

Protective efficacy of crude virus-like particle vaccine against HPAI H5N1 in chickens and its application on DIVA strategy

Jae-Keun Park,^{a,*} Dong-Hun Lee,^{a,*} Ha-Na Youn,^a Myeong-Seob Kim,^a Yu-Na Lee,^a Seong-Su Yuk,^a Tae-Hyun Lim,^a Jun-Hyuk Jang,^a Jung-Hoon Kwon,^a Byoung-Yoon Kim,^a Sang-Moo Kang,^b Baik-Lin Seong,^c Joong-Bok Lee,^a Seung-Yong Park,^a In-Soo Choi,^a Chang-Seon Song^a

^aAvian Disease Laboratory, College of Veterinary Medicine, Konkuk University, Seoul, Republic of Korea. ^bCenter for Inflammation, Immunity & Infection and Department of Biology, Georgia State University, Atlanta, GA, USA. ^cDepartment of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea.

Correspondence: Chang-Seon Song, PhD, Avian Disease Laboratory, College of Veterinary Medicine, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul, 143-701, Republic of Korea. E-mail: songcs@konkuk.ac.kr

*These authors contributed equally to this work.

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Background Currently, Asian lineage highly pathogenic avian influenza (HPAI) H5N1 has become widespread across continents. These viruses are persistently circulating among poultry populations in endemic regions, causing huge economic losses, and raising concerns about an H5N1 pandemic. To control HPAI H5N1, effective vaccines for poultry are urgently needed.

Objective In this study, we developed HPAI virus-like particle (VLP) vaccine as a candidate poultry vaccine and evaluated its protective efficacy and possible application for differentiating infected from vaccinated animals (DIVA).

Methods Specific pathogen-free chickens received a single injection of HPAI H5N1 VLP vaccine generated using baculovirus expression vector system. Immunogenicity of VLP vaccines was

determined using hemagglutination inhibition (HI), neuraminidase inhibition (NI), and ELISA test. Challenge study was performed to evaluate efficacy of VLP vaccines.

Results and Conclusions A single immunization with HPAI H5N1 VLP vaccine induced high levels of HI and NI antibodies and protected chickens from a lethal challenge of wild-type HPAI H5N1 virus. Viral excretion from the vaccinated and challenged group was strongly reduced compared with a mock-vaccinated control group. Furthermore, we were able to differentiate VLP-vaccinated chickens from vaccinated and then infected chickens with a commercial ELISA test kit, which offers a promising strategy for the application of DIVA concept.

Keywords Chicken, DIVA, HPAI, vaccine, VLP.

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Introduction

Highly pathogenic H5N1 avian influenza (HPAI), defined as a “notifiable” disease by the World Organization for Animal Health (OIE)¹, has caused fatal infections in poultry with severe economic impact worldwide². Especially, Asian lineage HPAI H5N1 has become widespread across continents, including Eurasia and Africa, and has become endemic in Southeast Asia in poultry since it was first identified in China in 1996³. Furthermore, to date, HPAI H5N1 has resulted in over 500 confirmed human cases with an approximately 60% fatality rate⁴, raising global public health concerns about its pandemic potential.

To control HPAI H5N1 outbreaks, several strategies have been applied including a high level of biosafety, movement control, stamping out, and vaccination. Although vaccina-

tion against HPAI in poultry is still a controversial topic and has been discouraged in the past, vaccination has also been recommended as an alternative HPAI control strategy^{5–8}, since mass culling that is frequently used to control outbreaks in poultry has not proved successful in HPAI H5N1 endemic regions⁹. To be used as part of effective HPAI control strategies, vaccination should prevent clinical disease and death, induce resistance to infection, decrease viral excretion from infected birds, and essentially, facilitate the differentiation of infected from vaccinated animals (DIVA)¹⁰, which has been a major inhibitor for the use of inactivated vaccine in AIV control¹¹.

Virus-like particles (VLPs), which resemble infectious virus particles in structure and morphology with multiple antigenic epitopes, have been suggested as a new generation of vaccine candidates against various viral infections with a

solid safety profile.¹² VLP vaccines were shown to be highly immunogenic probably due to its ability to stimulate a diverse set of host immune responses.¹³ As protective immune responses of influenza A VLPs against A/Udorn/72 (H3N2) have been described for the first time by Galarza *et al.*¹⁴, influenza A VLPs have been produced in different expression systems¹⁵, and the safety, immunogenicity, and protectivity have been studied in various animal models^{15–17}. Recently, several studies have demonstrated efficacy and safety of VLP vaccines against HPAI H5N1 using mouse^{18,19} and ferret^{20,21} models for the development of human vaccine. However, VLP vaccines against HPAI H5N1 for poultry species, in which HPAI H5N1 viruses are currently circulating and threatening public health and poultry industry, have not been studied.

Previously, a few studies demonstrated immunogenicity²² and protective efficacy²³ of LPVLP vaccine in ducks and chickens, respectively, that provided possibilities for the application of VLP vaccines against HPAI in poultry species, which play a critical role in the maintenance and spread of HPAI H5N1²⁴. In this study, for veterinary use, we developed a HPAI H5N1 VLP vaccine and evaluated its immunogenicity and protective efficacy against HPAI in specific pathogen-free (SPF) chickens for the first time. Furthermore, VLP vaccines were assessed for differentiating VLP-vaccinated chickens from vaccinated and then infected chickens, which may provide a useful method for serosurveillance in vaccinated flocks.

Materials and methods

Cloning of HA, NA, and M1 genes

Viral RNA was extracted from HPAI H5N1 A/chicken/Korea/ES/2003 (GenBank AY676035) using an RNeasy® Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instruction. For cDNA synthesis, reverse transcription (RT) was performed on extracted viral RNA using the Omniscript™ RT Kit (QIAGEN) with Uni12 primer as previously described.²⁵ The following primer pairs were used for the polymerase chain reaction (PCR) amplification of the HA, NA, and M1 genes, respectively: HA_ES03 (F) 5'-TGGATCCatgGAGAAAATAGTGC-3' and (R) 5'-TAAGCTTAGTAGAAACAAGG-3'; NA_ES03 (F) 5'-AGAATTCatgAATCCAAATCAGAAG-3' and (R) 5'-TAAGCTTAGTAGAAACAAGG-3'; M1_ES03 (F) 5'-AGAATTCatgAGTCTTCTAACCGAGG-3' and (R) 5'-TAAGCTTCACTTGAATCGCTGC-3' (ATG codons shown in lower case letters). PCR-amplified HA, NA, and M1 genes were cloned into TA cloning vector pGEM®-T (Promega, Madison, WI, USA), and each gene sequence was determined by DNA sequencing. The three resulting plasmid vectors containing influenza virus genes were designated as vHA, vNA, and vM1.

Construction of transfer vector

Plasmid vector vHA was digested by the restriction enzyme BamHI/HindIII, and the resulting HA DNA fragment was ligated into a BamHI-/HindIII-digested pFastBacT1 bacmid transfer vector (Invitrogen, Carlsbad, CA, USA). Similarly, plasmid vectors vNA and vM1 were digested by the restriction enzyme EcoRI/HindIII, and the resulting DNA fragments were ligated into an EcoRI-/HindIII-digested pFastBacT1 bacmid transfer vector. The three resulting transfer vectors containing influenza virus genes were designated as pHA, pNA, and pM1. A bacmid transfer vector encoding both NA and M1 genes, designated as pNAM1, was constructed by cloning a SnaBI-/HpaI-digested fragment from pM1 into the HpaI site of pNA. Finally, a bacmid transfer vector encoding three influenza genes, pNAM1HA, was prepared by cloning a SnaBI-/HpaI-digested fragment from pHA into the HpaI site of pNAM1. As a result, a transfer vector, pNAM1HA, encoding HA, NA, and M1 genes included within its own polyhedrin promoter and transcription termination sequences was constructed.

Generation of recombinant baculovirus

MAX Efficiency® DH10Bac™ competent *Escherichia coli* cells (Invitrogen) were transformed with the constructed transfer vector plasmids, pNAM1HA, to generate recombinant bacmids according to the instructions recommended by the manufacturer. The recombinant bacmid DNA was transfected into Sf9 cells seeded in 6-well plates, at a density of 8×10^5 cells/well, using Cellfectin® Reagent (Invitrogen) resulting in the release of recombinant baculovirus (rBV) into the culture medium. At 72 hours post-transfection, culture medium was harvested and inoculated into Sf9 cells for generation of high-titer rBV stock. Baculovirus titration was performed by plaque assay on Sf9 cells.²⁶

Production of HPAI H5N1 VLP and preparation of VLP vaccines

For VLP production, Sf9 cells, grown in Sf900III medium (GIBCO BRL, Grand Island, NY, USA), were infected with rBV expressing H5N1 A/chicken/Korea/ES/2003 HA, NA, and M1 proteins at a multiplicity of infection (MOI) of 3 for 72 hours. The culture medium containing VLPs was collected and clarified by low-speed centrifugation ($2000 \times g$, 30 minutes) to remove large cell debris, and culture supernatants were chemically treated with formalin at a final concentration of 0.2% for inactivation of baculovirus. After incubation for 48 hours at 37°C, formalin-treated culture supernatant was concentrated using Vivaspin 20 (100 000 MWCO PES; Sartorius, Göttingen, Germany) protein concentrator without further purification. As VLP antigen used in this study was not

sucrose-purified to reduce production cost for veterinary use, the VLP antigen contents were quantified by hemagglutination units (HAU) of concentrated culture medium as described in the OIE manual¹, rather than measuring total protein concentration.²³

Expression of influenza proteins in VLPs was confirmed by coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as well as Western blotting using mouse anti-H5 monoclonal antibody (Bionote, Inc., Hwaseong, Korea), rabbit anti-N1 antibodies (Immune Technology, NY, USA), and rabbit anti-M1 antibodies (Immune Technology) followed by the detection with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG (AbD Serotec, UK). H5N1 influenza VLP vaccine doses in this study were determined based on hemagglutination units (HAU) because of crude VLP vaccine preparations. VLP vaccines were prepared by emulsifying the escalating VLP concentration (2^8 , 2^9 , and 2^{10} HAU) of culture supernatants with Montanide ISA70 adjuvant (SEPPIC, France), which provided significant dose-sparing effect compared with the non-adjuvanted vaccine in our previous LPAI VLP study²³, at a ratio of 30:70 (w/w).

Immunization of animals and virus challenge

A total of 32 five-week-old SPF white leghorn chickens (Namduck Sanitec, Korea) were divided into four groups (8 chickens per group). Three groups of chickens were immunized with escalating dose (2^8 , 2^9 , and 2^{10} HAU) of HPAI H5N1 VLP vaccines with ISA70 adjuvant. As LPAI VLP vaccine with ISA70 adjuvant, which injected via intramuscular route, elicited high levels of HI antibody in chicken in our previous study²³, immunization was performed intramuscularly. Injection dose was 0.5 ml per chicken. As a mock-vaccinated control group, another eight SPF chickens were injected with an emulsified solution of conditioned SF900III medium with ISA70 in the same ratio as VLP vaccines. Three weeks after a single immunization, chickens were intranasally challenged with 100 μ l of $10^{6.0}$ EID₅₀/ml of A/chicken/Korea/ES/2003 (H5N1). The challenge study and all experiments with live viruses were conducted in a biosafety level 3 (BSL3) facility in Konkuk University. All animal procedures performed in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University.

Assessment of protection

As prevention of clinical signs (morbidity) and death (mortality) and reduction of viral shedding after HPAI challenge have been the most frequently used criteria to assess protective efficacy of HPAI vaccine^{27–31}, we observed mortality and clinical signs daily for 10 days post-challenge (dpc)

and determined viral shedding using real-time reverse transcriptase polymerase chain reaction (rRT-PCR).

To determine the viral shedding, oropharyngeal and cloacal swab samples were collected at 2, 3, 5, and 7 dpc and suspended in 1 ml of phosphate-buffered saline (PBS) supplemented with gentamycin (400 μ g/ml). Of this suspension, 200 μ l was used for RNA extraction using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instruction. The content of AIV RNA was quantified by cycle threshold (Ct) value using M gene-based rRT-PCR as previously described³².

For extrapolation of the Ct values to infectious units, known titers of A/chicken/Korea/ES/2003 (H5N1) viruses from egg allantoic fluid, measured in EID₅₀, were serially 10-fold diluted. Viral RNA was extracted from these dilutions and quantified by rRT-PCR as described above. For generating a standard curve, Ct values of each viral dilution were plotted against viral titers. The resulting standard curve was highly correlated ($r^2 > 0.99$) and was used to convert Ct values to EID₅₀.

Serology

To determine the immunogenicity of VLP vaccines, serum samples were collected prior to vaccination and 3 weeks after vaccination for hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests. Reverse genetics-derived influenza A/chicken/Korea/ES/2003 (clade 2.5), A/Indonesia/5/2005 (clade 2.1), and A/Vietnam/1194/2004 (clade 1) were formalin-inactivated and used as homologous or heterologous antigens. HI titers against homologous and heterologous strains were measured according to the OIE standard HI method¹.

The anti-NA antibody titers were measured by NI assay using the substrate 2'-(4-Methylumbelliferyl)- α -d-N-acetylneuraminic acid (4-MU-NANA; Sigma-Aldrich, St. Louis, MO, USA) and formalin-inactivated homologous antigen as enzyme source. Serum samples were twofold diluted with sodium acetate buffer (150 mM sodium acetate, 1 mM calcium chloride, pH 7.0), and 25 μ l of diluted samples was mixed with an equal volume of predetermined amount of inactivated homologous antigen (2^7 HAU). After incubation for 1 hour at 37°C, 50 μ l of substrate (100 μ M 4-MU-NANA in sodium acetate buffer) was added to each well. After additional 30 minutes incubation at 37°C, the reaction was stopped by addition of 100 μ l of stop solution (0.1 M glycine, 25% ethanol, pH 10.7). The fluorescence intensity was measured using SpectraMax Gemini EM fluorescent plate reader with excitation and emission wavelengths of 360 nm and 465 nm, respectively. Antibody-positive cut-off values were set as the mean value - 2x standard deviation (mean-2SD) of the fluorescence intensity in mock-vaccinated chickens. NI titers were expressed as the highest serum dilution factors that resulted fluorescence intensity lower than cut-off values.

For differentiating vaccinated chickens from vaccinated and then infected chickens, which may provide a useful method for serosurveillance in vaccinated flocks, serum samples were collected 3 weeks post-vaccination (wpv) and 10 dpc from 2^8 and 2^{10} VLP-vaccinated chickens. Collected samples were analyzed for AIV NP-specific antibody levels using a commercially available multispecies competitive NP-cELISA Kit (Bionote), which is pre-coated with AIV NP antigen into the wells of the ELISA plate. ELISA test was performed according to the manufacturer's instruction. Sample values were calculated [sample value = $1 - (\text{OD}_{450} \text{ sample} / \text{mean OD}_{450} \text{ negative})$], and sample value < 0.5 was considered negative for the presence of antibodies to NP. To compare changes in HI titers following infection with the results from NP-cELISA, 10 dpc HI titers from 2^8 and 2^{10} VLP-vaccinated chickens were measured using homologous antigen as described above.

Statistical analysis

Analysis of variance (ANOVA) with a Tukey–Kramer post-test was performed for serum HI antibody titers. Statistical analysis of differences between VLP-vaccinated groups was performed using Fisher's exact test. Results with P values < 0.05 were considered to be statistically significant.

Results

Immune responses to VLP vaccines

HPAI H5N1 VLPs containing HA, NA, and M1 protein derived from A/Chicken/Korea/ES/2003 (H5N1) virus were produced in Sf9 cells using the baculovirus expression vector system (BEVS). After formalin treatment, unconcentrated VLP-containing culture supernatant was found to have 2^7 HAU and concentrated to a final concentration of 2^8 , 2^9 , and 2^{10} HAU. Influenza proteins (HA, NA, and M1) of VLPs were detected by coomassie-stained SDS-PAGE (Figure 1A) and Western blotting (Figure 1B).

To evaluate the immunogenicity of HPAI H5N1 VLP vaccines, seroconversion was determined 3 weeks after a single immunization with a range of 2^8 to 2^{10} HAU of VLP vaccines. Throughout the experiment, none of the VLP-vaccinated chickens showed any signs of adverse effect (e.g. unusual local or systemic response) after administration of the crude VLP vaccine. As shown in Figure 2, significantly increased levels of HI antibody titers against homologous or heterologous antigens were induced in VLP-vaccinated chickens. As expected, both the heterologous HI titers (clades 2.1 and 1) were significantly lower than those against the homologous strain (clade 2.5). Additionally, heterologous HI titers against the clade 2.1 were observed at higher levels than the clade 1, reflecting the antigenic closeness. As shown in Figure 3, significantly increased levels of NI antibody titers against homologous antigen were

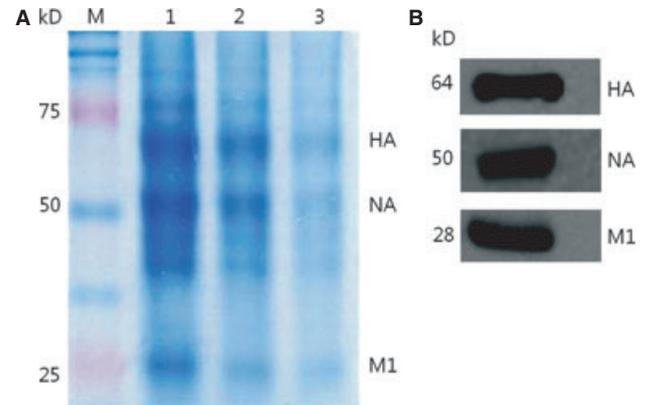


Figure 1. Identification of proteins in A/chicken/Korea/ES/2003 (H5N1) VLPs. (A) Coomassie-stained SDS-PAGE analysis of VLPs expressing HA, NA, and M1. M, a standard molecular size marker (in kilodaltons); Lane 1, 2^{10} HAU of VLP protein; Lane 2, 2^9 HAU of VLP protein; Lane 3, 2^8 HAU of VLP protein. (B) Analysis of VLPs by Western blot using mouse anti-H5 monoclonal antibody and rabbit polyclonal antibodies for N1 or M1. Molecular weights of expressed HA, NA, and M1 are indicated on the left.

induced in all VLP-vaccinated chickens. NI antibodies elicited by H5N1 VLP vaccine are expected to confer additional protective effect against HPAI challenge, because antibodies to the NA protein thought to reduce the amount of virus released from infected cells by aggregating influenza virus on the cell surface³³. Groups with higher antigen doses, 2^9 or 2^{10} HAU VLP vaccines, showed moderately higher levels of HI and NI antibody titers against homologous antigen than the 2^8 HAU VLP vaccine group although there were no statistical differences among vaccinated groups. However, no detectable levels of antibody responses were found in the mock-vaccinated control group that received an emulsified solution of conditioned SF900III cell culture medium with ISA70 adjuvant. These results suggest that crude HPAI H5N1 VLP vaccination is safe and can induce anti-H5 and anti-N1 functional antibody responses in chickens.

Protection against HPAI H5N1 and reduced viral shedding in VLP-vaccinated chickens

To examine the protectivity and efficacy of H5N1 VLP vaccines, a challenge infection with a high dose of HPAI A/chicken/Korea/ES/2003 (H5N1) was performed 3 weeks after a single immunization. Mock-vaccinated chickens showed severe clinical signs and 100% mortality within 3 days after challenge [mean death time (MDT) = 2.4], which insured that proper challenge was accomplished. However, 100% of the VLP-vaccinated chickens were protected from mortality, and only mild clinical signs, including swollen head and diarrhea, were observed in two chickens each in 2^8 and 2^9 VLP-vaccinated groups

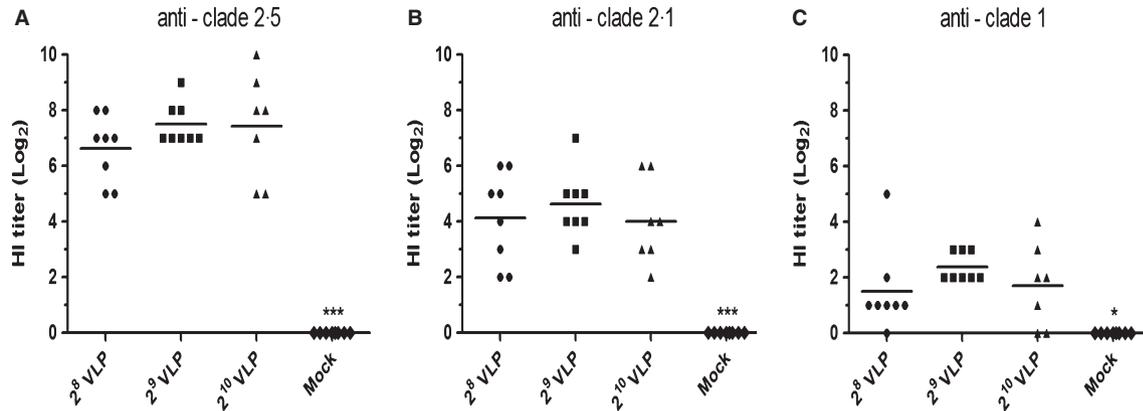


Figure 2. Mean serum hemagglutination inhibition (HI) titers (\log_2) induced in specific pathogen-free (SPF) chickens after a single dose of virus-like particle (VLP) vaccine with ISA70 adjuvant. A total of 32 five-week-old SPF chickens (8 per group) were intramuscularly immunized with HPAI H5N1 VLP vaccines. HI titers against the homologous antigen (A) A/chicken/Korea/ES/2003 (clade 2-5) and heterologous antigen (B) A/Indonesia/5/2005 (clade 2-1) and (C) A/Vietnam/1194/2004 (clade 1) were determined 3 weeks after vaccination. Mock – emulsified solution of conditioned SF900III medium with ISA70. * $P < 0.05$ and *** $P < 0.001$ by anova with Tukey–Kramer post-test compared with other groups.

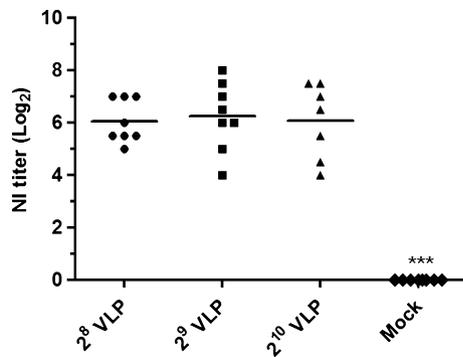


Figure 3. Mean serum neuraminidase inhibition (NI) titers (\log_2) induced in specific pathogen-free (SPF) chickens after a single dose of virus-like particle (VLP) vaccine with ISA70 adjuvant. A total of 32 five-week-old SPF chickens (8 per group) were intramuscularly immunized with HPAI H5N1 VLP vaccines. NI titers against the homologous antigen were determined 3 weeks after vaccination. Mock – emulsified solution of conditioned SF900III medium with ISA70. *** $P < 0.001$ by anova with Tukey–Kramer post-test compared with other groups.

(Table 1). No clinical signs were observed in 2^{10} VLP-vaccinated chickens throughout the study.

Oropharyngeal and cloacal swab samples collected at 2, 3, 5, and 7 dpc were tested for viral RNA quantification by rRT-PCR. All swab samples from mock-vaccinated chickens yielded positive results with Ct values ranging from 27.2 to 23.3 for oropharyngeal swabs, representing infectious titers of approximately 3.1 to 4.6 \log_{10} EID₅₀/ml, respectively, and 27.5 to 23.6 for cloacal swabs, corresponding to 3.0 to 4.5 \log_{10} EID₅₀/ml.

All groups of VLP-vaccinated chickens not only survived, but showed significantly reduced viral excretion compared with mock-vaccinated chickens. In oropharyngeal swabs from VLP-vaccinated groups, samples showed reduced levels

of viral excretion with the lowest mean Ct value of 32.2 (1.1 \log_{10} EID₅₀/ml). Even though there were no statistical differences between groups, oropharyngeal shedding rate of challenge virus was generally lower in 2^9 and 2^{10} VLP-vaccinated groups compared with the 2^8 VLP-vaccinated group, at 2 and 7 dpc (Table 2), which is probably due to moderately higher levels of HI and NI antibody titers of 2^9 and 2^{10} VLP-vaccinated groups (Figures 2 and 3). Moreover, one chicken that received a 2^{10} VLP vaccine was negative for oropharyngeal excretion throughout the study (Table 2). The cloacal excretion of challenge virus from VLP-vaccinated chickens was less frequent compared with oropharyngeal specimens with the lowest mean Ct value of 33.1 (0.8 \log_{10} EID₅₀/ml) (Table 3). Cloacal excretion of challenge virus was not detected at 7 dpc in all groups of VLP-vaccinated chickens.

Table 1. Morbidity and mortality in VLP- and mock-vaccinated chickens intranasally challenged with 10^5 EID₅₀ of A/chicken/Korea/ES/2003 (H5N1) highly pathogenic avian influenza (HPAI) virus

Group*	Mortality [number dead/total (MDT**)]	Morbidity (number ill/total)
2^8 VLP	0/8	1/8
2^9 VLP	0/8	1/8
2^{10} VLP	0/7***	0/7
Mock	8/8 (2.4)	6/8

*A dose of VLP vaccine as indicated by units of hemagglutination activity.

**MDT – mean death time denoted in days.

***One bird died before challenge study because of cannibalism.

Table 2. Challenge virus excretion from oropharyngeal swab samples

Day post-challenge	2 ⁸ VLP		2 ⁹ VLP		2 ¹⁰ VLP		Mock vaccinated	
	No. of positive/total	Avg Ct (logEID ₅₀ /ml*)	No. of positive/total	Avg Ct (logEID ₅₀ /ml)	No. of positive/total	Avg Ct (logEID ₅₀ /ml)	No. of positive/total	Avg Ct (logEID ₅₀ /ml)
2	3/8	32.8 (0.88)	1/8	32.2 (1.11)	1/7	34.7 (0.09)	8/8	24.8 (4.05)
3	8/8	33.6 (0.57)	8/8	33.8 (0.47)	6/7	33.8 (0.47)	3/3	23.8 (4.45)
5	3/8	34.4 (0.25)	3/8	34.0 (0.42)	3/7	34.2 (0.30)	–	N/A
7	1/8	34.4 (0.24)	0/8	–	0/7	–	–	N/A

N/A, not applicable.

*log EID₅₀ equivalents were determined with the use of real-time reverse transcriptase polymerase chain reaction (rRT-PCR). Numbers in parentheses are averages of viral titers shed from chickens in each group.

Especially, in 2 dpc, rRT-PCR test failed to detect AIV from oral and cloacal swab samples in a large number of VLP-vaccinated birds, while all mock-vaccinated chickens at 2 dpc showed positive test results with low Ct values (Tables 2 and 3). Kinetic of shedding in the vaccinated group might be delayed, and/or viral shedding was suppressed probably because of effective immune responses elicited by VLP vaccine. It is believed that viral shedding level in a large number of challenged birds at 2 dpc was below the detection limit of rRT-PCR test used in this study. Lower frequency of viral shedding in earlier days, 2 dpc in this study, could be seen in data from other study³⁴. These results suggest that a single immunization of chickens with H5N1 VLP vaccines is effective in reducing viral shedding of challenge virus.

Differentiation of vaccinated chickens from vaccinated and then infected chickens

Serum samples were collected from VLP-vaccinated chickens before and 10 days post-challenge for serological test-

ing. As expected, all pre-challenge sera from VLP-vaccinated chickens were negative by the NP-cELISA ($1-[S/N] < 0.5$). However, as shown in Figure 4A, 6 of 8 and 1 of 7 post-challenge sera from 2⁸ VLP- and 2¹⁰ VLP-vaccinated chickens, respectively, showed a positive immune response to influenza NP ($1-[S/N] \geq 0.5$). Therefore, VLP vaccination and the companion DIVA test, NP-cELISA, could differentiate vaccinated group from vaccinated and then infected group. Interestingly, the 2⁸ VLP-vaccinated group, which resulted higher NP-cELISA test positive rate, showed moderately increased mean HI titer at 10 dpc compared with pre-challenge sera whereas the 2¹⁰ VLP-vaccinated group showed similar mean HI titers between pre- and post-challenge sera (Figure 4B).

Discussion

In the present study, even a single immunization with HPAI H5N1 VLP vaccine elicited high levels of functional

Table 3. Challenge virus excretion from cloacal swab samples

Day post-challenge	2 ⁸ VLP		2 ⁹ VLP		2 ¹⁰ VLP		Mock vaccinated	
	No. of positive/total	Avg Ct (logEID ₅₀ /ml*)	No. of positive/total	Avg Ct (logEID ₅₀ /ml)	No. of positive/total	Avg Ct (logEID ₅₀ /ml)	No. of positive/total	Avg Ct (logEID ₅₀ /ml)
2	0/8	–	0/8	–	0/7	–	8/8	25.6 (3.72)
3	6/8	34.1 (0.37)	5/8	34.3 (0.29)	4/7	34.6 (0.15)	3/3	25.4 (3.79)
5	2/8	34.6 (0.15)	2/8	33.1 (0.76)	1/7	34.0 (0.42)	–	N/A
7	0/8	–	0/8	–	0/7	–	–	N/A

NA, not applicable.

*log EID₅₀ equivalents were determined with the use of rRT-PCR. Numbers in parentheses are averages of viral titers shed from chickens in each group.

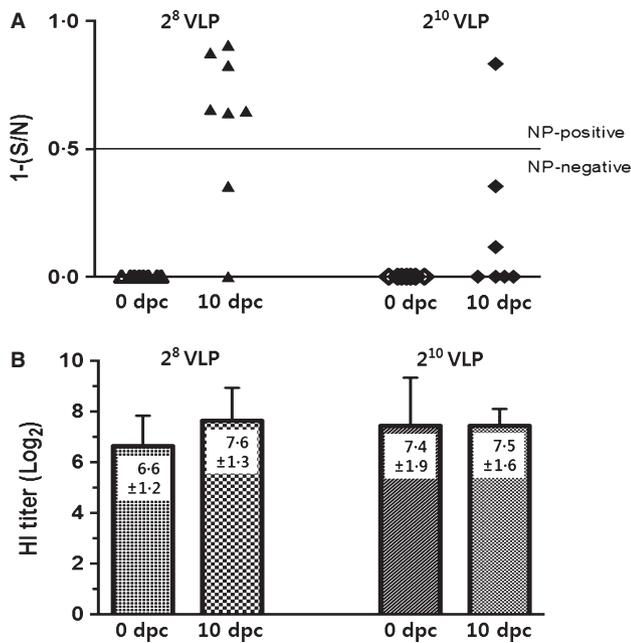


Figure 4. Differentiation of vaccinated chickens from vaccinated and then infected chickens and changes in hemagglutination inhibition (HI) titers following infection. Serum samples were taken 3 weeks post-vaccination from 2⁸ and 2¹⁰ VLP-vaccinated chickens. 10 days post-challenge (dpc) infection, serum samples were taken from 2⁸ and 2¹⁰ VLP-vaccinated and then infected chickens. (A) NP antibody levels from each serum samples were tested with commercially available NP-cELISA Kit. Each dot represents the NP-specific antibody value of each chicken. (B) Hemagglutination inhibition (HI) titers (log₂) against homologous antigen at 10 dpc were compared with pre-challenge sera. Data shown are the mean titers of each group ± standard deviation.

antibodies and fully protected chickens from a lethal HPAI challenge with a very small number of chickens showing minor clinical signs. Serology tests that revealed the lack of detecting seroconversions against AIV NP in 8 of 15 chickens indicated that virus replication was strongly suppressed, especially in the 2¹⁰ VLP-vaccinated group. These results are in accordance with other studies^{27–28,35} showing missing seroconversion after HPAI challenge in vaccinated chickens, which could possibly confirm protective efficacy of the HPAI VLP vaccines developed in this study.

For effective vaccination programs, adequate serological or virological surveillance is essential to determine whether the field virus is circulating in vaccinated flocks.³⁶ Moreover, for both trade and surveillance purposes, it is important not only to differentiate naturally infected and vaccinated birds, but also to identify vaccinated birds that become infected with field virus.¹¹ As expected, the HPAI VLP vaccine developed in this study did not induce antibodies against AIV NP. However, after H5N1 challenge, antibodies against AIV NP were detected using NP-cELISA in VLP-vaccinated and then infected groups, which allowed us to differentiate VLP-vaccinated chickens from

VLP-vaccinated and then infected chickens. Classical agar gel immunodiffusion (AGID) test that targets AIV NP is inexpensive, simple, and most widely used diagnostic tool to detect influenza infection. As VLP vaccine does not induce antibodies to NP, VLP vaccination accompanied by AGID test is expected to allow DIVA strategy without unusual supplies or expensive equipment. Interestingly, in the previous^{27,28,35} and present studies, antibody responses were not detectable in some vaccinated and then HPAI H5N1-infected chickens. Lack of seroconversion in some chickens, occurring as a result of high levels of protection, is a potential issue for all DIVA vaccination strategies except for the use of unvaccinated sentinel birds in vaccinated flocks. Therefore, DIVA strategies based on serosurveillance should only be utilized on a flock basis and not for individual birds.

In terms of safety and antigenic similarity, VLP vaccine technology against HPAI has potential advantages over egg-based inactivated influenza vaccines, because VLPs can be rapidly produced from regional HPAI strains without the need for high biocontainment facilities, which is essentially required for the use of HPAI strains for vaccine production. Although inactivated vaccines prepared from LPAI H5 strains have been frequently used in HPAI H5N1 vaccination programs and have been shown to confer resistance to HPAI H5N1 infection in vaccinated birds^{28,31}, these vaccines could be less effective for control of HPAI because of antigenically distinct properties.^{37,38} Recently, reverse genetics-derived inactivated vaccine, allowing high antigenic similarity, has been reported to efficiently protect chickens against HPAI challenge.³⁹ However, these vaccines could have limitations on DIVA strategy, which highlights advantage of VLP-based vaccine use for HPAI H5N1 control in endemic region. Currently, HPAI H5N1 viruses undergo continuous antigenic mutations that allow viruses to drift away from current vaccine strains. Because VLP vaccine technology allows rapid vaccine production, it could provide easy updates against mutated strains. Moreover, VLP antigens can be modified easily to construct so-called chimeric VLPs that make VLP technology attractive for novel vaccine design and development. In this study, HPAI H5N1 VLP vaccine elicited significantly lower HI titers against different clades of HPAI H5N1 antigen than the HI titers against homologous antigen. Broadened immune responses of chimeric VLPs that include molecular adjuvant⁴⁰ or multiple subtypes of HA⁴¹ have been studied previously. Using similar techniques, including incorporation of various Toll-like receptor (TLR) ligands into VLPs or co-expression of HA proteins from multiple clades of H5N1 within a VLP, HPAI H5N1 VLP vaccines providing high level of cross-clade protection with increased vaccine efficacy might be designed for HPAI control in different endemic regions and need to be further developed. Addi-

tionally, VLP manufacturing facilities for poultry vaccine could be rapidly used for human vaccine production in case of H5N1 pandemic outbreak.

Cost of vaccine is one of the main concerns that might seriously impede the use of vaccination in poultry species. In our previous report²³, as performed by other VLP studies^{18–20}, VLP antigens were purified by sucrose density gradient purification requiring expensive equipment (e.g. ultracentrifuge) and time-consuming process, which would potentially increase the vaccine production cost. In this study, however, we prepared VLP antigen without further purification except for low centrifugation to remove large cell debris. As we prepared VLP antigen without expensive and time-consuming purification process, it is evident that VLP vaccine used in this study costs less to produce compared with highly purified VLP vaccines reported in previous studies^{18–20,23}. Although VLP antigen was prepared without sucrose gradient purification, all groups of VLP-vaccinated chickens showed high levels of HI antibody titers without any signs or symptoms attributable to crude VLP antigen. These results provide method for decreasing production cost of VLP vaccines in poultry use.

In conclusion, HPAI H5N1 VLP vaccine developed in this study was safe, immunogenic, and fully protected SPF chickens from lethal HPAI infection with strongly reduced viral shedding, even without costly purification. Furthermore, we could differentiate VLP-vaccinated chickens from vaccinated and then infected chickens, supporting the use of VLP vaccine as an effective DIVA vaccination strategy. The results in this study demonstrate that VLP vaccination in poultry species is a promising strategy for the control of HPAI H5N1.

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