

## Research Article

# One-Day Old Chicks Vaccinated with Inactivated H5 Antigen are Protected against Highly-Pathogenic Avian Influenza Virus

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H5-subtype highly pathogenic avian influenza viruses cause considerable economic losses to the poultry industries in many countries, including China, South Korea, and Vietnam. Vaccination is the most important strategy to control highly pathogenic avian influenza viruses in poultry. We studied the efficacy of inactivated H5 antigen in one-day-old chicks. One-day-old chicks were immunized in the pectoral muscles with a single dose (1.0 µg) of inactivated H5 antigen derived from the highly pathogenic H5N8 avian influenza virus. They were then intranasally challenged at 3 weeks and 16 weeks-post-vaccination by the highly pathogenic H5N8 or H5N6 avian influenza viruses. The protective antibody and immunity against homologous H5N8 virus was effective in the vaccinated chickens until 16 weeks-post-vaccination. Vaccinated chickens challenged with H5N8 virus did not show any clinical signs of infection, nor did they shed virus through the trachea or cloaca. Meanwhile, vaccinated chickens challenged with the heterologous H5N6 virus did not survive, and shed lots of viruses from the trachea and cloaca. Vaccination of one-day-old chicks with inactivated H5 antigens is an effective preventive measure to minimize the economic burden of the highly pathogenic H5 avian influenza viruses on of the poultry industry.

**Keywords:** Highly pathogenic avian influenza virus; Vaccine; H5N8; H5N6**Introduction**

Influenza viruses belong to family *Orthomyxoviridae*, and are divided into four types: A, B, C and D, based on nucleoprotein (N) and matrix (M) genes [1,2]. Influenza A viruses can infect a broad range of hosts, including humans, pigs, horses, dogs, cats, and whales [3-9]. Avian influenza viruses belong to type A, and circulate in water birds [7]. Most avian influenza viruses circulating in water birds exhibit low pathogenicity. Highly-pathogenic (HP) H5 avian influenza viruses which infect poultry in many countries, including China, South Korea, and Vietnam, were originally derived from A/goose/Guangdong/1/1996 (H5N1)-like HP avian influenza virus [10-12]. In South Korea, H5N1 HP avian influenza viruses have infected poultry since 2003. After 2014, H5N8 and H5N6 HP avian influenza viruses became prevalent in poultry, and caused huge economic losses to the poultry industry [10,13-16].

Avian influenza viruses possess negative-sense, single-stranded, eight-segmented genomes containing polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic (PA), hemagglutinin (HA), and nucleoprotein (NP), neuraminidase (NA), matrix (M), and non-structural (NS) genes that encode 10 proteins: PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, and NS2 [7]. Of these 10 viral proteins, HA is regarded as a major determinant of pathogenicity in poultry, containing polybasic amino acids such arginine (R) and lysine (K) at the cleavage site, which are responsible for systemic infections in poultry [7,14].

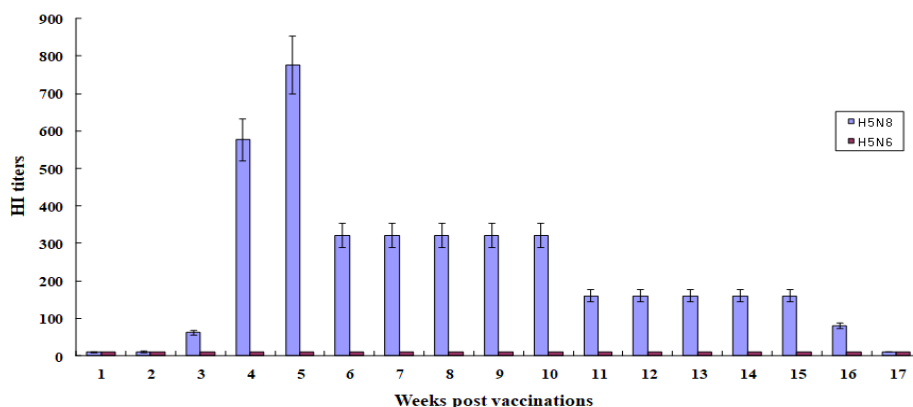
Vaccination is one of tools used to control HP avian influenza viruses in poultry. Several vaccine strategies are being studied. Virus-like particle-based vaccine protects mule and pekin duck from infections of homologous H5N8 HP avian influenza virus [17]. The combined H5ND inactivated vaccine including the inactivated reassortant H5N1 avian influenza virus and Newcastle disease virus, can protect chickens from HP H5N1 avian influenza virus and virulent Newcastle disease virus [18]. The recombinant turkey herpesvirus expressing H5 antigen protects chickens from HP H5N8 avian influenza virus [19].

Here, we studied the vaccine efficacy in one-day-old chicks immunized with a vaccine consisting of inactivated H5 antigen of HP H5N8 avian influenza virus, and tested how long the protective immunity lasted.

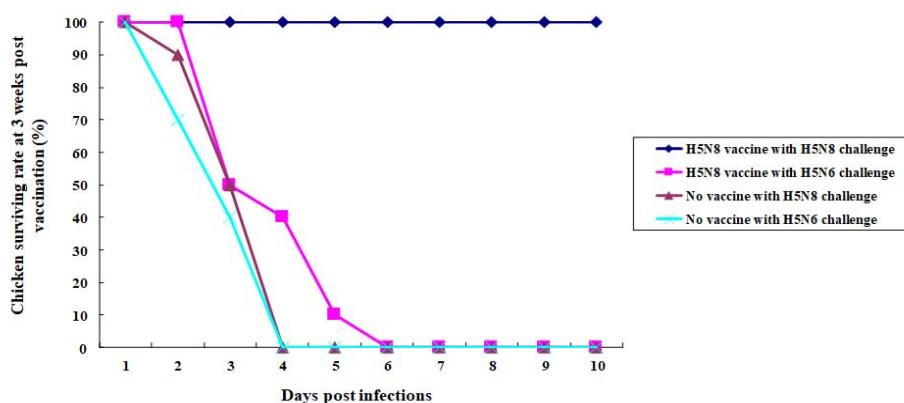
**Material and Methods****Viruses and animals**

Highly Pathogenic (HP) avian influenza viruses, A/Waterfowl/S005/Korea/2014 (H5N8) (clade 2.3.4.4) and A/Waterfowl/Korea/S57/2016 (clade 2.3.4.4.) were amplified by inoculation into 10-day-old fertilized eggs. This work with HP H5 viruses were performed in an animal BSL-3 facility approved by the Korean government.

Chicks (White Leghorn) were hatched from fertilized eggs purchased from the local farm in Korea. One-day-old chicks were used to inoculate H5 inactivated antigen.



**Figure 1:** Antibody responses in one-day-old chicks. One-day old chicks ( $n=10$  per group) were immunized with 300  $\mu$ l of one-dose 1.0  $\mu$ g of inactivated H5RG antigens with oil adjuvant. The immunized chickens were bred in one-week intervals until 17 weeks p.v. Antibody titers in sera were measured by HI assay against A/Waterfowl/Korea/S57/2016 (H5N6) or A/Waterfowl/S005/Korea/2014 (H5N8). Data are the mean HI titers  $\pm$  standard deviations.



**Figure 2:** Efficacy of inactivated H5 antigens in 3-week-old chickens. The immunized chickens ( $n=10$  per group) were i.n. infected with 100  $\mu$ l of  $10^5$  EID<sub>50</sub>/ml of A/Waterfowl/Korea/S57/2016 (H5N6) or A/Waterfowl/S005/Korea/2014 (H5N8) on 3 weeks p.v. The surviving rates were recorded.

**Animal rights statement:** Chungnam National University (CNU) Internal Animal Use Committee approved the protocol for the chicken vaccine study and collection of clinical chicken samples.

The authors declare that there is no conflict of interests.

### Preparation of H5 vaccine antigen

Vaccine virus, H5N8-RG-CNUK4-2014 (H5RG), which was constructed using an A/PR/8/34 (H1N1) backbone and H5 of A/Waterfowl/S005/Korea/2014 (H5N8) using reverse genetics in our laboratory [20] was used to prepare the vaccine antigen.

Allantoic fluid was harvested from 10-day-old embryonated eggs which were inoculated with H5RG virus. The egg fluid was concentrated to 1/10 volume by an Amicon concentrator. The concentrated fluid was purified by a 20% to 75% continuous sucrose gradient by centrifugation at 26,000 rpm for 2 h. The purified viruses were inactivated by treatment with 0.01% formalin at 4 °C for over 14 h. The protein concentration of the purified H5RG vaccine was measured by the Bradford method (Bio-Rad) with a bovine serum albumin (BSA) standard curve.

### Vaccination and infection of one-day-old chicks

One-day old chicks ( $n=10$  per group) were injected into pectoral

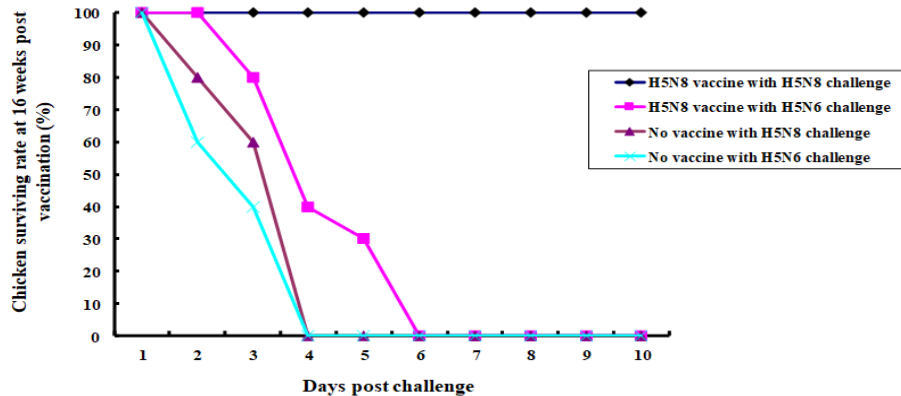
muscles with 300  $\mu$ l of 1.0  $\mu$ g of inactivated H5RG antigens with oil adjuvant (70% oil and 30% antigens in PBS) (Montanide IMS 1313 NPR, SEPPIC Co., France). Immunized chickens were intranasally infected with 100  $\mu$ l of  $10^5$  EID<sub>50</sub>/ml of A/Waterfowl/Korea/S57/2016 (clade 2.3.4.4) or A/Waterfowl/S005/Korea/2014 (H5N8) (clade 2.3.4.4) at 3 weeks and 16 weeks post-vaccination.

### Measurement of antibody titers

Hem Agglutination-Inhibition (HI) assay was performed to determine antibody titers in chickens. The collected sera from the vaccinated chickens were treated with Receptor-Destroying Enzyme (RDE) (DENKA SEIKEN, Tokyo, Japan) before the sera were serially 2-fold diluted in PBS (pH 7.4) in 96-well plates (V bottom). Twenty five microliters (eight HA units) of A/Waterfowl/Korea/S57/2016 (clade, 2.3.4.4) or A/Waterfowl/S005/Korea/2014 (H5N8) (clade, 2.3.4.4) virus was added to the diluted sera, and was incubated for 15 min at room temperature. Then, 50  $\mu$ l of 0.5% turkey red blood cells in PBS (pH 7.4) was added and was incubated at room temperature for 40 min. HI titer was read as reciprocal dilutions which inhibit hem agglutination.

### Determination of virus titer

Swabs were performed of trachea and cloacae in the surviving



**Figure 3:** Efficacy of inactivated H5 antigens in 16-week-old chickens. The immunized chickens (n=10 per group) were i.n. infected with 100  $\mu$ l of  $10^6$  EID<sub>50</sub>/ml of A/Waterfowl/Korea/S57/2016 (H5N6) or A/Waterfowl/S005/Korea/2014 (H5N8) on 16 weeks p.v. Surviving rates were recorded.

chickens infected with HP H5N6 or H5N8 avian influenza virus. Tracheal and cloacal swab samples were suspended in 0.5 ml and 1 ml of PBS (pH 7.4) supplemented with Antibiotic-Antimitotic solution (SIGMA, St. Louis, MO, USA), respectively, and were serially diluted (10 fold) in PBS (pH 7.4). Each sample was inoculated into four 10-day-old fertilized eggs. Virus presence in the inoculated eggs was determined by hem agglutination assay using 0.5% turkey red blood cells in PBS (pH 7.4). Viral titers were expressed by  $\log_{10}$  egg infectious dose 50/ml ( $\log_{10}$  EID<sub>50</sub>/ml). The detection limit was 1.0  $\log_{10}$  EID<sub>50</sub>/ml.

Chicken lung tissues (1g) from vaccinated 16-week-old chickens challenged with HP H5N6 or H5N8 avian influenza virus on day 3 p.c. were homogenized and suspended in PBS (pH 7.4). Virus titer was measured by  $\log_{10}$  EID<sub>50</sub>/ml.

### Histopathology of chicken lung

Lung tissues were collected from vaccinated 16-week-old chickens (n=3 per group) challenged with HP H5N6 or H5N8 avian influenza virus on 3 days p.c., were fixed in 10% neutral-buffered formalin, and were embedded in paraffin. Thin sections (5  $\mu$ m) were made and were stained with H&E. The stained tissues were observed under an Olympus DP70 microscope (Olympus Corporation, Tokyo, Japan).

### Statistical analysis

The statistical significance was analyzed by the Mann-Whitney U test. A P value of less than 0.05 (P<0.05) was considered significant.

## Results

### Antibody response

We vaccinated one-day-old chicks (n=10 per group) with the inactivated H5 antigen derived from HP H5N8 avian influenza virus to determine the possibility on whether early-age vaccination could protect chicks from HP avian influenza virus. One-day-old chicks were immunized with one-dose (1.0  $\mu$ g) of inactivated H5 antigen with oil adjuvant. Sera were collected from 1 to 17 weeks post-vaccination (p.v.). Antibody titers against homologous H5N8 and heterologous H5N6 avian influenza viruses were measured by HI assays (Figure 1). Specific antibody titers against homologous H5N8 avian influenza virus were detected from 3 weeks p.v. and lasted until 16 weeks p.v., while no specific antibody titers were detected against heterologous H5N6 avian influenza virus. The mean HI titers against

homologous H5N8 avian influenza virus at 3 weeks and 16 weeks p.v. were 61 and 80, respectively.

### Protective efficacy in 3-week-old chicks

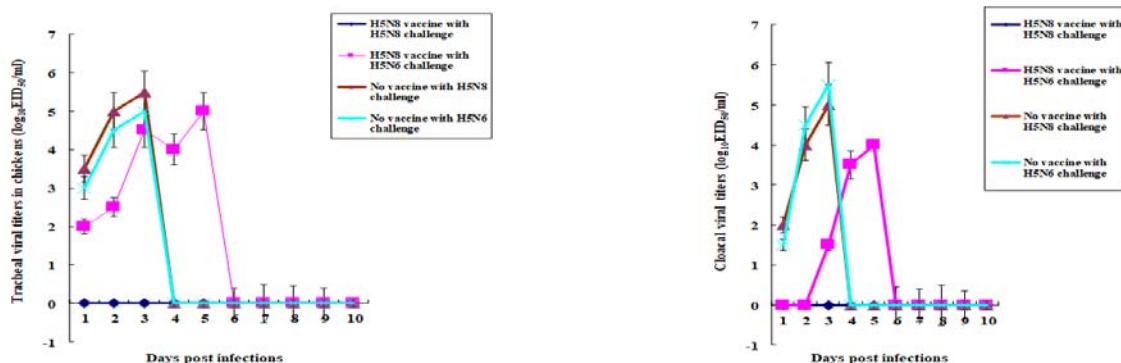
We intranasal challenged the immunized chicks (n=10 per group) at 3 weeks p.v. using homologous HP H5N8 or heterologous H5N6 avian influenza viruses (Figure 2). All vaccinated chicks challenged with homologous HP H5N8 avian influenza virus survived without apparent clinical signs, but all vaccinated chicks challenged with heterologous HP H5N6 avian influenza virus died until day 6 post-challenge (p.c.). All unvaccinated chicks challenged with either homologous HP H5N8 or heterologous HP H5N6 avian influenza virus died until day 4 p.c.

### Protective efficacy in 16-week-old chickens

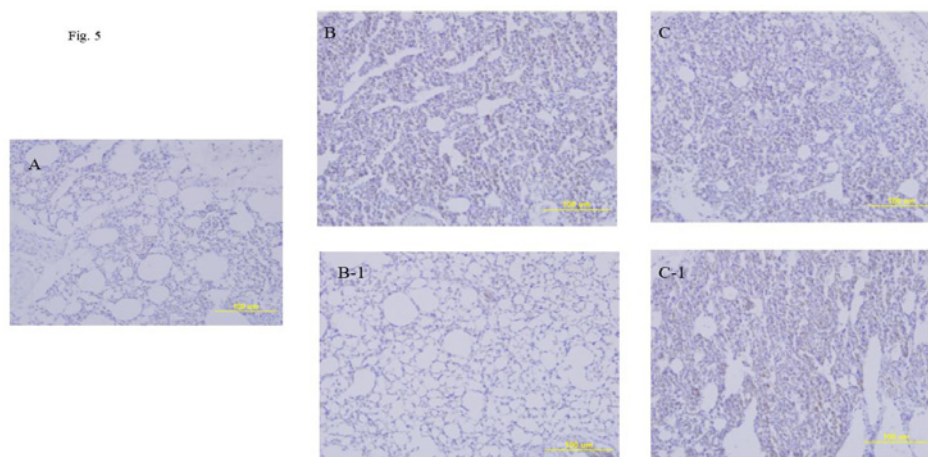
We were interested in finding out the protective duration provided by the vaccination against HP avian influenza viruses in chicks by one dose at one day of age (Figure 3). One-day-old chicks (n=10 per group) were immunized with 1.0  $\mu$ g of inactivated H5 antigen with oil adjuvant, and at 16 weeks p.v., chickens were challenged with homologous HP H5N8 or heterologous HP H5N6 avian influenza virus. All vaccinated chickens challenged with homologous HP H5N8 avian influenza virus survived, while all vaccinated chickens challenged with heterologous HP H5N6 avian influenza virus died until day 6 p.c. All unvaccinated chickens challenged with either homologous HP H5N8 or heterologous H5N6 avian influenza virus died until day 4 p.c.

### Virus titers in 16-week-old chickens

Chickens challenged at 16 weeks p.v. with homologous HP H5N8 or H5N6 avian virus were swabbed in the trachea and cloaca until day 10 p.c. Virus titers in the swabbed samples were measured by  $\log_{10}$  EID<sub>50</sub>/ml (Figure 4). In tracheas, no viruses were detected in vaccinated chickens challenged with homologous HP H5N8 avian influenza virus. In contrast, the range of detected virus titers in vaccinated chickens challenged with heterologous HP H5N6 avian virus was from 2.0 to 5.0 EID<sub>50</sub>/ml (Figure 4A). The range of virus titers detected in the unvaccinated chickens challenged with either homologous HP H5N8 or heterologous H5N6 avian influenza virus was 3.0 to 5.5 EID<sub>50</sub>/ml (Figure 4A). In cloacae, the virus was not detected in the vaccinated chickens challenged with homologous



**Figure 4:** Viral titers in 16-week-old infected chickens. Swabs were performed in tracheas and cloacae in PBS in the surviving chickens infected with HP H5N6 or H5N8 viruses in the experiment in Figure. 3. Viral titers were measured by  $\log_{10}EID_{50}/ml$ . Data are the mean viral titers  $\pm$  standard deviations. A, Tracheal swabs; B, Cloacal swabs.



**Figure 5:** Lung histopathology in the infected 16-week-old chickens. The immunized chickens were i.n. infected with  $100 \mu l$  of  $10^6 EID_{50}/ml$  of A/Waterfowl/Korea/S57/2016 (H5N6) or A/Waterfowl/S005/Korea/2014 (H5N8) on 16 weeks p.v. Chickens were euthanized on 3 days after infections prior to the collections of lung tissues. Lung tissues were stained with H&E.

A, Uninfected lung tissue of chicken; B, Lung tissue of unvaccinated infected chicken with H5N8 virus; B-1, Lung tissue of vaccinated infected chicken with H5N8 virus; C, Lung tissue of unvaccinated infected chicken with H5N6 virus; C-1, Lung tissue of vaccinated infected chicken with H5N6 virus.

HP H5N8 avian influenza virus, while the range of virus titers in the vaccinated chickens with heterologous HP H5N6 avian influenza virus was 1.5 to 4.0  $EID_{50}/ml$  (Figure 4B). The range of virus titers in the unvaccinated chickens challenged with either homologous HP H5N8 or heterologous H5N6 avian influenza virus was 1.5 to 5.5  $EID_{50}/ml$  (Figure 4B).

### Lung histopathology

Lung tissues were collected from 16-week-old chickens challenged with either homologous HP H5N8 or heterologous H5N6 avian influenza virus on day 3 p.c., and these were stained with hematoxylin and eosin (H&E) (Figure 5). The lung tissue of the vaccinated chicken which was challenged with homologous HP H5N8 avian influenza virus did not have pneumonia (Figure 5B-1), while that of vaccinated chickens challenged with heterologous HP H5N6 avian influenza virus showed severe interstitial pneumonia with accumulation of inflammatory cells (Figure 5C-1). The lung tissues of unvaccinated chickens challenged with either homologous HP H5N8 or H5N6 avian influenza virus showed the severe interstitial pneumonia (Figure 5B, 5C). Lung tissue of uninfected chicken did not show any

sign of pneumonia (Figure 5A).

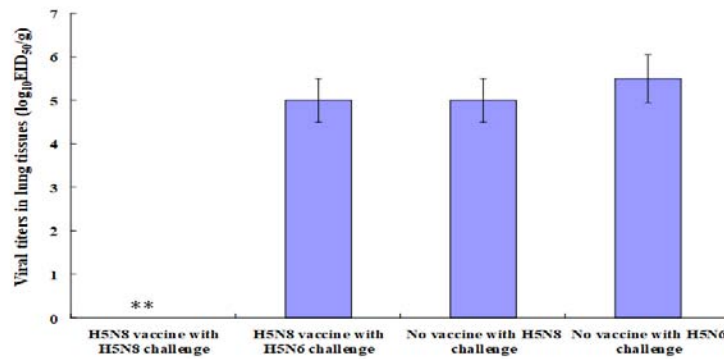
### Virus titers in lung tissues in vaccinated 16-week-old chickens

Lung tissues from vaccinated chickens ( $n=3$  per group) challenged with either homologous HP H5N8 or Heterologous HP H5N6 avian influenza virus on 16 weeks p.v. were collected on day 3 p.c., and were homogenized in phosphate-buffered saline (PBS) (pH 7.4). Virus titers in the homogenized lung tissues were measured by  $\log_{10}EID_{50}/ml$  (Figure 6). Virus was not detected in lung tissues of the vaccinated chickens challenged with homologous HP H5N8 avian influenza virus, while the virus titer in the lung tissues of vaccinated chickens challenged with heterologous HP H5N6 avian influenza virus was 5.0  $EID_{50}/g$ . The mean virus titer in the lung tissues of unvaccinated chickens challenged with homologous HP H5N8 or Heterologous H5N6 avian influenza virus was 5.0 and 5.5  $EID_{50}/g$ , respectively.

### Discussion

HP avian influenza viruses cause tremendous economic losses to the poultry industry around the world. Vaccination is the most





**Figure 6:** Viral titers of lung tissues in the infected 16-week-old chickens. Viral titers in the lung tissues from Figure. 5 were measured by log<sub>10</sub>EID<sub>50</sub>/g. Data were the mean viral titers (n=3 per group) ± standard deviations. Statistical analysis was performed between the mean viral titer in the lungs of vaccinated chickens challenged with homologous HP H5N8 avian influenza virus and that in the lungs of vaccinated chickens challenged with heterologous HP H5N6 avian influenza virus.

\*\*P<0.001

important means to protect poultry from infections of HP avian influenza viruses. We studied the efficacy of a one-dose vaccine consisting of inactivated H5 antigen in one-day-old chicks against HP H5N8 and H5N6 avian influenza viruses. Protective immunity was conferred against homologous HP H5N8 avian influenza virus.

Our study showed that the protective antibody titers and protective immunity lasted until 16 weeks p.v. in one-day-old chicks immunized with one-dose (1.0 µg) of inactivated H5 antigen. Previous studies showed that the protective antibody lasted only 12 weeks p.v. in six-week-old chickens immunized with one-dose (5.0 µg) of inactivated H5N1 antigen [21]. In a study of short-and long-term protective efficacy against H5N2 HP avian influenza virus, Santos et al [22] showed that two-day-old turkeys primed with the recombinant α-replicon-expressing HA gene of A/Gyrfalcon/Washington/41088-6/2014 (H5N2) and boosted with oil-adjuvanted inactivated H5N2 antigen of A/Gyrfalcon/Washington/41088-6/2014 (H5N2), were protected against HP H5N2 avian influenza virus on 16 weeks p.v.

Protective immunity was mainly conferred against homologous HP H5N8 avian influenza virus in one-day-old chicks immunized with inactivated H5 antigen with oil adjuvant. In the study with Herpesvirus turkey (HVT) vector-based live recombinant expressing the H5 gene of a clade 2.2 HP H5N1 avian influenza virus [HVT-AI (H5)], when one-day-old broilers and layers were immunized with HVT-AI (H5) and challenged with heterologous 2017 Hungarian HP H5N8 avian influenza virus, they were protected and exhibited reduced shedding of viruses [19]. The lack of protective immunity against heterologous HP avian influenza virus in our study may be due to the lack of cellular immunity inductions by inactivated H5 antigen.

We showed that inactivated H5 antigen could provide the protective immunity to chickens against HP H5 avian influenza virus. Previous studies also showed that the inactivated vaccine could be efficient at protecting chickens from lethal infections of HP avian influenza viruses. Peng et al. [23] showed that inactivated PR8-H7/H5 vaccine containing HA proteins of A/chicken/Guangdong/RZ/2017 (H7N9) and A/chicken/Fujian/5/2016 (H5N6) considerably reduced viral shedding in the infected chickens, and protected chickens

from the lethal infections of H5 and H7N9 avian influenza viruses. The study with oil-emulsion-inactivated H5 avian influenza vaccine protected broiler chickens and ducks from HP H5N1 avian influenza viruses [24].

## Conclusion

Our results suggest that vaccination of one-day-old chicks with the inactivated H5 antigens should be applied to prevent chickens from the infections of HP avian influenza viruses.

## Acknowledgments

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