1 *Running title:* Immunity against avian influenza

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4 **Vaccination with virus-like particles containing H5 antigens from three H5N1**
5 **clades protects chickens from H5N1 and H5N8 influenza viruses**
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Abstract

Highly pathogenic avian influenza (HPAI) viruses, especially H5N1 strains, represent a public health threat and cause widespread morbidity and mortality in domestic poultry. Recombinant virus-like particles (VLPs) represent a promising novel vaccine approach to control avian influenza including HPAI strains. Influenza VLPs contain viral hemagglutinin (HA), which can be expressed in cell culture within highly immunogenic VLPs that morphologically and antigenically resemble influenza virions, except VLPs are non-infectious. Here we describe a recombinant VLP containing HA proteins derived from three distinct clades of H5N1 viruses as an experimental, broadly protective H5 avian influenza vaccine. A baculovirus vector was configured to co-express the H5 genes from recent H5N1 HPAI isolates A/chicken/Germany/2014 (clade 2.3.4.4), A/chicken/West Java/Subang/29/2007 (clade 2.1.3) and A/chicken/Egypt/121/2012 (clade 2.2.1). Co-expression of these genes in Sf9 cells along with influenza neuraminidase (NA) and retrovirus gag genes resulted in production of triple-clade H555 VLPs that exhibited hemagglutination activity and morphologically resembled influenza virions. Vaccination of chickens with these VLPs resulted in induction of serum antibody responses and efficient protection against experimental challenges with three different viruses including the recent U.S. H5N8 HPAI isolate. We conclude that these novel triple-clade VLPs represent a feasible strategy for simultaneously evoking protective antibodies against multiple variants of H5 influenza virus.

Keywords:

Avian Influenza

Virus-Like Particle

VLP Vaccine

Trivalent

1. Introduction

Avian influenza (AI) is a highly contagious, agriculturally-relevant disease that can severely affect poultry industry and the national food supply and safety [1]. The AI also represents an acute threat to public health [2, 3]. The viruses vary widely in their pathogenicity and the ability to cause disease among birds. Wild species usually do not develop clinical disease, while some AI viruses cause severe illness or death in domestic birds such as chickens and turkeys. The viruses are divided into low-pathogenic AI (LPAI) and highly pathogenic AI (HPAI) strains. HPAI viruses, especially H5N1 strains, cause widespread morbidity and mortality in domestic bird populations. Furthermore, with an increasing number of human infections of HPAI of the H5N1 subtype, there is a [concern](#) that this virus could cause a pandemic [3, 4].

The severity [of the AI in the poultry species](#) ranges from inapparent illness to rapidly fatal disease. HPAI strains of the virus can propagate very rapidly, particularly in young chickens, and there may be no clinical signs other than sudden death. LPAI causes milder symptoms but can evolve into HPAI [5, 6]. Outbreaks of HPAI have resulted in significant economic losses in the poultry industry. For example, from 1997 to 2007, more than 250 million birds have been killed or culled, compared to only 23 million in the previous 40 years [7]. A major outbreak of HPAI occurred in the northeastern U.S. in 1983-84 [6, 8]. The economic impact of H5N1 HPAI outbreaks in Asia has exceeded \$575 million [9]. Low pathogenic strains of AI virus are always present and cause losses in the poultry industry as well [5, 6].

Multiple subtypes along with the constant threat of antigenic drift and shift represent challenges for AI vaccine development [2]. One strategy to control AI including H5N1 HPAI is the development of effective vaccines for the use in poultry industry. Recombinant virus-like particles (VLPs) represent a promising novel vaccine approach to control influenza including HPAI. Influenza VLPs include viral hemagglutinin (HA) and neuraminidase (NA), which can be co-expressed in cultured *Spodoptera frugiperda* (Sf9) cells along with matrix (M1) or retrovirus

gag protein as highly immunogenic VLPs that morphologically and antigenically resemble influenza virions, except VLPs are non-infectious. Recombinant VLPs have advantages in safety, efficacy, and manufacturing because they circumvent problems of the conventional egg-based vaccine production such as slow virus growth, unpredictable yields, and virus mutations during egg adaption.

Recently, a major outbreak of HPAI was detected in wild birds and commercial poultry in the U.S. [10-12]. During December 2014-June 2015, 21 states reported Asian-origin HPAI of H5N2, H5N8 and H5N1 subtypes in commercial premises, backyard flocks, and wild birds [13]. More than 50 million birds died or were slaughtered during this time, renewing interest in the development of vaccines against these viruses.

A VLP vaccine that induces immunity to multiple strains of H5 including recent H5N8 HPAI viruses would be important for AI control in poultry. The approach can also be of importance to the development of pandemic preparedness strategies as such vaccines could decrease the severity of disease and save lives during a pandemic until a specific pandemic vaccine is made [2, 3, 14]. Inclusion of several clades of H5 HA can potentially improve vaccine coverage. However, it is not feasible to make a polyvalent H5 vaccine using current commercial technology. Classic inactivated vaccines represent mixtures of inactivated viruses [3, 4, 15]. Each virus is grown separately in fertilized eggs, inactivated, and then combined with two other strains to make a trivalent vaccine [3, 15]. The need for individual preparation of vaccine for each strain significantly increases vaccine cost. The use of eggs in vaccine production is another weakness; in the event of an outbreak of AI or other agricultural disease that affects chicken flocks, the supply of eggs could be limited.

Recently, we have shown that VLPs can accommodate multiple subtypes of HA in their envelopes and that such multi-subtype VLPs induce protective immune responses against multiple strains of influenza [16]. In this study, we configured VLPs to express HA proteins derived from the three distinct clades of H5N1 HPAI viruses and demonstrated that vaccination

with such triple-clade VLPs protects chickens from experimental challenges with three distinct variants of H5 AI viruses including H5N8 virus from the recent outbreak.

2. Materials and methods

2.1. HPAI viruses, plasmids and cells

HA gene sequences were derived from H5N1 HPAI strains A/chicken/Germany/2014 (clade 2.3.4.4), A/chicken/West Java/Subang/29/2007 (clade 2.1.3) and A/chicken/Egypt/121/2012 (clade 2.2.1). Influenza NA gene was from A/chicken/Egypt/121/2012 [17]. As inner core for influenza VLPs we used BIV gag protein sequence (R-29) obtained from GenBank, accession number AAA42763. All genes were codon-optimized and synthesized (Genscript, Piscataway, NJ) for high-level expression in *Spodoptera frugiperda* (Sf9) cells (Life Technologies, Carlsbad, CA). In order to generate triple-clade H555 VLPs, three full-length H5 HA genes, as well as N1 NA and BIV gag genes were cloned within a single baculovirus transfer vector, each gene within its own transcriptional cassette that included a polyhedrin promoter upstream from each gene (Fig. 1A). Recombinant baculovirus (rBV) expressing H5 genes, as well as NA and BIV gag genes was generated using a Bac-to-Bac baculovirus expression system (Life Technologies). The titers of rBV preparations were determined by standard plaque assay in Sf9 cells.

The virus stocks of A/Gryfalcon/Washington/2014 H5N8 (clade 2.3.4.4) (GyrF), A/Ck/West Java Subang/2007 H5N1 (clade 2.1.3) (Ck/WJ) and A/Ck/Egypt/2010 H5N1 (clade 2.2.1) (Ck/Egypt) were used for viral challenges. A 50% egg infectious doses (EID₅₀/ml) were determined using standard assay methods as previously described [18]. All experiments with H5N1 HPAI viruses were performed in negative pressure biosafety level 3 laboratories with enhancements as outlined in the Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th edition (<http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf>).

2.2. Genetic stability study

The rBV expressing three H5 HA genes, NA, and BIV gag genes was passed 5 times in Sf9 cells at an MOI of ~0.01. Then, Sf9 cells were infected at MOI of 3 for 72 hours with passage P1, P2, and P5 rBV viruses to confirm the presence of H5 HA, NA and BIV gag genes by hemagglutination assay, SDS-PAGE, and western blot.

2.3. Preparation of triple-clade H555 VLPs

Sf9 cells were maintained as suspension cultures in SF900II-SFM insect serum free medium (Life Technologies) at $27 \pm 2^{\circ}\text{C}$. For production of H555 VLPs, 2L of Sf9 cells at concentration 2×10^6 cells/ml were infected at a multiplicity of infection (MOI) of 3.0 with rBV expressing three indicated H5 HA genes, as well as NA and BIV gag genes. VLPs were harvested at 72 h postinfection from the growth medium supernatant, sterile filtered using 0.2 μm filter and concentrated by using tangential flow filtration (TFF, 500 000 MWCO) prior to purification. VLPs were purified by using ion exchange chromatography followed by ultracentrifugation. Anion exchange chromatography was used to remove major impurities such as baculovirus and host cell DNA [19]. Then, ultracentrifugation was used to purify and concentrate VLPs as described elsewhere [20]. VLP preparations were characterized and stored at $2-8^{\circ}\text{C}$ in a PBS buffer until vaccinations.

The SDS-PAGE was done in 4-12% polyacrylamide gels (Life Technologies) followed by staining with GelCode Blue stain (Pierce, Rockford, IL). Western blots were carried out by using anti-H5(H5N1) mouse IgG2a, clone 268D8, H5-specific monoclonal antibody (MAb) (Immune Technology, New York, NY) followed by the alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (KPL, Gaithersburg, MD).

2.4. Hemagglutination assay

VLPs were serially diluted in PBS at twofold increments in 50 µl volume in a 96-well plate. Turkey red blood cells (RBC) are recommended by the WHO as a reagent for animal influenza diagnosis and surveillance [21]. To each VLP dilution, 50 µl of 1% turkey RBC working solution was added, mixtures of VLPs and RBCs were gently mixed and the plate was incubated at room temperature for 30-60 minutes. Negative hemagglutination results appeared as dots in the centers of the wells. The titer was calculated as the highest dilution factor that produced a positive reading.

2.5. Electron microscopy of H555 VLPs

VLP samples were adsorbed onto a freshly discharged 400 mesh carbon parlodion-coated copper grids (Poly-Sciences, Warrington, PA). The grids were rinsed with buffer containing 20 mM Tris, pH 7.4, and 120 mM KCl and negatively stained with 1% phosphotungstic acid, then dried by aspiration. The VLPs were visualized on a Hitachi H-7600 transmission electron microscope (Hitachi High Technologies America, Schaumburg, IL) operating at 80 kV and digitally captured with a CCD camera at 1k×1k resolution (Advanced Microscopy Techniques Corp., Danvers, MA).

2.6 Serology and antibody assays

Hemagglutination inhibition (HI) assay was performed using standard methods [20]. Homologous HPAI GyrF (H5N8), Ck/WJ (H5N1) and Ck/Egypt (H5N1) viruses were used as antigens.

2.7. Vaccination and viral challenges

VLP H555 vaccine was formulated with a commercial adjuvant (SEPPIC, Montanide 70/30, Fairfield, New Jersey) to contain 1536 HA units per dose of H555 VLPs. *Because all three H5 genes were expressed from the identical expression cassettes in Sf9 cells, it is expected that H5 clades are present at similar levels in the H555 VLPs, although precise quantitation of VLP components will require additional studies.* Specific Pathogen-Free (SPF) chickens (n=30) were vaccinated subcutaneously with 0.2 ml of H555 VLP at day 1 of age and 0.5 ml at day 21 of age. Control birds received PBS. Birds were arbitrarily placed into groups of 10 and challenged intranasally (10^6 EID₅₀ per bird) with one of the following HPAI isolates at day 35: GyrF H5N8 (clade 2.3.4.4); Ck/WJ H5N1 (clade 2.1.3) or Ck/Egypt H5N1 (clade 2.2.1). After challenges, birds were monitored for clinical signs daily, serum was taken at day 0 and day 14 post challenge, and oral swabs were taken to measure virus shedding.

2.8. Determination of virus shedding

Oropharyngeal and cloacal swabs were collected in sterile brain heart infusion medium and kept frozen at -70°C. Viral RNA was extracted using Trizol LS reagent (Invitrogen, Calsbad, CA) and the MagMAX AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX). Quantitative real time RT-PCR (qRRT-PCR) was performed as previously described (Pantin-jackwood, 2013). Briefly, qRRT-PCR targeting the influenza M gene was conducted using AgPath-ID one-step RT-PCR Kit (Ambion) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Calsbad, CA). For viral quantification, a standard curve was established with viral RNA extracted from the titrated challenge virus, GyrF H5N8, Ck/WJ H5N1 or Ck/Egypt H5N1. Results were reported as EID₅₀/ml equivalents and the lower limit of detection being $10^{0.9}$ EID₅₀/ml for samples from chickens.

2.9. Statistical analysis

Kaplan-Meier survival curves were generated with Prism 5 (GraphPad Co., San Diego, CA). The Mantel-Cox log-rank test was used to compare survival curves between the experimental groups (Prism 5). Statistical differences in mean and standard error between HI titers were analyzed using ANOVA (Prism 5). Lower case letters indicate statistical significance between compared groups. The Student t-test was used for pair-wise comparison of virus titers from oral and cloacal swabs (Prism 5). All statistical tests were performed using $P < 0.05$.

3. Results

3.1. Expression of triple-clade H555 VLPs

For preparation of triple-clade H555 VLPs, the HA proteins were derived from three clades of H5N1 HPAI avian-origin influenza viruses, strains A/chicken/Germany/2014 (clade 2.3.4.4), A/chicken/West Java/Subang/29/2007 (clade 2.1.3) and A/chicken/Egypt/121/2012 (clade 2.2.1). These H5 genes encoded polypeptides of 563 aa, 564 aa, and 567 aa in length with predicted average molecular masses of 63.72 kilodalton (kDa), 63.71 kDa and 64.26 kDa, respectively. The H5 HA gene from A/chicken/West Java/Subang/29/2007 was modified to delete the furin cleavage site. The NA and BIV gag are 449 aa and 476 aa in length and have expected molecular masses of 49.02 kDa and 53.47 kDa, respectively. HA and NA have been previously shown to be important structural constituents of influenza VLPs [16, 20] and therefore were included in the expression construct. In a previous study, we have shown that influenza VLP can co-express multiple subtypes of HA within the same particle [16]. Therefore, in order to prepare triple-clade H555 VLPs containing HAs from the three H5N1 HPAI viruses, the rBV

vector was configured for co-expression of three H5 genes, as well as NA and BIV gag genes (Fig. 1a). To prepare triple-clade H555 VLPs, Sf9 cells were infected with rBV at an MOI=3 to allow expression of genes. VLPs were harvested from culture supernatant. The functional ability of H555 VLPs to agglutinate turkey RBCs was confirmed by hemagglutination assay using turkey RBCs. The hemagglutination activity with the titer of 1:256 per 50 µl of the medium supernatant containing VLPs confirmed that the incorporated HA proteins retained their functional stability and RBC binding activities (Fig. 1b). Contribution of each H5 clade to hemagglutination activity remains to be determined.

3.2. Vector rBV genetic stability study

The genetic stability of the expression rBV construct has been studied by rBV passages in Sf9 cells followed by expression tests. Expression of H5 in the media from Sf9 cells infected with passage P1, P2 and P5 rBV vectors has been confirmed by SDS-PAGE, western blot and HA assay (Fig. 1c). No considerable loss of H5 expression was observed in VLP samples prepared by using P1, P2, or P5 rBV. Thus, the rBV appeared to be genetically stable at least for five passages in Sf9 cells.

3.3. Preparation and characterization of VLPs

The VLPs were harvested from culture supernatant on day 3 post infection. The growth medium from infected Sf9 cells was clarified by centrifugation and 0.2 µm filtration and VLPs were concentrated by TFF and purified by ion exchange chromatography. The presence of BIV gag and HA proteins was confirmed by stained SDS-PAGE and by western blot using H5-specific antibody (Fig. 2a). The H5 clades have similar molecular weights and are predicted to

migrate as a single band on SDS-PAGE. Furthermore, the H5 proteins are expected to be expressed at similar levels from the polyhedrin expression cassettes (Fig. 1a); however, the exact ratio of each H5 clade within H555 VLPs remains to be determined. Consistent with our previous observations [22], HAs within VLPs represented uncleaved HA0 polypeptides of approximately 62-64 kDa. A band of approximately 55 kDa corresponding to BIV gag protein was also detected by SDS-PAGE (Fig. 2a). No considerable cell- or baculovirus-derived impurities were detected in the purified H555 VLP preparations. The HA reacted with H5-specific antibody in western blot (Fig. 2a). Expression of NA was monitored by functional neuraminidase enzyme assay (data not shown).

The ability of purified VLPs to agglutinate turkey RBCs was confirmed by hemagglutination assay (Fig. 2b). The hemagglutination titer of concentrated VLPs was 1:16384 per 50 μ l of VLP suspension. By transmission negative-staining electron microscopy, the H555 VLPs were identified as largely spherical, influenza-like enveloped particles approximately 150-200 nm in diameter with characteristic HA spikes protruding from the VLP envelope (Fig. 2c).

3.4. Immunogenicity and protective efficacy of triple-clade H555 VLPs in chickens

Following vaccination with VLPs and prior to challenge, antibody levels against the vaccine antigen were determined by HI assay. As demonstrated in Fig. 3, all groups of vaccinated birds exhibited high HI titers ($\geq 9 \log_2$) two weeks after a second application of VLP vaccine when the homologous vaccine antigen was used in the assay.

Sham and vaccinated birds were challenged with one of three HPAI viruses two weeks after vaccination. Pre-challenge and post-challenge HI antibody titers to challenge viruses are shown on Fig. 4a and Fig. 4b, respectively. High HI titers were detected before challenge. The observed comparable HI titers to each virus strain suggest the presence of three H5 clades in the VLPs. As expected, the HI titers increased after challenge (Fig. 4b).

Vaccine protection was measured by survival and reduction in viral shedding in chickens following virus challenges with three indicated H5 viruses (Fig. 5, 6). All birds in vaccine groups 1, 2, or 3, corresponding to GyrF (H5N8), Ck/Egypt (H5N1) and Ck/WJ (H5N1) challenge, respectively, survived (Fig. 5). No clinical signs of disease were observed in any of the vaccine-challenge birds. In contrast, all sham-vaccinated birds died following challenge with mean death times of 4, 3 and 3 in GyrF, Ck/Egypt, and Ck/WJ challenge groups, respectively. Thus, the VLPs provided complete protection against each of three challenge AI isolates tested including H5N8 virus. All sham vaccinated birds were dead by 5 days post challenge, indicating a significant level of protection in VLP vaccinated birds.

Virus shedding was significantly reduced against the three H5 isolates tested (Fig. 6). Following virus challenges with 10^6 EID₅₀ or PFU in each challenge experiments, all three H5N1 viruses replicated efficiently in the upper respiratory tract of unimmunized control chickens. In comparison, immunized birds displayed a significant reduction in H5N1 viral load on each day post challenge. Similar data were observed in cloacal swabs (Fig. 6).

Taken together, these data demonstrate that triple-clade H555 VLP immunization offered significant protection against lethal disease following challenges with two distinct isolates of H5N1 AI virus, as well as challenge with H5N8 HPAI virus.

4. Discussion

In the current study, we prepared novel recombinant VLPs as a vaccine to protect against AI viruses of H5 subtype. Baculovirus-produced VLPs have been developed previously as agricultural vaccines including VLP vaccine against porcine circovirus type 2 (PCV2) [23, 24]. Expression of PCV2 ORF2 in insect cells resulted in VLPs that were structurally and antigenically similar to PCV2 capsids and were highly immunogenic [25]. The AI outbreaks affect millions of commercial birds worldwide causing significant damage to poultry industry

affecting food supply and safety. Human contact with AI infected poultry can also result in a human infection that can lead to hospitalization or death, especially in the elderly, the very young and those with underlying health conditions. Furthermore, AI in poultry can threaten the supply of eggs that are used for manufacturing of egg-dependent vaccines worldwide including those against influenza, yellow fever, mumps and measles viruses.

Multiple AI viruses continue to circulate in birds including H5 subtypes. Vaccines capable of protecting against multiple AI especially HPAI strains are needed. The majority of current AI vaccines for poultry are traditional inactivated whole-virus vaccines. These vaccines utilize viruses that are grown in embryonated eggs, a process that requires large biocontainment facilities for highly pathogenic viruses. However, preparation of multivalent vaccine against several HPAI viruses using current commercial manufacturing technology causes biosecurity concerns and is economically not feasible. Recombinant multi-clade H555 VLPs described in this study represent a conceptually novel trivalent influenza vaccine, which is designed to simultaneously elicit specific immunity to three distinct clades of H5 HPAI. This triple-clade H555 VLP vaccine does not require blending of individual vaccines to prepare a trivalent formulation. Essentially, the triple-clade VLPs contain multiple clades of H5 molecules on their surfaces and combine [protective](#) epitopes from three H5N1 HPAI viruses. Our results suggest that the protection was likely due to the production of antibodies directed against the HA. [The role of cell-mediated immunity remains to be studied.](#) The presence of BIV gag in the VLPs can be important when differentiation of infected from vaccinated animals (DIVA) is important [26, 27]. DIVA is needed to allow serologic surveillance to be used to determine if field virus is still circulating in poultry flocks which have been vaccinated. DIVA can be used as a tool to assure commercial partners that exported poultry are free from infection. In addition to BIV gag, the VLP vaccines have another built-in DIVA component, specifically that they do not contain the internal viral proteins, including the nucleoprotein (NP). Since commercial flock surveillance for

AI includes serologic testing via ELISA, a test based on NP antigen can be used to demonstrate DIVA, and ensure to trading partners that flocks that have been vaccinated are free of infection.

Since this is a novel approach, more research is needed. Additional data are needed regarding assembly mechanism of multi-clade VLPs, as well as the ratio of distinct HAs within the VLPs. We quantitated the total HA content in the vaccine by the HA assay; however, we did not quantitate content of each H5 clade within the VLPs. Since each H5 protein was expressed from the polyhedrin promoter, equivalent expression of the H5 proteins is expected. The observed comparable HI titers and protection for each challenge virus confirm the presence of similar quantities of each H5 clade in the VLPs. If one of the H5 proteins was under-expressed, a drop in HI antibody titers and likely a reduced level of protection would be expected. In the previous study, we demonstrated that the content of each HA within triple-subtype VLPs varies, ranging between 23-41% of the total HA content [16]. This ratio was sufficient to protect against three challenge viruses [16]. In the current study, complete protection against three H5 AI viruses was demonstrated. The HI reactivity and protection *in vivo* can be considered as evidence of co-localization of distinct H5 proteins within the vaccine, and potentially, as the release criteria for the vaccine manufacturing.

Alternative approaches for preparation of broadly protective influenza vaccine include the use of the consensus sequences for HA protein and the “universal” vaccines based on highly conserved influenza epitopes, for example the ectodomain of M2 ion channel protein [28-30]. Universal vaccine approaches also include induction of immune response to conservative regions of HA [31-33]. However these approaches require additional studies to fully evaluate their safety and efficacy in poultry species. Our research shows that triple-clade H555 VLPs is a promising candidate for a safe and highly immunogenic vaccine against AI. This generic approach can also be applicable to other LPAI and HPAI viruses, as well as for the development of human influenza vaccines.

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Conflict of interest statement: The authors declare that they have no competing financial interests.

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Figure Legends

Fig. 1. Preparation of triple-clade H555 VLPs containing H5 HA proteins from three clades of H5N1 HPAI viruses. (a) Recombinant baculovirus (rBV) for expression of triple-clade H555 VLPs in *Spodoptera frugiperda* (Sf9) cells. Influenza HA gene sequences were derived from A/chicken/Germany/2014 (clade 2.3.4.4), A/chicken/West Java/Subang/29/2007 (clade 2.1.3) and A/chicken/Egypt/121/2012 (clade 2.2.1). NA and BIV gag genes were also included (see Materials and methods for details). The genes were combined within recombinant rBV in a tandem fashion. Polyhedrin promoters are indicated with arrows. (b) Hemagglutination assay of H555 VLPs in the growth medium of rBV-infected Sf9 cells, by using 1% turkey RBC. NC, PBS negative control. (c) Genetic stability of rBV vectors, by detection of H5 expression in the medium supernatant of Sf9 cells infected with passage P1, P2, and P5 rBV vectors. VLPs were concentrated 100-fold by ultracentrifugation. Expression was detected by stained SDS-PAGE (left panel) and western blot with anti-H5 MAb (center). HA assay was done using VLP-containing growth medium without concentration using two fold dilutions starting with 1:8 dilution (right panel). Lanes 1, 2 and 3 indicate expression from Sf9 cells infected with P1, P2 and P5 rBV vectors, respectively.

Fig. 2. Purification and characterization of triple-clade H555 VLPs. VLPs were harvested from the culture medium of rBV-infected Sf9 cells on day 3. VLPs were sterile filtered using 0.2 μ m filtration, concentrated by TFF (500 000 MWCO) and purified using ion exchange chromatography. (a) Stained SDS-PAGE gel and western blot using anti-H5 (H5N1) mouse IgG2a MAb. Protein molecular weight standards (M) and positions of HA and BIV gag are indicated. (b). Hemagglutination assay using 1% turkey RBC. NC, PBS negative control. (c). Negative stain transmission electron microscopy. For electron microscopy, VLPs were stained with 1% phosphotungstic acid. Bar, 100 nm.

Fig 3. Antibody response in birds vaccinated with H555 VLP against vaccine antigen. Chickens were arbitrarily designated into three challenge groups 1, 2 and 3 (n=10 per group) corresponding to GyrF, Ck/Egypt and Ck/WJ challenge, as described in the text. All birds were vaccinated at 1-day and 21 days-of-age with 1536 HA units of H555 VLP vaccine per bird. Serum was taken at five weeks of age. Pre-challenge HI antibody titers are shown separately for each challenge group, as well as for control unvaccinated group (sham). Statistical significance between mean titers was determined with ANOVA using the Tukey's Multiple Comparison Test ($p < 0.05$). Sham: unvaccinated.

Fig 4. Individual HI titers ($\log_2$) and standard error for bird groups against the challenge virus, (a) five weeks post-vaccination (prechallenge) and (b) two weeks post-challenge.

Fig. 5. Kaplan-Meier survival plots for protection of H555 VLP vaccinated chickens against HPAI H5N1 or H5N8 challenge. Chickens were unvaccinated (Sham, solid line) or vaccinated with H555 VLP at one day-of-age and 21 days of age (dashed line), then randomly split into three groups, and each group was challenged (10^6 EID₅₀ per bird) at 35 days of age with one of the indicated HPAI H5 isolates: A/Gryfalcon/Washington/2014 H5N8 (clade 2.3.4.4), A/Ck/WJSubang/2007 H5N1 (clade 2.1.3) or A/Ck/Egypt/2010 H5N1 (clade 2.2.1).

Fig 6. Viral titers from oral and cloacal swabs on day 2 and 4 post-challenge. Birds were vaccinated with H555 VLP at 1 and 21 days of age, and challenged at day 35 with indicated H5 viruses. Viral titers are expressed as $\log_{10}$ EID₅₀ per ml. The lower limit of detection is 0.9 $\log_{10}$ EID₅₀ per ml. (a) Viral titers after challenge with A/Gryfalcon/Washington/2014 H5N8 (clade 2.3.4.4) virus; (b) Viral titers after challenge with A/Chicken/Egypt/2010 H5N1 (clade 2.2.1) virus; (c) Viral titers after challenge with A/Chicken/WJSubang/2007 H5N1 (clade 2.1.3) virus.

Fig. 1

Fig. 2

Pre challenge HI antibody titers to vaccine antigen H555

Fig. 3

b Post challenge HI antibody titers to challenge virus

Fig. 4

Fig. 5

a

A/Gryfalcon/Washington/2014 H5N8 (clade 2.3.4.4)

Fig. 6a

b

A/Chicken/Egypt/2010 H5N1 (clade 2.2.1)

Fig. 6b

C

A/Chicken/WJSubang/2007 H5N1 (clade 2.1.3)

Fig. 6c