protection obtained in the in vivo experiment for each vaccine/challenge virus combination is shown in percentage and color code, with high and low protection shown as green and red, respectively. The amino acids located in the antigenic sites A (cyan), B (orange), C (magenta), D (g in reen), E (yellow) or not (X or X) were...
identified. The “red” X is the epitope previously listed as important for virus escape, and the “black” X as unknown in the literature. B) Ribbon diagram and surface of H5 HA. Only monomers are shown. The amino acids listed in table A are shown in the HA structure. Predicted antigenic sites A (cyan), B (orange), C (magenta), D (green), and E (yellow) are labeled for each challenge viruses. Amino acid position highlighted by the yellow circle is only observed if the threshold for vaccine protection is 70% or above.
Table 1. Avian influenza vaccine strains and challenge viruses tested, clade and accession number of HA gene.

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<th>GenBank access number HA gene</th>
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*GISAID accession number
Table 2. HA1 amino acid identity (%) between vaccine strains and challenge viruses.

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<td>84.7</td>
<td>85.6</td>
<td>100.0</td>
</tr>
</tbody>
</table>

on January 29, 2021 by guest http://jvi.asm.org/ Downloaded from
Figure 1

A) Vaccine 1 (1.1/Camb/07)  
Challenge viruses

B) Vaccine 2 (2.1.3.2/Indo/05)

C) Vaccine 3 (2.2.1.1/E3300/08)

D) Vaccine 4 (2.3.2.1a/Hub/10)

E) Vaccine 5 (2.3.4/Anh/05)

F) Vaccine 6 (7.1/Vn016/08)

Days post-challenge

Percent survival

Days post-challenge

Percent survival

Days post-challenge

Percent survival

Days post-challenge

Percent survival

Days post-challenge

Percent survival

Days post-challenge

Percent survival
Figure 2

Differences in protection between vaccine groups

Vaccines

Challenge viruses
1. 1.1/Vn118/08
2. 2.1.3/W29/07
3. 2.2.1/Ind/10
4. 2.2.1.1a/Egy106/10
5. 2.3.2.1/Vn398/10
6. 2.3.2.1b/Vn672/11
7. 2.3.4/Anh/05
8. 7.1/Vn016/08

Protection (%)
Figure 3

A) Vaccine 1 (1.1/Camb/07)  

B) Vaccine 2 (2.1.3.2/Indo/05)  

C) Vaccine 3 (2.2.1.1/E3300/08)  

D) Vaccine 4 (2.3.2.1a/Hub/10)  

E) Vaccine 5 (2.3.4/Anh/05)  

F) Vaccine 6 (7.1/Vn016/08)
Figure 4
A) Vaccine 1 (1.1/Camb/07): challenge virus as antigen
B) Vaccine 2 (2.1.3.2/Indo/05): challenge virus as antigen
C) Vaccine 3 (2.2.1.1/E3300/08): challenge virus as antigen
D) Vaccine 4 (2.3.2.1a/Hub/10): challenge virus as antigen
E) Vaccine 5 (2.3.4/Anh/05): challenge virus as antigen
F) Vaccine 6 (7.1/Vn016/08): challenge virus as antigen

Challenge viruses:
1. 1.1/Vn118/08
2. 2.1.3/WJ29/07
3. 2.2.1/Egy102d/10
4. 2.2.1.1a/Egy1063/10
5. 2.3.2.1/Vn398/10
6. 2.3.2.1b/Vn672/11
7. 2.3.4/HK8825/08
8. 7.1/Vn016/08
Figure 5
<table>
<thead>
<tr>
<th>Strain/Clade</th>
<th>Amino Acid Position</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1/Camb/07</td>
<td>E69K</td>
<td>80.0</td>
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<tr>
<td>2.1.3.2/Indo/05</td>
<td>L71N</td>
<td>50.0</td>
</tr>
<tr>
<td>2.2.1.1/E3300/08</td>
<td>N72D</td>
<td>60.0</td>
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<tr>
<td>2.3.2.1a/Hub/10</td>
<td>T140K</td>
<td>70.0</td>
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<tr>
<td>2.3.4/Anh/05</td>
<td>S140G</td>
<td>80.0</td>
</tr>
<tr>
<td>3.2.1/Egy102d/10</td>
<td>T140S</td>
<td>90.0</td>
</tr>
<tr>
<td>5.1/016/08</td>
<td>S140N</td>
<td>80.0</td>
</tr>
<tr>
<td>6.1/VN016/08</td>
<td>E140N</td>
<td>90.0</td>
</tr>
<tr>
<td>7.1/VN016/08</td>
<td>T140M</td>
<td>100.0</td>
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<tr>
<td>8.1/HK8825/08</td>
<td>R162S</td>
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<tr>
<td>9.1/VN118/08</td>
<td>S124E</td>
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<tr>
<td>10.1/016/08</td>
<td>H165K</td>
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<td>11.1/016/08</td>
<td>T140N</td>
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<tr>
<td>12.1/016/08</td>
<td>K162S</td>
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<td>13.1/016/08</td>
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<td>14.1/016/08</td>
<td>Q169K</td>
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<td>15.1/016/08</td>
<td>D183N</td>
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<td>16.1/016/08</td>
<td>P194S</td>
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<td>17.1/016/08</td>
<td>E270G</td>
<td>100.0</td>
</tr>
<tr>
<td>18.1/016/08</td>
<td>G398E</td>
<td>100.0</td>
</tr>
</tbody>
</table>

**Protection:**

- **High:** 90.0%
- **Medium:** 80.0%
- **Low:** 70.0%
- **None:** 60.0%
Figure 6

B)

Challenge 1 (1.1/Vn118/08)
Challenge 2 (2.1.3/WJ29/07)
Challenge 4 (2.2.1.1a/Egy1063/10)
Challenge 5 (2.3.2.1/Vn398/10)
Challenge 6 (2.3.2.1b/Vn672/11)
Challenge 7 (7.1/Vn016/08)