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H9N2 avian influenza virus-like particle vaccine provides protective immunity and a strategy for the differentiation of infected from vaccinated animals

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Abstract

In the present study, virus-like particles (VLPs) were evaluated as a candidate poultry vaccine against avian influenza virus (AIV) subtype H9N2. Specific pathogen-free chickens received a single injection of the VLP vaccine expressing HA and M1 protein of AIV H9N2 (H9 HA VLP) at escalating doses in the presence or absence of ISA70 water-in-oil adjuvant. At 3 weeks post vaccination, we performed hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA) to determine serological immune responses, and challenge studies using SPF chickens. A single dose of H9 HA VLP vaccine induced high levels of HI antibodies and lowered frequencies of virus isolation after the wild-type virus challenge. The addition of ISA70 adjuvant significantly increased the immunogenicity of H9 HA VLP vaccines. Furthermore, it allows differentiation of AIV-infected chickens from vaccinated chickens with an ELISA using nucleocapsid antigen, which offers a promising strategy to differentiate infected from vaccinated animals (DIVA). These results provide support for continued development of the VLP as an animal vaccine against influenza virus.

Keywords

Virus-like particle; Avian influenza; Vaccine; DIVA

1. Introduction

Low pathogenic avian influenza (LPAI) H9N2 viruses have been circulating in multiple avian species in Eurasia resulting in great economic losses due to declined egg production or moderate to high mortality [1–6]. Furthermore, the H9N2 LPAI virus is known to possess human-like receptor specificity [7] and transmissibility to mammalian species including humans, raising public concerns about increasing pandemic potential [8,9].

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H9N2 LPAI viruses have been endemic in domestic poultry farms in Korea since 1996 and have often cause slight to moderate mortality (5–30%) with apparent clinical signs that is characterized by depression, edema of head, cyanosis of comb and legs, drop in egg production [10]. Since then, the H9N2 viruses that belong to the Korea group have been prevalent in chickens and have continuously evolved through reassortment with isolates circulating in the wild bird and live bird market [5,11,12].

In order to control the LPAI outbreaks, since 2007, the Korean veterinary authority has permitted the use of inactivated oil-adjuvant H9N2 LPAI vaccine [13,14], but only a few inactivated commercial vaccines have been assessed in chicken. In addition, one limitation of the inactivated vaccines is that vaccinated birds cannot be differentiated serologically from naturally infected birds using the commonly available diagnostic tests. Therefore, several different strategies to differentiate infected from vaccinated animals (DIVA) have been proposed for avian influenza to overcome this limitation [15,16].

Influenza virus-like particles (VLPs) have been suggested and developed as a new generation of non-egg-based cell culture-derived vaccine candidates against influenza infection [17]. VLP vaccines containing influenza hemagglutinin (HA) and neuraminidase (NA) antigens are produced easily in insect or mammalian cells via the simultaneous expression of HA and NA along with a viral core protein, such as influenza matrix (M1) [18]. The protective mechanism of influenza VLP vaccine is known to be similar to that of the commercial influenza vaccines inducing neutralizing antibodies and hemagglutination inhibition (HI) activities [17]. Furthermore, VLP vaccine has strong immunogenicity and protective efficacy in pandemic and highly pathogenic influenza challenge models [19–22]. In previous studies, H5N3 VLP vaccine was applied to duck species and provided the possibility of reliable use of VLP vaccine in poultry species [23,24].

In the present study, we developed H9 influenza VLP vaccine and evaluated its immunogenicity and protective efficacy in specific-pathogen-free (SPF) chickens. Furthermore, we assessed VLP vaccines by using a companion nucleocapsid protein (NP) coated enzyme linked immunosorbent assay (ELISA) test, which may provide a useful method to discriminate between chickens infected naturally and chickens vaccinated only.

2. Materials and methods

2.1. Preparation of avian influenza H9 HA VLPs

Influenza H9 VLPs containing HA and M1 proteins were produced by following a procedure previously described [25]. Briefly, to generate the recombinant baculoviruses (rBVs) expressing influenza proteins, full length HA cDNA derived from influenza A/Chicken/Korea/01310/2001 (H9N2) virus and M1 were cloned into pFastBac (Invitrogen, Carlsbad, CA) and transferred into Bacmid BV DNA (rAcNPV) followed by transformation with DH10Bac cells (Invitrogen, Carlsbad, CA). The rBVs expressing HA and M1 proteins were generated by transfecting sf9 insect cells with Bacmid recombinant BV DNA, and plaque isolation from culture supernatants of transfected sf9 insect cells. To generate influenza H9 VLPs, sf9 insect cells (ATCC, CRL-1711) were co-infected with rBVs expressing H9 HA and M1 at a multiplication of infection of 3 and 1 respectively. Culture

media were collected and clarified by low speed centrifugation (2000 × *g*, 30 min, 4 °C) at 2 days post infection. Culture supernatants were concentrated and filtrated by Quixstand bench-top system (GE Healthcare, Waukesha, WI) using hollow fiber cartridge (300,000 Da of nominal molecular weight cut-off). Further purification was performed by 30 and 60% sucrose layer gradient ultracentrifugation (28,000 × *g*, for 60 min) following dialysis with HEPES buffered saline solution (HBS) and then H9 VLP solution was concentrated by Viva spin (Sartorius, Bohemia, NY) protein concentrator. The final protein concentration of H9 VLPs was quantified by a protein assay kit (Bio-rad, Irvine, CA) and biological activity was determined by a hemagglutination assay as previously described [26]. Units of hemagglutination activity are presented as a factor of dilution that prevents the precipitation of red blood cells.

2.2. Vaccine and viruses

Vaccines, with or without adjuvant, were prepared by emulsifying the escalating concentration (2 μg, 5 μg, 10 μg, and 20 μg) of VLP antigen solution with Montanide ISA70 (SEPPIC, France) in the ratio 30:70 (v/v). To evaluate vaccine efficacy, chickens were challenged intranasally with 10^6 EID_{50} of homologous H9N2 influenza A virus (A/Chicken/Korea/01310/2001).

2.3. Animals and experimental design

Sixty 6-week-old SPF chickens (10 chickens per group) were divided into six groups. Chickens were immunized with escalating dose of (2 μg, 5 μg, 10 μg, or 20 μg) VLP vaccines with adjuvant or 20 μg of VLP without adjuvant, via an intra-muscular route. Ten SPF chickens were vaccinated with commercial H9N2 whole-virus inactivated vaccine "Chicken-Vac" (DaeSung, Korea), containing the 2^{12}HAU concentration of inactivated allantoic fluid with mineral-oil adjuvant in the ratio 30:70 (v/v), according to the manufacturer's instruction (0.5 ml injection via intra-muscular route). As a non-vaccinated control group, another 10 SPF chickens were injected with emulsified solution of distilled water with ISA 70 in the same ratio with VLP vaccines.

At 3 weeks after a single dose of vaccination, under Animal Biosafety Level 2 enhanced conditions, chickens were challenged intranasally with the H9N2 homologous virus. To determine the replication of challenge influenza virus at day 5 post-challenge as previously described [14], trachea and cecal tonsil tissues were collected from each chicken and homogenized. 10% (w/v) of tissue homogenates were prepared in PBS containing 400 μg/ml gentamycin, then the suspensions were centrifuged and a clear liquid supernatant at the topper layer was collected. All specimens were inoculated into the allantoic cavity of 9–11-day old SPF chicken embryonated eggs. After 72 h of incubation, the eggs were chilled and allantoic fluids were harvested and tested for hemagglutination activity.

For AIV-infected sera, fifteen 6-week-old SPF chickens were inoculated intranasally with the same challenge virus, and serum samples were collected from AIV infected chickens at 2 weeks after challenge and used to determine antibodies in comparison with vaccine derived antibodies.

2.4. Serology

In order to determine the immunogenicity of VLP vaccines, serum samples were collected prior to vaccination and 3 weeks after the vaccination for HI test and ELISA. HI tests were performed as described in the OIE standard HI method using formalin-inactivated homologous antigen. AIV NP-specific antibody levels were measured using a commercially available multispecies competitive NP-ELISA kit (Bionote, Korea), which have been implemented to detect antibodies against AIV NP in different avian species, according to manufacturer‘s instruction.

2.5. Statistical analysis

Statistical analysis of differences between vaccinated groups and unvaccinated control group was performed using a Fisher’s exact test. ANOVA with Tukey–Kramer post-test was performed for statistical test of serum antibody titers. Statistical significance was designated for differences with p-values less than or equal to 0.05.

3. Results

3.1. Immune responses to vaccination with influenza H9 HA VLPs

Influenza H9 VLPs containing the HA protein derived from A/Chicken/Korea/01310/2001 (H9N2) virus were produced in insect cells by the rBV expression system. Purification procedures were performed by ultra-filtration and sucrose gradient ultra-centrifugation. Purified H9 HA VLPs were found to have approximately 8000 units of hemagglutination activity (HAU) at the protein concentration of 1.2 mg per milliliter. This result indicates that the HA protein incorporated into VLPs maintains a biologically active conformation in the receptor binding site. The native conformation in the receptor binding site in the influenza VLPs was recently shown to be important for maintaining the immunogenicity of HA VLPs and inducing protective immune responses [27].

To evaluate the immunogenicity of H9 HA VLP vaccines, groups of chickens were intramuscularly immunized and immune responses in sera were determined 3 weeks after a single dose of vaccination. As shown in Fig. 1, as low as 2 μg VLP vaccine induced significant levels of virus-specific antibodies that are equivalent to $2^{7.1}$ HI units. However, any detectable levels of antibody responses were not observed in the control group that received mock vaccination with ISA70 adjuvant. Levels of virus-specific antibody responses showed an increase up to 10 μg VLPs in a dose-dependent manner. Importantly, H9 HA VLP vaccines (5 or 10 μg dose) showed antibody responses that are comparable to the whole-viral vaccine control. Without ISA70 adjuvant, low antibody responses equivalent to $2^{4.3}$ HI units were induced even with 20 μg VLPs. Therefore, the inclusion of ISA70 adjuvant in the H9 VLP vaccination showed a significant immune enhancing effect on VLP vaccines by over 10 fold. These results suggest that influenza H9 HA VLP vaccines can induce virus-specific functional antibody responses.

3.2. Reduced viral shedding in chickens vaccinated with influenza H9 HA VLPs

The mock control group showed the highest frequency of virus detection in the trachea and cecal tonsil as 9 out of 10 chickens’ trachea and 8 out of 10 chickens’ tonsil were positive

for viral replication (Table 1). However, chickens vaccinated with 2 μg of VLPs with ISA70 adjuvant showed the virus detection in the trachea and cecal tonsil as 1 out of 10 chickens' trachea and 2 out of 10 chickens' tonsil were positive for viral replication. Chickens vaccinated with 5 μg of VLPs with ISA70 adjuvant showed the virus detection in the trachea and cecal tonsil as 2 out of 10 chickens' trachea and 2 out of 10 chickens' tonsil were positive for viral replication. Additionally, no virus was isolated from the tracheal or cecal tonsil tissues of chickens vaccinated with over 10 μg of VLP with ISA70 adjuvant or whole-virus inactivated vaccine. Moreover, vaccination with 20 μg VLP without adjuvant also significantly reduced viral shedding from cecal tonsil compared to the unvaccinated control group.

3.3. Differentiation of infected chickens from the vaccinated with a H9 HA VLP vaccine

As expected, all serum samples of chickens vaccinated with VLP vaccine ($n = 10$) were negative by the NP-cELISA [1 − (S/N) ≤0.5] (Fig. 2). In contrast, the sera of chickens vaccinated with whole virus inactivated vaccine ($n = 10$) were all positive for NP immune responses [1 − (S/N) ≥0.5]. We tested AIV-infected sera ($n = 15$) using the NP-cELISA and were all positive for NP immune responses [1 − (S/N) ≥0.5]. Therefore, VLP vaccine and the companion DIVA test, NP-ELISA, could allow the utilization of the DIVA strategy.

4. Discussion

An advantage of a VLP vaccine approach is a virus mimicking particle with multiple viral antigens and epitopes that stimulate a diverse set of immune responses [28,29]. Influenza VLP vaccines have been shown to be very immunogenic and safe in various animal models [17,21,23,28,29]. In our previous studies, antibody responses to M1 were found to be minimal even after prime-boost of two immunizations with influenza M1 core VLPs [30]. Also, mice immunized with M1 VLPs alone were not protected [31], indicating that immune responses to M1 do not play a significant role in inducing protective immunity. In the present study, we demonstrated that H9 HA VLP vaccine elicited high levels of antibody responses to the virus as shown by hemagglutination inhibition activity and also lessened the number of chickens with viral shedding from respiratory and gastrointestinal tract. To our knowledge, this study is the first work intended to generate and evaluate poultry H9 HA VLP vaccines in chickens, and to provide a promising DIVA strategy. Recently, Prel et al. introduced VLP vaccine by using a triple baculovirus recombinant coding for HA, NA, and M1 proteins from H5N3 AIV and showed protection conferred by these VLPs in Muscovy duck [23]. Here, we evaluated H9 HA VLP vaccine in chickens which are known to be more susceptible species to AIV than duck species. Our results should further support for the possibility of reliably immunizing VLP vaccines in poultry species.

The use of VLP vaccines would help in limiting the safety concerns compared to live-attenuated and inactivated whole-virus vaccines. For example, a strong possibility was suggested that the introduction of highly pathogenic H5N2 of American lineage into Japan was due to a transfer of live virus from improperly inactivated vaccine or from contaminated vaccine [32]. Because of the noninfectious nature of VLPs and their lack of viral genomic material, the VLP vaccine represents a desirable safety feature as a vaccine candidate [17].

In addition, during AIV circulation in poultry population, HA and NA surface antigens undergo progressive amino acid substitutions which could cause evasion of the previously acquired immunity by vaccination. Because VLP vaccine approach allows rapid vaccine production, it could provide easy updates of the mutated or newly emerged strains.

ISA 70 has already been demonstrated as a safe and effective adjuvant in numerous poultry disease models with enhancing antibody and/or cell-mediated immune responses [33]. Enhanced immune reactions induced by ISA70 adjuvant were characterized by proliferation of macrophages, epithelial cells, and fibroblasts around the small cysts in the muscle [34]. Based on our results, 10 μg of VLP with the ISA70 adjuvant provide significantly higher mean antibody titers than the same or high dose of VLP vaccine without adjuvant. Furthermore, a lower dose of VLP vaccines (2 μg) with an ISA70 adjuvant provided significantly enhanced antibody titers than a high dose of VLPs (20 μg) in the absence of adjuvant. The combined effects of vaccine doses and increased HI titers observed suggest that the immune enhancing effects on VLP vaccine antigens by the addition of ISA70 adjuvant are significant. These results suggest that influenza VLPs can confer effective protection in chickens and that inclusion of ISA70 adjuvant significantly enhances the protective immunity by influenza VLPs. Therefore, the addition of ISA70 adjuvant to the VLP vaccination could provide enhanced protective immune response against AIV even with vaccination with low doses of VLP vaccines.

Vaccination against AIV, an effective control measure for the eradication of LPAI or HPAI, may result in issues related to surveillance programs and international trades of poultry and poultry products [15,35]. Therefore, several different DIVA strategies have been suggested using appropriate vaccines and companion serologic tests for discriminating between naturally infected and vaccinated-only animals [16,35,36]. Particularly, many different types of subunit vaccines, including virus vectored vaccines and vaccines using proteins expressed in culture systems, have been shown to provide protection against AIV and allow DIVA strategy [15,23,37]. The present study provides evidence that H9 HA VLP vaccination can be a promising DIVA strategy by companion NP-coated ELISA tests.

Vaccine cost has been thought to be an important issue that influences the feasibility of recombinant subunit vaccines including VLP for poultry. Based on our results, VLP vaccine has potential advantages over egg-based vaccine, particularly for providing significant protection even with low doses of VLP vaccines compared to unvaccinated animals. In addition, purified influenza VLP vaccines have very high-yield and hemagglutination activity (2^{13} HAU), which are expected to be advantageous for manufacturing not only cost-effective vaccines but also diagnostic antigens of HPAI. Based on our analysis, the cost of VLP production would be equal to or less than the conventional inactivated whole virus vaccine. Further, the cost may be reduced if the purification procedures are improved and minimized in the future. In addition, during HPAI outbreaks, vaccine manufacturers using VLP vaccine technology could produce vaccines without the need for expensive high-bio-safety-requiring biocontainment facilities.

In conclusion, H9 HA VLP vaccine developed in this study was safe, immunogenic, and vaccination with H9 VLPs lessened the number of chickens with viral shedding from

respiratory and gastrointestinal tract. Furthermore, we could differentiate VLP vaccinated chickens from AIV infected chickens, supporting that VLP vaccine allows an effective DIVA strategy. These results provide support for continued development of the VLP as a poultry vaccine against AIV.

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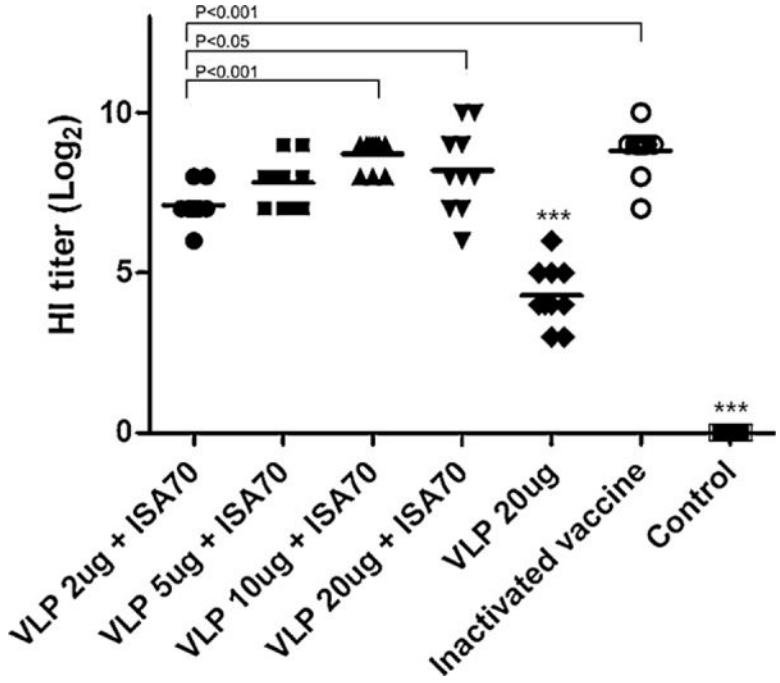

Fig. 1.

Mean serum HI titers (log 2) induced in SPF chickens after a single dose of vaccination. Sixty 6-week-old SPF chickens (10 chickens per group) were intramuscularly immunized with influenza H9 HA VLPs (2, 5, 10, or 20 μg total protein) or inactivated whole viral vaccine (0.5 ml dose) commercially manufactured HI titers against the homologous antigen [A/chicken/Korea/310/2001(H9N2)] were determined at 3 weeks after vaccination. ISA70: an adjuvant; control: ISA70 adjuvant only without vaccine. ***$p < 0.001$ by ANOVA with Tukey–Kramer post-test compared to other groups.

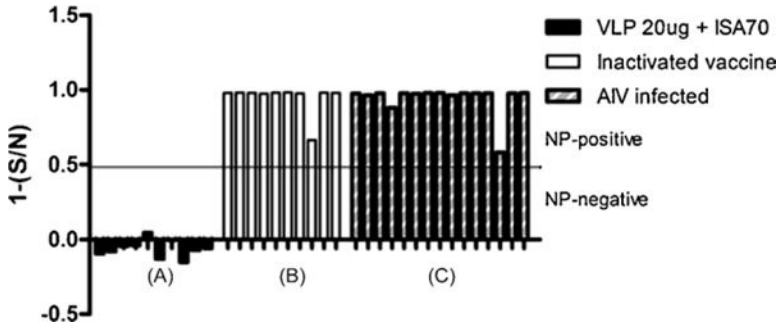

Fig. 2.

NP serum antibody levels induced by VLP vaccine, inactivated whole virus vaccine, or AIV infection by NP ELISA. SPF chickens were immunized with 20 μg of VLP antigen with ISA70 adjuvant (A) or whole virus inactivated vaccine (B). Antibody levels were determined by NP-cELISA at 3 weeks post vaccination. AIV infected (C) sera were collected at 2 weeks post H9N2 virus challenge. Each bar represents the NP specific antibody value of each chicken.

Table 1

Vaccination with H9 HA VLP vaccine lowers frequencies of virus isolation from SPF chickens challenged with wild type H9N2 AIV.[a]

Group	Virus isolation[b]/total	
	Trachea	Cecal tonsil
VLP 2 μg + ISA70	1/10 **	2/10 *
VLP 5 μg + ISA70	2/10 **	2/10 *
VLP 10 μg + ISA70	0/10 ***	0/10 ***
VLP 20 μg + ISA70	0/10 ***	0/10 ***
VLP 20 μg only	7/10	3/10 *
Commercial vaccine[c]	0/10 ***	0/10 ***
Challenge control	9/10	8/10

[a] Groups of SPF chickens were challenged intranasally with 10^6 EID_{50} of homologous H9N2 virus at 3 weeks post vaccination.

[b] Virus re-isolation was done by chicken embryo inoculation at 5 days post challenge.

[c] Commercial vaccine contains identical AIV strains and mineral-oil adjuvant.

* $p < 0.05$ by Fisher's exact test, compared to challenge control group.

** $p < 0.01$ by Fisher's exact test, compared to challenge control group.

*** $p < 0.001$ by Fisher's exact test, compared to challenge control group.