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A computationally designed H5 antigen shows immunological breadth of coverage and protects against drifting avian strains

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ABSTRACT

Since the first identification of the H5N1 Goose/Guangdong lineage in 1996, this highly pathogenic avian influenza virus has spread worldwide, becoming endemic in domestic poultry. Sporadic transmission to humans has raised concerns of a potential pandemic and underscores the need for a broad cross-protective influenza vaccine. Here, we tested our previously described methodology, termed Computationally Optimized Broadly Reactive Antigen (COBRA), to generate a novel hemagglutinin (HA) gene, termed COBRA-2, that was based on H5 HA sequences from 2005 to 2006. The COBRA-2 HA virus-like particle (VLP) vaccines were used to vaccinate chickens and the immune responses were compared to responses elicited by VLP's expressing HA from A/whooper swan/Mongolia/244/2005 (WS/05), a representative 2005 vaccine virus from clade 2.2. To support this evaluation a hemagglutination inhibition (HAI) breadth panel was developed consisting of phylogenetically and antigenically diverse H5 strains in circulation from 2005 to 2006, as well as recent drift variants (2008 – 2014). We found that the COBRA-2 VLP vaccines elicited robust HAI titers against this entire breadth panel, whereas the VLP vaccine based upon the recommended WS/05 HA only elicited HAI responses against a subset of strains. Furthermore, while all vaccines protected chickens against challenge with the WS/05 virus, only the human COBRA-2 VLP vaccinated birds were protected (80%) against a recent drifted clade 2.3.2.1B, A/duck/Vietnam/NCVD-672/2011 (VN/11) virus. This is the first report to demonstrate seroprotective antibody responses against genetically diverse clades and sub-clades of H5 viruses and protective efficacy against a recent drifted variant using a globular head based design strategy.

1. Introduction

Since its emergence in 1996 in Guangdong, China, H5N1 highly pathogenic avian influenza (HPAI) viruses of A/goose/Guangdong/1/1996 (Gs/GD) lineage have spread widely across Asia, Europe, Africa, and more recently in North America (2014–2015) infecting domestic and wild birds, and occasionally spilling over into mammals including humans with a diagnosed case fatality rate of 53% for the H5 hemagglutinin (HA) subtype [1–5]. Over time, the H5N1 HPAI virus has diverged into multiple phylogenetically and antigenically distinct clades and subclades based on the H5 HA gene [6]. Such genetic and antigenic diversity has created challenges in maintaining relevant H5 seed strains for poultry vaccines and for human pre-pandemic preparedness [7].

Vaccination against influenza viruses is the most effective strategy to prevent infection and transmission of the virus [8]; however, there are currently no vaccine seed strains which can elicit protective immunity across the various clades and subclades of H5 Gs/GD lineage of influenza A virus [3,9]. This has resulted in World Health Organization listing 35 pre-pandemic vaccine seeds representing 19 different H5 clades and subclades [9], with an additional 5 CVVs in preparation representing 1 additional subclade. The extreme antigenic and genetic diversity of H5 strains

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makes the development of a broadly protective H5 influenza A vaccine that can provide coverage of antigenically distinct co-circulating viruses as well as future drift variants highly desirable. The previously described methodology termed Computationally Optimized Broadly Reactive Antigen (COBRA) was utilized to generate novel H5 hemagglutinin (HA) consensus sequences [10,11]. Two different COBRA-2 HA sequences were previously generated, the Human COBRA-2 (Hu COBRA-2) variant, based on human isolates from 2005 to 2006 covering clade 2, and a Human-Avian COBRA-2 (Hu-Av COBRA-2) variant, which further added avian clade 2 HA sequences to the human clade 2 consensus [11]. In previous studies using mice, ferrets, and cynomolgus macaques, COBRA-2 HA antigen vaccines protected against lethal challenge with homologous H5N1 (WS/05) HPAI virus, showing more efficient viral clearance and eliciting broader antibody responses against different clades and sub-clades than mono- or polyvalent vaccines [10,12,13]. In the current work, the goal was to expand on these earlier findings using an established avian model of infection to demonstrate both homologous and heterologous efficacy with the COBRA vaccines.

In the present study, chickens were used to determine the protective efficacy against homologous and heterologous H5N1 HPAI viruses, as well as the level of functional immunity as measured by hemagglutination inhibiting (HI) antibodies against not only H5 clades 1, 1.1, 2.1, 2.2, 2.3.2, 2.3.2.1, 2.3.4, 4, and 7; but also more recent clade 2.3.4.4 strains that infected poultry flocks and wild birds in North America, Asia, and Europe in 2014 [3,14,15]. In this report, Hu COBRA-2 HA proteins and Hu-Av COBRA-2 HA proteins were displayed on the surface of virus-like particles (VLPs) and used to vaccinate chickens co-administered with an adjuvant. The elicited immune responses were directly compared to a VLP expressing the wild type (WT) HA of WS/05, a vaccine virus representative of clade 2.2 selected for years 2005–2007 [16]. Vaccinated birds were challenged with either the WS/05 (homologous) virus, or the more recently drifted VN/11 (heterologous) virus representative of a 2011 clade 2.3.2.1B strain. The Hu COBRA-2 HA VLP not only elicited broader HI antibody responses against a panel of avian and human H5 viruses and VLPs expressing HA, but also protected chickens against both challenge viruses more efficiently than the homologous WS/05 delivered as a VLP. These results are the first to demonstrate that a computationally designed HA can elicit broadly-reactive functional antibody responses targeting the HA globular head against avian and human viruses from different H5 clades over a 10-year period.

2. Materials and methods

2.1. Animals

Three-week-old specific pathogen free (SPF) White Leghorn chickens from U.S. National Poultry Research Center (USNPRC) in-house flocks were used. Each group of birds was housed separately in negative pressured isolators with high efficiency particulate air (HEPA)-filtered air within the animal biosafety level 2 (ABSL-2) facilities of USNPRC during vaccination period, and then subsequently transferred to animal biosafety level 3 enhanced (ABSL-3E) facilities for the challenge period. Feed and water were provided *ad libitum*. All procedures were performed according to the requirements of the Institutional Laboratory Animal Care and Use Committee, and Institutional Biosafety Committee.

2.2. Vaccine preparation

The following vaccines were prepared: human-avian COBRA-2 HA H5N1 VLP (Hu-Av COBRA-2 VLP), human COBRA-2 HA H5N1 VLP (Hu COBRA-2 VLP), and A/whooper swan/Mongolia/244/2005 HA H5N1 VLP (WS/05 VLP).

VLP vaccines were prepared as previously described [10,12,13]. Briefly, mammalian 293T cells were transfected with one of three plasmids expressing either the influenza neuraminidase (N1 NA, A/Thailand/2004), the HIV p55 Gag sequences and one of the various H5N1 wild-type, Hu COBRA-2, or Hu-Av COBRA-2 HA expressing plasmids on previously described mammalian expression vectors [17]. Following 72 h of incubation at 37 °C, supernatants from transiently transfected cells were collected, centrifuged to remove cellular debris, and filtered through a 0.22 μm pore membrane. VLPs obtained from mammalian cells were purified and sedimented by ultracentrifugation on a 20% glycerol cushion at 135,000*g* for 4 h at 4 °C. VLPs were suspended in phosphate buffered saline (PBS) and total protein concentration assessed by conventional bicinchoninic acid assay. Hemagglutination activity of each preparation of VLPs was determined by adding an equal volume of horse red blood cells (RBCs) to a V-bottom 96-well plate and incubating with serially diluted volumes of VLPs for 30 min incubation at room temperature. The highest dilution of VLP with full agglutination of RBCs was considered the endpoint HA titer. Purified VLP vaccines were prepared as 3ug dose per 0.5 ml when mixed (30/70) with Montanide ISA VG70 oil emulsion (Seppic Inc. Fairfield, NJ) according to the manufacturer's recommendations.

2.3. Determination of HA content

A high-affinity, 96-well flat bottom ELISA plate was coated with 5–10 μg of total protein of VLP. Serial dilutions of a recombinant H5 antigen (rHA H5 Indo/05/2005, Protein Sciences, Meriden, CT) were made using ELISA carbonate buffer (50 mM carbonate buffer, pH 9.5) and plated. The plate was incubated overnight at 4 °C on a rocker. The next morning, plates were washed with PBS containing 0.05% Tween-20 (PBST), then non-specific epitopes were blocked with 1% bovine serum albumin in PBST solution for 1 h at room temperature. Buffer was removed and a stalk-specific Group 1 influenza A virus antibody (IT-003-001 M14, Immune-tech) was added to the plate, which was then incubated for 1 h at 37 °C. Plates were washed and probed with goat anti-human IgG horseradish-peroxidase-conjugated secondary antibody (1030-05, Southern Biotech, Birmingham, AL) for 1 h at 37 °C. Plates were washed and freshly prepared o-phenylenediamine dihydrochloride (P8287, Sigma, City, State, USA) substrate in citrate buffer (P4922, Sigma) was added to wells followed by 1 N H_2SO_4 stopping reagent. Plates were read at 492 nm absorbance using a microplate reader (Powerwave XS, Biotek, Winooski, VT) and background was subtracted from negative wells. Linear regression standard curve analysis was performed using the known concentrations of recombinant standard antigen to estimate HA content in VLP lots.

2.4. Experimental design

Chickens were randomly distributed into ten groups (n = 10/-group) and vaccinated as indicated in Table 1. All the groups were twice-vaccinated intramuscularly (D0 and D14, 3 and 5 weeks of age, respectively). Sham-vaccinated birds received a vaccine with Montanide ISA VG70 containing sterile phosphate buffered saline and referred to as "sham". At 7 weeks of age (D28/0 days post-challenge (dpc)), all the birds were bled for serum collection to evaluate antibody titers prior to challenge. Chickens were challenged by the intranasal route, via inoculation into the middle nasal chamber through the choanal slit in the roof of the mouth, with 10^6 mean egg infectious dose (EID_{50}) of either WS/05 (groups 1 to 5) or VN/11 (groups 6 to 10) (Table 1). WS/05 challenge virus represented the homologous virus whereas VN/11 represented the heterologous virus since it was resistant to some poultry vaccine

Table 1
Study design.

N	Vaccine D0	Vaccine D14	Dose	Challenge D28[1]
10	Hu-Av COBRA-2 VLP	Hu-Av COBRA-2 VLP	3 μg	WS/05 (H5N1, clade 2.2)
10	Hu COBRA-2 VLP	Hu COBRA-2 VLP	3 μg	WS/05 (H5N1, clade 2.2)
10	WS/05 VLP	WS/05 VLP	3 μg	WS/05 (H5N1, clade 2.2)
10	Sham	Sham		WS/05 (H5N1, clade 2.2)
10	Hu-Av COBRA-2 VLP	Hu-Av COBRA-2 VLP	3 μg	VN/11 (H5N1, clade 2.3.2.1b)
10	Hu COBRA-2 VLP	Hu COBRA-2 VLP	3 μg	VN/11 (H5N1, clade 2.3.2.1b)
10	WS/05 VLP	WS/05 VLP	3 μg	VN/11 (H5N1, clade 2.3.2.1b)
10	Sham	Sham		VN/11 (H5N1, clade 2.3.2.1b)

[1] VN/11, A/duck/Vietnam/NCVD-672/2011; WS/05, A/whooper swan/Mongolia/244/2005.

seed strains [18]. The inoculum titers were subsequently verified by back titration as $10^{6.3}$ and $10^{6.1}$ EID_{50}/0.1 ml of WS/05 and VN/11, respectively. Chickens were monitored daily for clinical signs and mortality. Oropharyngeal swabs were collected at 2 and 4 dpc in brain-heart infusion medium (BBL™ Brain Heart Infusion, Becton-Dickinson and Company, Sparks, MD) with antibiotics (100 μg/ml gentamicin, 100 units/ml penicillin, and 5 μg/ml amphotericin B). At the end of the experiment (14 dpc, 9 weeks of age), surviving birds were euthanized by intramuscular anesthesia with ketamine (10 g/kg body weight, Imalgene® 1000, Merial, Lyon, France) followed by cervical dislocation.

2.5. HAI assay

The HAI assay was used to assess functional antibodies to inhibit agglutination of horse erythrocytes by the presence of either virus or VLPs (Table S1). The VLPs were prepared and characterized as described above for the vaccine preparations. The HAI protocols were adapted from the WHO laboratory influenza surveillance manual [19]. To inactivate nonspecific inhibitors, sera were treated with receptor-destroying enzyme (RDE) (Denka Seiken, Co., Japan) prior to being tested. Briefly, three parts of RDE was added to one part of sera and incubated overnight at 37 °C. RDE was inactivated by incubation at 56 °C for ~30 min. RDE-treated serum were diluted in a series of two-fold serial dilutions in in V-bottom microtiter plates. An equal volume of each H5N1 virus or VLPs, adjusted to approximately 4 HAU/25 μl, was added to each well. The plates were covered and incubated at room temperature for 20 min, and then 0.8% horse erythrocytes (Lampire Biologicals, Pipersville, PA, USA) in PBS were added. Red blood cells were stored at 4 °C and used within 72 h of preparation. The plates were mixed by agitation and covered, and the RBCs were allowed to settle for 1 h at room temperature. The HAI titer was determined by the reciprocal dilution of the last well that contained non-agglutinated RBCs. Positive and negative serum controls were included for each plate. All chickens were negative (HAI $\leq$ 1:10 = $\log_2$ 3.32) for pre-existing antibodies to HPAI viruses prior to vaccination, Seroprotection was defined as HAI titer >1:40 (Log_2 5.32) and seroconversion as a 4-fold increase in titer compared to baseline, as per the WHO and European Committee for Medicinal Products to evaluate influenza vaccines [20].

2.6. Viruses

H5N1 viruses representing avian and human strains that circulated in Asia, Africa, Europe, and North America between the years of 2004 to 2014 were obtained through either the Centers for Disease Control (CDC), the International Reagent Resource (IRR), or University of Alberta. Viruses were passed once in the same growth conditions as they were received, in either embryonated chicken eggs or semi-confluent Madin-Darby canine kidney cell culture as per the instructions provided by the WHO [19]. The HA titer for all virus lots was determined using horse erythrocytes, and viruses were aliquoted for single-use applications. Strains used as virus or VLP in the HAI panel and for challenge are listed in S1.

2.7. Viral RNA quantification in oropharyngeal swabs

Oropharyngeal swab samples were processed for quantitative real-time reverse transcriptase polymerase chain reaction (qRRT-PCR) to determine viral RNA quantity. Viral RNA was extracted using MagMAX™-96 AI/ND Viral RNA Isolation Kit® (Ambion, Inc., Waltham, MA). The resulting viral RNA extracts were quantified by one-step qRRT-PCR which targets the influenza virus matrix gene using 7500 FAST Real-time PCR System (Applied Biosystems, Foster City, CA) and the AgPath-ID OneStep RT-PCR kit (Ambion, Inc.). The standard curve for viral RNA quantification was established with RNA extracted from dilutions of the same titrated stock of the challenge viruses and compared to titers in embryonated chicken eggs, which was run in each plate. The limit of detection was 2.1 and 1.9 $\log_{10}$ EID_{50}/ml for WS/05 and VN/11, respectively. Prior studies have shown good correlation (r = 0.972) between qRRT-PCR results and infectious titer generated in embryonated chickens eggs for respiratory swab samples from chicken challenge studies [21]. For statistical purposes, qRRT-PCR negative samples were treated as 2.0 and 1.8 $\log_{10}$ EID_{50}/ml, respectively.

2.8. Statistical analysis

Mortality and number of birds shedding virus were tested for statistical significance with Fisher's exact test. Antibody levels were tested for statistical significance with Kruskal-Wallis and Mann-Whitney tests (GraphPad Prism™ Version 5 software). A *P*-value of <0.05 was considered to be significant.

2.9. H5 phylogenetic tree

H5 hemagglutinin protein sequences were downloaded from the Influenza Virus Resource at the National Center for Biotechnology Information [22]. Unique HA sequences were aligned using MAFFT [23] and a subset of residues associated with published antibody epitopes, 65C6 [24], AVFFluIg03 [25], 100F4 [24], H5.3

[26] and H5M9 [27,28] was used to infer a maximum-likelihood phylogenetic tree using FastTree2 [29]. Dendroscope3 [30] was used for visualization and the tree was rooted using A/Goose/Guangdong/1/1196 (clade 0).

3. Results

3.1. VLPs elicit antibody responses in vaccinated chickens

To expand previous findings comparing COBRA HA vaccines to the clade 2.2, WS/05 HA-based vaccines [10,12,13], White Leghorn chickens (n = 60) were twice-vaccinated intramuscularly with either purified Hu COBRA-2 VLPs, Hu-Av COBRA-2 VLPs, or WS/05 VLPs (Table 1). All antigens were formulated with an oil emulsion adjuvant. Serum was drawn from all chickens on D28 and analyzed for the ability to block HA-receptor binding to RBCs (HAI) against a panel of 20 diverse H5 viruses or VLPs representing avian and human strains that circulated in Asia, Africa, Europe, and North America between the years of 2004 to 2014 (S1). Chickens injected with adjuvant only (sham) showed no HAI activity against any of the H5 test antigens as expected (data not shown). Overall, Hu COBRA-2 VLP vaccinated groups showed a higher frequency of chickens with seroprotective HAI titers (>1:40, $\log_2$ 5.32) against the breadth panel representing clades 1, 2.1, and 2.2 in circulation between the years 2004 and 2011 (Figs. 1 and 2). In contrast, chickens vaccinated with either Hu-Av COBRA-2 VLP or WS/05 VLP exhibited HAI activity against fewer clade 1, 2.1 and 2.2 H5 viruses from this time period (Fig. 1). Furthermore, chickens vaccinated with the Hu COBRA-2 VLP vaccine generated antibodies with HAI activity against more recent drifted HAs from 2014 representing both 2.3.2 and 2.3.4 subclades, with an average geometrical mean HAI titer of 1:320 ($\log_2$ 8.32) (Fig. 1). In contrast, sera from Hu-Av COBRA-2 VLP and WS/05 VLP vaccinated chickens recognized few clade 2.3.2 HAs (Fig. 1), with less than 50% of chickens demonstrating a seroprotective titer >1:40 ($\log_2$ 5.32) (Fig. 2). Taken together, the Hu COBRA-2 VLP vaccinated chickens showed higher HAI titers and greater rate of seroconversion (HAI titer 1:40, $\log_2$ 5.32) against the entire H5 breadth panel than the other vaccine candidates (Fig. 2). Importantly, this computationally designed HA was able to elicit potent HAI responses against antigenically distinct recent drift variants that were not included in its design.

3.2. Challenge with H5N1 HPAI viruses

To confirm protective efficacy against H5N1 HPAI virus infection, vaccinated chickens were challenged with a lethal dose of the wild-type clade 2.2 isolate WS/05 or a more recently drifted clade 2.3.2.1B isolate from 2011, VN/11. All VLP-vaccinated chickens were protected from disease and death by WS/05 challenge virus, while all sham-vaccinated chickens died by 3 dpc (Table 2). Chickens vaccinated with any of the three vaccines did not show a significant reduction in the number of birds shedding virus by the oropharyngeal route compared to the sham group, but all groups had a significant reduction in virus shedding titers as compared to the sham group (Table 2). Similar viral titers were recovered from the oropharyngeal swabs of the three vaccinated groups, both at 2 dpc and 4 dpc (Table 2).

Challenge with the heterologous drifted strain VN/11 resulted in 80% survival with Hu COBRA-2 VLP and 0% survival for Hu-Av COBRA-2 VLP, WS/05 VLP, and sham vaccines (Table 2). The mean death time (MDT) of chickens from Hu-Av COBRA-2 VLP (4.8 dpc MDT), Hu COBRA-2 VLP (6 dpc MDT), WS/05 VLP (5.1 dpi MDT) groups were delayed as compared to the sham group (2.1 dpc MDT) (Table 2). Similar to the homologous WS/05 challenge, chickens vaccinated with any of the three vaccines did not show a significant reduction in the number of birds shedding virus by the oropharyngeal route compared to the sham group, but all groups had a significant reduction in virus shedding titers as compared to the sham group (Table 2) at 2 dpc. Similarly on 4 dpc, viral titers were still reduced from the oropharyngeal swabs of the three vaccinated groups, but with the Hu COBRA-2 VLP group shedding significantly lower viral titers as compared to the other vaccinated groups (Table 2).

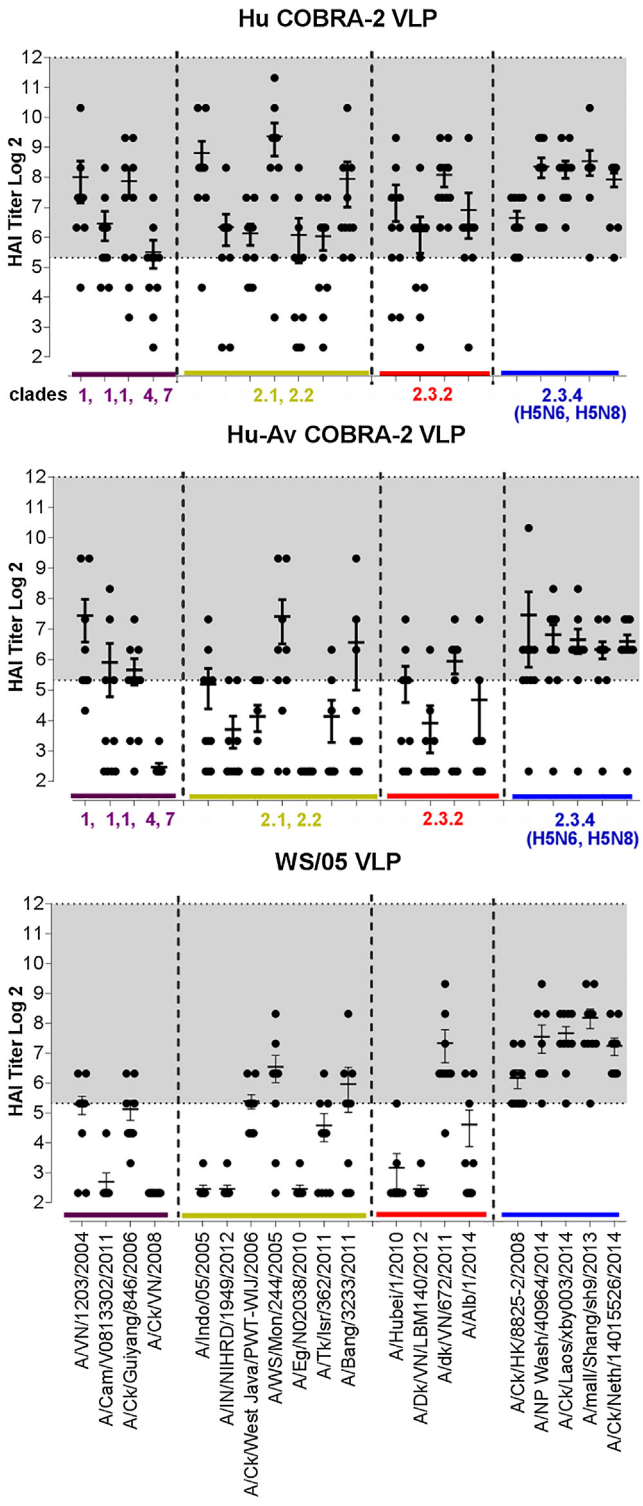


Fig. 1. Hemagglutinin inhibition (HAI) titers. D28 (0 dpc) HAI antibodies were assessed against a breadth panel of viruses and VLPs covering diverse clades and subclades. HAI titer values were $\log_2$ transformed. Grey box highlights titers ≥1:40.

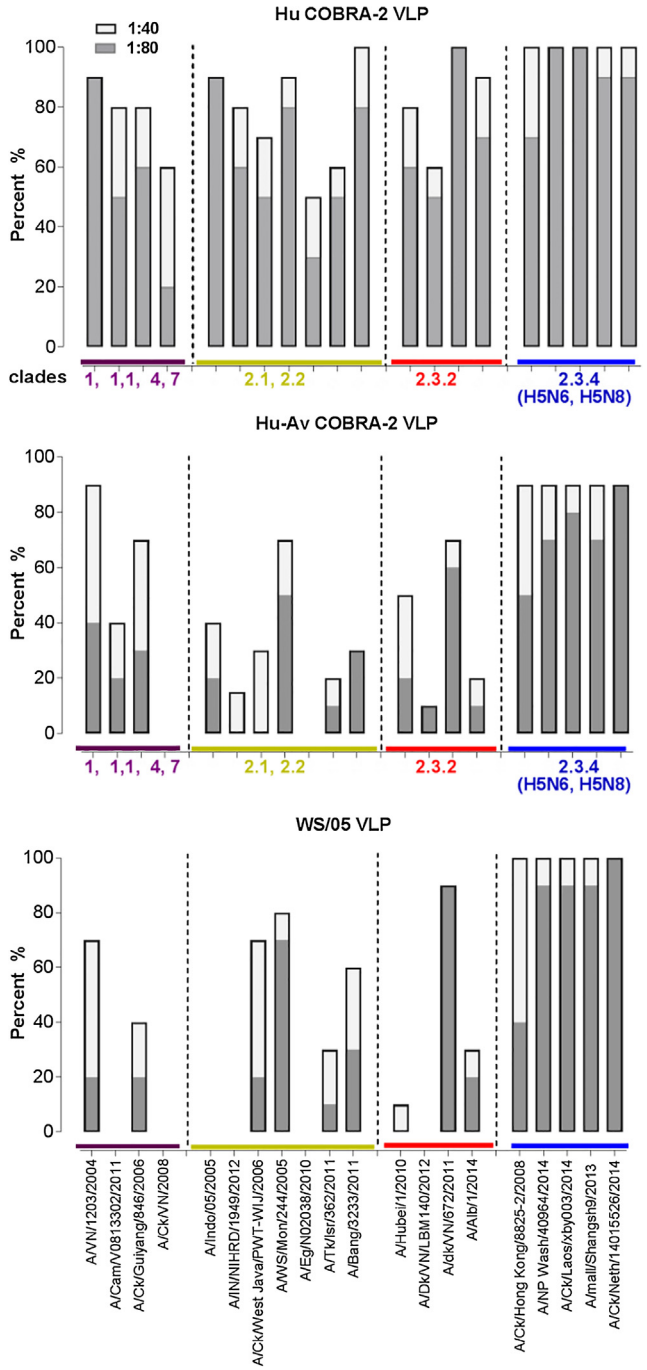


Fig. 2. Seroconversion rates. Seroconversion corresponds to negative pre-vaccination serum converting to an HAI titer >1:40 (white boxes) and HAI titer >1:80 (grey boxes) at D28 (0 dpc).

4. Discussion

Avian influenza caused by H5 viruses of the Gs/GD lineage has spread globally and outbreaks have impacted poultry and wild birds, and caused sporadic disease in humans with a case fatality rate of ~53% in individuals diagnosed with H5 infection [4,5]. Novel HPAI virus reassortants like H5N6, H5N8, and H5N9 are becoming increasingly prevalent in China [31–33]. The absence of preexisting immunity to H5 influenza A in humans, the severity of disease when infection occurs, and the increased global circulation of these viruses raises a legitimate concern with regard to the increasing pandemic potential for H5 influenza A viruses [34–36]. These concerns are compounded by the fact that, as with other influenza A virus subtypes, H5 viruses continually mutate to evade host immunity in a process known as antigenic drift [6,37]. As a result, there is continual circulation of newly evolved strains in poultry in endemic countries against which preexisting immunity has limited or no impact. These concerns underscore the need for an effective broadly protective H5 influenza vaccine that can provide expanded coverage of co-circulating, antigenically distinct variants in poultry and as a pre-pandemic vaccine for humans. The development of more broadly protective influenza A vaccines, even within a subtype would mitigate stockpiling of strain specific pre-pandemic vaccines where there is limited breadth potential.

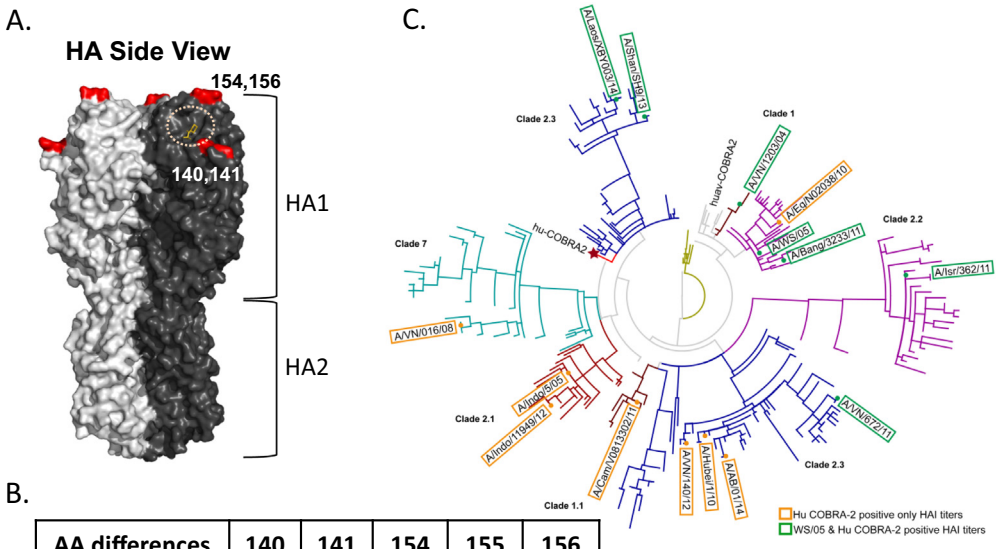
In this report, the immunogenicity and protective efficacy of two computationally designed COBRA H5 HAs was evaluated. The COBRA approach to antigen design is reliant on a layered consensus building technique described previously [10,11,13]. The two COBRA HAs described here were designed based on H5 sequences representing subclades 2.2, 2.1, and 2.3, and which were isolated between the years of 2005 and 2006 from either humans (Hu COBRA-2) or from both birds and humans (Hu-Av COBRA-2). The COBRA HAs were generated as VLPs and compared to an HA derived from the recommended vaccine strain of this time period, WS/05, delivered as a VLP vaccine in chickens. Previously shown in mice, ferrets, and non-human primates the COBRA-2 VLP prototypes induce broadly reactive HAI titers against multiple antigenically distinct H5 Gs/GD clades and show protective efficacy against challenge with HPAI viruses from 2005 [10,11,13]. The study presented herein represents the first attempt to demonstrate efficacy against antigenically distinct HPAI virus strains in an established avian model of influenza A virus infection using a highly lethal challenge dose.

Following vaccination of chickens, COBRA-2 VLPs elicited broadly reactive HAI antibody titers against genetically distinct H5 clades and subclades. Overall, the HAI profile observed in chickens immunized with the COBRA-2 VLP variants were similar, however the magnitude in HAI responses were higher for the Hu COBRA-2 VLP than the Hu-Av COBRA-2 VLP, resulting in broader seroprotection. Interestingly, there are four amino acid differences clustered around the HA receptor binding domain (RBD) (AA positions140-141 and 154–156) between Hu COBRA-2 and Hu-Av COBRA-2 that may explain differences in the HAI antibody responses and/or potency of antibodies elicited [11] (Fig. 3A, B). In addition, antibodies elicited by the Hu COBRA-2 VLP recognized more viruses from clades 1, 4, 7, and subclades 2.1, 2.2, 2.3.2, and 2.3.4 than antibodies elicited in chickens vaccinated with the WS/05 VLP (Fig. 3C). Amino acid differences between Hu COBRA-2 and the WS/05 HA sequence at these same 140–141 and 154–156 sites suggest that these regions around the RBD appear instrumental in the serological breadth observed in antisera elicited by the Hu COBRA-2 VLP vaccine over the WS/05 vaccine (Fig. 3B). Hu COBRA-2 HA harbors a potential glycosylation site on the globular head that is absent in both Hu-Av COBRA-2 and WS/05 proteins. Glycosylation can impact HA immunogenicity by masking epitopes. In the case of Hu COBRA-2, glycosylation may lead to refocusing of the immune response to conserved epitopes, and may have contributed to the increased breadth elicited by Hu COBRA-2 VLP [38–40]. The positions of the amino acid differences around the RBD reside in two of the previously identified sites targeted by broadly neutralizing antibodies on the H5 globular head [41,42]. Natural infection or vaccination in humans can elicit potent neutralizing antibodies targeting conserved amino acids around the RBD of the virus and blocking infection [41,42]. Further interrogation of the antigenic sites on the HA globular head will ultimately contribute to designing more effective broadly protective vaccines.

Table 2
Efficacy and mean viral shedding from chickens vaccinated and challenged with clade 2.2 (homologous) and clade 2.3.2.1b (heterologous) H5N1 HPAI viruses.

Vaccine D0	Vaccine D14	Challenge D28	Survival (MDT*)	Viral RNA detection† (log_{10} EID_{50}/ml)	
				OP swabs, 2 dpc	OP swabs, 4 dpc
Hu-Av COBRA-2 VLP	Hu-Av COBRA-2 VLP	WS/05	$10/10^{a}$	$9/10^{a}$ (3.7^{A})	$8/10^{a}$ (3.3^{A})
Hu COBRA-2 VLP	Hu COBRA-2 VLP		$10/10^{a}$	$9/10^{a}$ ($2.8^{A,B}$)	$7/10^{a}$ (2.8^{A})
WS/05 VLP	WS/05 VLP		$10/10^{a}$	$6/10^{a}$ (2.5^{B})	$7/10^{a}$ (2.7^{A})
Sham	Sham		$0/10^{b}$ (2.2)	$10/10^{a}$ (6.7^{C})	–
Hu-Av COBRA-2 VLP	Hu-Av COBRA-2 VLP	VN/11	$0/10^{b}$ (4.8)	$10/10^{a}$ (3.5^{A})	$9/9^{a}$ (4.9^{A})
Hu COBRA-2 VLP	Hu COBRA-2 VLP		$8/10^{a}$ (6)	$6/10^{a}$ (2.7^{A})	$8/10^{a}$ (3.1^{B})
WS/05 VLP	WS/05 VLP		$0/10^{b}$ (5.1)	$10/10^{a}$ ($3.8^{A,B}$)	$10/10^{a}$ (5.4^{A})
Sham	Sham		$0/10^{b}$ (2.1)	$10/10^{a}$ (7.3^{C})	–

MDT, mean death time; dpc, days post challenge; VN/11, A/duck/Vietnam/NCVD-672/2011; WS/05, A/whooper swan/Mongolia/244/2005.
For statistical purposes, 2.0 log_{10} EID_{50}/ml and 1.8 log_{10} EID_{50}/ml were given to WS/05 and VN/11 qRT-PCR negative samples, respectively.
Parameters in our study were not distributed normally according to The D'Agostino & Pearson test.
Different superscript lowercase letters denote significant difference for number of positive birds on qRRT-PCR between groups; Fisher's exact test.
Different superscript uppercase letters inside the parenthesis denote significant difference for mean viral titers between groups within a day; Kruskal-Wallis test.
* #dead birds × dpc/total dead birds (expressed as dpc).
† #positive at qRRT-PCR for *M* gene/total.



AA differences	140	141	154	155	156
WS/05	R	S	D	N	A
Hu COBRA-2	S	P	N	N	T
Hu-Av COBRA-2	K	S	N	S	A

Fig. 3. Hu COBRA-2 VLP vaccine prototype elicits breadth against antigenically diverse strains over a decade of drift. A. Homology model of Hu COBRA-2 HA generated with Rosetta Biosoftware [46] using PDB ID: 2IBX [47] as template. Amino acids thought to contribute to the expanded breadth elicited by Hu COBRA-2 (positions 140–141 and 154–156) are highlighted in red around the RBD (ligand in gold). B. Amino acid differences noted at the specified positions between WS/05, Hu COBRA-2, and Hu-Av COBRA-2 sequences. C. Maximum-likelihood tree based on a subset of residues associated with published epitopes recognized by broadly neutralizing antibodies. Tree is rooted on A/Goose/Guangdong/1/1996 (Clade 0). Older strains are positioned toward the center and more recent drifted strains are located towards the edges of the dendogram. Strains recognized by anti-sera from WS/05 and Hu COBRA-2 VLP vaccinated chickens are highlighted by green boxes, whereas strains recognized only by anti-sera from Hu COBRA-2 VLP vaccinated chickens are highlighted by orange boxes.

To evaluate the ability of these vaccine candidates to mediate protective immunity against homologous and heterologous challenge strains, vaccinated chickens were challenged with a circulating virus (clade 2.2 WS/05) from 2005, the same time period the COBRA immunogen was designed from (2005–2006), as well as a more recent drift variant (clade 2.3.2.1b, VN/11) from 2011. COBRA-2 VLP, the Hu-Av variant, and WS/05 VLP vaccine protected chickens from disease and death after homologous WS/05 challenge. These results suggest that the COBRA HA antigens were in protecting chickens against a homologous virus, as observed in previous studies using mice, ferrets, and cynomolgus macaques [10,12,13]. Furthermore, chickens receiving either of the COBRA vaccines or WS/05 VLP vaccine had significantly reduced WS/05 virus shedding compared to sham birds.

Challenge with the VN/11 provided the first opportunity to differentiate the vaccines based on efficacy under an antigenic drift variant. The Hu COBRA-2 VLP vaccine provided the best protection (80%) against heterologous VN/11 challenge, which is an antigenic variant field virus resistant to some H5 vaccine seed strains [18]. Chickens vaccinated with either Hu-Av COBRA-2 VLP or WS/05

VLP completely succumbed to infection (0% survival). Protection was also observed at the level of reduced cloacal virus shedding as compared to the sham group. The Hu COBRA-2 VLP vaccinated group had the lowest level of viral RNA load of all groups at both 2 and 4 dpc showing partial protection in virus shedding from the oropharynx. Further investigation would be needed to assess vaccine formulation and complete protection of oral virus shedding in chickens. Taken together, these data demonstrate that a vaccine based on the Hu COBRA-2 antigen would have been more resistant to drift than vaccines based on the wild-type WS/05 strain.

It should be noted that HAI titers are not absolutely predictive of efficacy for a particular H5 strain in preclinical animal models, including chickens [13,43,44]. Indeed, we observed that a robust HAI response elicited by WS/05 VLPs against VN/11 (Fig. 1) did not translate to protective efficacy upon challenge with this virus. Considering that the Hu COBRA 2 VLP-mediated HAI responses against VN/11 are only marginally higher than the HAI response elicited by WS/05 VLPs, it is apparent that HAI titers alone are not sufficient to explain the substantial difference in efficacy. The difference in efficacy is almost certainly mediated by the HA itself, as all other viral antigens in the VLP formulations are identical. Potential mechanisms may include neutralizing antibodies that do not mediate HAI or perhaps cell-mediated effector mechanisms (e.g., ADCC-mediating antibodies, T cell responses) [45]. The influenza field is still gaining further evidence of additional effector mechanisms and their relative level of importance to assist with forecasting protective efficacy in humans. Currently the HAI assay is the established *in vitro* correlate of protection in humans and was used in these studies.

In conclusion, the present study demonstrates that globular head-based COBRA HA antigen vaccines elicited a broad HAI antibody response and provided complete protection against both a co-circulating virus that was not included in its design (WS/05), as well as against a more recent drift variant (VN/11). This study provides evidence that the COBRA methodology can be used to generate immunogens that elicit broadly reactive antibodies against future antigenically distinct drift variants and hold promise as a strategy for generating broadly protective influenza vaccines for veterinary and human pre-pandemic and seasonal influenza vaccines.

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Conflict of interest

Select authors are employees of SP and Boehiringer Ingelheim and may hold stock of their respective companies.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.03.018.

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