

Live attenuated H5N1 vaccine with H9N2 internal genes protects chickens from infections by both Highly Pathogenic H5N1 and H9N2 Influenza Viruses

Nguyen Tai Nang,^{a,b} Byung Min Song,^{a,b} Young Myong Kang,^{a,b} Heui Man Kim,^{a,b} Hyun Soo Kim^c and Sang Heui Seo^{a,b}

^aLaboratory of Influenza Research, ^bInstitute for Influenza Virus, ^cLaboratory of Public Health, College of Veterinary Medicine, Chungnam National University, Yuseong Gu, Daejeon, Korea.

Correspondence: Sang Heui Seo, Laboratory of Influenza Research, College of Veterinary Medicine, Institute for Influenza virus, Chungnam National University, 220 Gung Dong, Yuseong Gu, 305-764 Daejeon, Korea. E-mail: seos@cnu.ac.kr

Accepted 3 March 2012. Published Online 9 April 2012.

Background The highly pathogenic H5N1 and H9N2 influenza viruses are endemic in many countries around the world and have caused considerable economic loss to the poultry industry.

Objectives We aimed to study whether a live attenuated H5N1 vaccine comprising internal genes from a cold-adapted H9N2 influenza virus could protect chickens from infection by both H5N1 and H9N2 viruses.

Methods We developed a cold-adapted H9N2 vaccine virus expressing hemagglutinin and neuraminidase derived from the highly pathogenic H5N1 influenza virus using reverse genetics.

Results and Conclusions Chickens immunized with the vaccine were protected from lethal infections with homologous and heterologous H5N1 or H9N2 influenza viruses. Specific antibody against H5N1 virus was detected up to 11 weeks after vaccination (the endpoint of this study). In vaccinated chickens, IgA and IgG antibody subtypes were induced in lung and intestinal tissue, and CD4+ and CD8+ T lymphocytes expressing interferon-gamma were induced in the splenocytes. These data suggest that a live attenuated H5N1 vaccine with cold-adapted H9N2 internal genes can protect chickens from infection with H5N1 and H9N2 influenza viruses by eliciting humoral and cellular immunity.

Keywords H5N1, H9N2, influenza virus, live vaccine.

Please cite this paper as: Nang et al. (2013) Live attenuated H5N1 vaccine with H9N2 internal genes protects chickens from infections by both Highly Pathogenic H5N1 and H9N2 Influenza Viruses. *Influenza and Other Respiratory Viruses* 7(2) 120–131.

Introduction

Influenza A virus is a genus of the *Orthomyxoviridae* family and contains 8 RNA segmented genomes: PB2, PB1, PA, HA, NP, NA, M, and NS.¹ The hemagglutinin (HA) protein is a major determinant of disease in humans and animals. There are 16 HA and 9 neuraminidase (NA) subtypes circulating in aquatic birds,^{1,2} and H5N1, H7N7, and H9N2 subtypes are established in poultry, causing significant economic losses to the poultry industry.²

H9N2 influenza virus was first detected in turkeys in the United States in 1966³ and subsequently disseminated globally. The H9N2 virus is widespread in many countries, including China, Egypt, India, Pakistan, Israel, South Korea, and Bangladesh.^{4–11} In South Korea, infection of chickens by H9N2 was first reported in 1996,¹² and H9N2 has since become endemic in Korean chickens.^{9,12,13}

Three lineages of H9N2 virus (Chicken/Beijing/1/94, Quail/Hong Kong/G1/97, and Duck/Hong Kong/Y439/

97) are currently circulating in poultry worldwide.^{14–16} The H9N2 virus represents an appreciable cause of economic loss for the poultry industry, resulting in a 20–60% mortality rate of chickens and reducing egg production in laying flocks.^{6,9} Furthermore, transmission of H9N2 from infected poultry to humans was reported in Hong Kong in 2003 and 2009,^{17,18} raising the possibility of a pandemic infection if the virus was able to successfully cross the poultry–human species barrier.

Highly pathogenic (HP) H5N1 influenza virus was first detected in sick geese in the Guangdong Province of China in 1996.¹⁹ However, in 1997, humans and poultry in Hong Kong were found to be infected with HP H5N1 virus, in which the HA and NA genomes were derived from A/goose/Guangdong/1/96 (H5N1), and the PB2, PB1, PA, NP, M, and NS internal genes were derived from either H6N1 or H9N2 viruses.^{20,21} Since 2003, the HP H5N1 virus has spread to poultry in many countries, including Indonesia, Japan, South Korea, Thailand, and Vietnam.^{22–26}

In May 2005, unprecedented outbreaks of HP H5N1 virus in migratory birds occurred in Qinghai Lake in China.²⁷ Moreover, HP H5N1 virus has continued to infect humans, and 329 human deaths from a total of 562 infections have been reported since 2003 http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_06_22/en/index.html.

Both H9N2 and H5N1 viruses cause considerable economic loss to the poultry industry and have the potential to infect humans and cause pandemic infections. Thus, development of an effective vaccine is necessary to protect poultry and minimize human infections. Several approaches, including live attenuation and inactivation, have been carried out to develop a vaccine capable of protecting poultry from infection by H5N1 or H9N2 viruses.^{28–43} An attractive approach for the vaccination of poultry would be a live attenuated vaccine, which can be inoculated on a large scale, and induces IgA antibodies that are important for mucosal immunity.

The aim of this study was to develop a live attenuated influenza vaccine—based on cold-adapted H9N2 internal genes and HP H5N1-derived HA and NA genes—to protect chickens from infections by both H5N1 and H9N2 viruses. We also studied whether the vaccine could induce specific cellular immunity, such as CD4+ and CD8+ T lymphocytes, in immunized chickens.

Methods

Viruses

Wild-type H9N2 (A/Chicken/Korea/S21/04) and cold-adapted H9N2 (A/Chicken/Korea/S1/03)⁴¹ influenza viruses were propagated in 10-day-old chicken eggs. HP H5N1 influenza viruses, A/Vietnam/1203/04 (clade 1) and A/Vietnam/HN31244/2007 (clade 2.3.4), were kindly provided by the World Health Organization Collaborating Center for Influenza and United States Centers for Disease Control and Prevention and grown in 10-day-old chicken eggs. All experiments involving HP H5N1 viruses were performed in a Korean government-approved BSL-3 facility. *In vivo* experiments were approved by an internal animal ethics committee at Chungnam National University.

Generation of an attenuated H5N1 vaccine virus based on cold-adapted H9N2 internal genes

RNA was extracted from allantoic fluid containing the CNUK-1 attenuated H5N1 vaccine strain, in which the polybasic cleavage site was deleted,³⁶ or from the cold-adapted H9N2 strain (A/Chicken/Korea/S1/03)⁴¹ using the RNeasy kit (Qiagen, Valencia, CA, USA). cDNA was synthesized with a uni12 primer (5'-AGCAAAAGCAGG-3') and ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's

instructions. The six internal genes (PB2, PB1, PA, NP, M, and NS) of the cold-adapted H9N2 virus were amplified from cDNA using universal primers as previously described⁴⁴ and were cloned into the pHW2000 vector using an In-Fusion Advantage PCR Cloning kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. The pHW2000 plasmids containing the HA gene, in which polybasic amino acids were deleted, and the NA gene from A/Vietnam/1203/04 (H5N1, clade 1) have been described previously.^{36,45}

To rescue the live attenuated H5N1 vaccine virus based on a cold-adapted H9N2 backbone, plasmids containing six internal genes (PB2, PB1, PA, NP, M, and NS) of the cold-adapted H9N2 virus and the HA and NA genes of the HP H5N1 influenza virus were transfected into 293T cells using TransIT-LT1 transfection reagent (PanVera, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 293T cells were prepared in a 6-well tissue culture plate (Greiner Bio-One, Kremsmünster, Czech Republic), and 1 µg of each plasmid and 18 µl of TransIT-LT1 transfection reagent were mixed in Opti-MEM I medium (Invitrogen, Carlsbad, CA, USA) to a final volume of 200 µl. The mixture was incubated at room temperature for 45 min, added to 1 ml of Opti-MEM I, and the resulting mixture was added to each well. Following a 6-h incubation, the transfection mixture was replaced with 3 ml of Opti-MEM I, and cells were incubated for 72 h. An aliquot (300 µl) of each supernatant was collected, treated with 1-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (1 µg/ml), and 200 µl was inoculated into the allantoic cavity of 10-day-old chicken eggs. The presence of rescued virus in the allantoic fluid of inoculated eggs was confirmed by both genetic analysis and a hemagglutination inhibition (HI) assay using H5N1-specific antibodies. The rescued vaccine virus was designated as cold-adapted H5N1 with internal H9N2 (CaH5N1/H9N2backbone).

Determination of the cold-adapted and temperature-sensitive phenotype of CaH5N1/H9N2backbone

Wild-type H9N2 virus (A/Chicken/Korea/S21/04), cold-adapted H9N2 virus (A/Chicken/Korea/S1/03), and rescued vaccine virus (CaH5N1/H9N2backbone) were inoculated into five eggs per virus [2' 10⁵ EID₅₀/ml (50% egg infectious dose/ml)] and incubated at 25, 33, or 41°C for 72 h. The allantoic fluid was collected, and the viral titers were determined by log₁₀EID₅₀/ml in 10-day-old chicken eggs at 33°C, as described previously.⁴⁶ The sensitivity of viruses to temperature was subsequently determined. Each virus (200 µl of 2' 10⁵ EID₅₀/ml) was inoculated into five eggs, which were incubated at 25, 33, or 41°C for 72 h before viral titers were determined by log₁₀EID₅₀/ml.

Immunization of chickens with CaH5N1/H9N2backbone

Groups of 3-week-old specific pathogen-free (SPF) chickens (Hy-Line, USA) ($n = 10$ per group) were intranasally (i.n.) immunized with 0.5 ml of 1×10^6 EID₅₀/ml of CaH5N1/H9N2backbone. Mock-vaccinated chickens ($n = 10$) were inoculated i.n. with 0.5 ml of phosphate-buffered saline (PBS, pH 7.4). A boost inoculation was performed 3 weeks after the first inoculation using the same dose and route. Tracheal and cloacal swabs of immunized chickens were collected at 3, 5, and 7 days post-vaccination to determine viral shedding in the immunized chickens.

Determination of antibody titers in the sera of chickens immunized with CaH5N1/H9N2backbone

Sera were collected from chickens ($n = 10$ per group) immunized with two doses of CaH5N1/H9N2backbone, treated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) for 20 h, and absorbed by turkey red blood cells to remove substances causing non-specific hemagglutination. The treated sera were serially diluted 2-fold in PBS in V-bottom 96-well plates, and 25 μ l of A/Vietnam/1203/04 (H5N1, clade 1) diluted to contain 8 hemagglutination (HA) units was added to each well. The plates were incubated for 15 min at room temperature, and 50 μ l of 0.5% turkey red blood cells in PBS was subsequently added. After a 40-min incubation at room temperature, the HI titers were expressed as reciprocal dilutions that completely inhibited hemagglutination.

Determination of vaccine virus shedding in chickens immunized with CaH5N1/H9N2backbone

To evaluate shedding in chickens ($n = 10$ per group) immunized with 1 or two doses of CaH5N1/H9N2backbone, chickens were euthanized by cervical dislocation at 1, 3, or 5 days after immunization and bled. Tissues from the nasal turbinate, tracheas, lungs, and intestines were collected in PBS (1 g/ml) and homogenized, and the supernatants were serially 10-fold diluted in PBS. Each diluted sample was inoculated into four 10-day-old chicken eggs, and the presence of virus in the inoculated eggs was determined by HA assay using 0.5% turkey red blood cells. Viral titers were expressed as log₁₀EID₅₀/ml.

Detection of antibody subtypes in chickens immunized with CaH5N1/H9N2backbone

Sera and tissue samples (1 g/ml in PBS) from the nasal turbinate, tracheas, lungs, and intestines were collected from mock-immunized chickens ($n = 10$) or from chickens ($n = 10$) immunized with 1×10^6 EID₅₀/ml (0.5 ml) of CaH5N1/H9N2backbone at 3 weeks (1 dose) or 4 weeks (two doses) after vaccination. The homogenized tissue supernatants or sera were diluted 5-fold in PBS containing

5% horse serum and 0.05% Tween 20 (PBS-Tween 20), and an enzyme-linked immunosorbent assay (ELISA) was performed to determine the subtypes of antibodies in immunized chickens. The wells of ELISA plates (Greiner Bio-One) were coated with 100 μ l of 0.05 g of purified and inactivated H5N1 antigens of A/Vietnam/1203/04 (H5N1, clade 1) diluted in 1 ml of carbonate-bicarbonate buffer (pH 9.6). To each well, 100 μ l of prepared tissue supernatants or sera were added. Plates were incubated at room temperature for 1 h and washed 3 times with PBS-Tween 20. Subsequently, 100 μ l (1:1000 diluted in PBS-Tween 20) of horseradish peroxidase-conjugated goat anti-chicken IgM, IgG, or IgA (KPL, Gaithersburg, MD, USA) was added to each well. Plates were incubated at room temperature for 1 h and washed 3 times with PBS-Tween 20. Finally, 100 μ l of ABTS peroxidase substrate (KPL) was added to each well, and the plates were incubated at room temperature for 30 min before adding ABTS peroxidase stop solution (KPL) to terminate the reaction. The optical density was read at 405 nm using an ELISA microplate reader (Tecan Systems, San Jose, CA, USA).

Challenge of CaH5N1/H9N2backbone-immunized chickens with HP H5N1 or H9N2 viruses

Immunized chickens ($n = 10$ per group) were challenged with 1×10^3 50% chicken lethal dose (CLD₅₀/ml) of homologous HP H5N1 virus [A/Vietnam/1203/2004 (clade 1)], heterologous HP H5N1 virus [A/Vietnam/HN31244/2007 (clade 2)], or wild-type H9N2 virus (A/chicken/Korea/S21/2004). Mortality was observed for 21 days after challenge, and the tracheal and cloacal swabs were collected at 3, 5, 7, 9, and 14 days after challenge for virus titration.

Titration of virus in CaH5N1/H9N2backbone-immunized chickens challenged with HP H5N1 or H9N2 viruses

Swabbed samples were diluted in 0.5 ml of PBS supplemented with 2' antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA) and serially 10-fold diluted in PBS (pH 7.4). The diluted samples were inoculated into 10-day-old chicken eggs. Viral titers (log₁₀EID₅₀/ml) in the inoculated allantoic fluids were confirmed by HA assay using 0.5% turkey red blood cells in PBS.

Detection of CD4+ or CD8+ T lymphocytes expressing interferon-gamma (IFN- γ) in chickens immunized with CaH5N1/H9N2backbone

Lymphocytes were isolated by Histopaque 1083 (Sigma-Aldrich) from the spleens of chickens ($n = 5$) immunized with one or two doses of 1×10^6 EID₅₀/ml (0.5 ml) of CaH5N1/H9N2backbone. The isolated splenocytes were suspended at a density of 2×10^7 /ml in RPMI 1640 medium supplemented with 5% fetal bovine serum and 1'

antibiotic–antimycotic solution (Sigma-Aldrich) and were stimulated overnight with H9N2 virus at a multiplicity of infection (MOI) of 0.001. Stimulated splenocytes (1×10^7 /ml) were incubated with 5 μ g/ml of fluorescein isothiocyanate (FITC)-labeled mouse anti-chicken CD4+ or CD8+ or rabbit anti-chicken IFN- γ in PBS supplemented with 5% horse serum and 1% sodium azide at room temperature for 30 min. Cells were washed three times with PBS containing 5% horse serum and 1% sodium azide and incubated with R-phycoerythrin-labeled sheep anti-rabbit IgG in PBS supplemented with 5% horse serum, 1% sodium azide, and 0.5% Tween 20 (for permeabilization). Cells were fixed with 2% paraformaldehyde for 10 min at room temperature. The stained splenocytes were analyzed by flow cytometry using a FACSCalibur apparatus (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis

Statistical analysis was performed using the Statistical Product and Services Solutions, version 10.0 (SPSS, Cary, NC, USA). Anova analysis was performed by comparing the data from immunized chickens with those from unimmunized chickens. A *P*-value of <0.05 was considered statistically significant.

Results

Characterization of a live attenuated H5N1 vaccine strain containing the internal genes of cold-adapted H9N2 virus

We first determined the sensitivity of the CaH5N1/H9N2backbone vaccine virus to temperatures of 25, 33, and 41°C (Fig. 1). Eggs ($n = 10$) were inoculated with 200 μ l of wild-type H9N2 virus (A/Chicken/Korea/S21/04), cold-adapted H9N2 virus (A/Chicken/Korea/S1/03), or CaH5N1/H9N2backbone vaccine virus. The eggs were incubated at the different temperatures, and viral titers (\log_{10} EID₅₀/ml) were determined at 33°C. Both the CaH5N1/H9N2backbone vaccine virus (mean titer, 6.7 \log_{10} EID₅₀/ml) and cold-adapted H9N2 virus (mean titer, 7.3 \log_{10} EID₅₀/ml) grew well at 25°C, whereas the wild-type H9N2 virus (mean titer, <1.0 EID₅₀/ml) did not grow well at 25°C. The viral titers at 33°C were similar for all three virus types. At 41°C, the viral titers of both CaH5N1/H9N2backbone (5.3 \log_{10} EID₅₀/ml) and cold-adapted H9N2 (5.2 \log_{10} EID₅₀/ml) viruses were lower than that of the wild-type H9N2 virus (7.0 \log_{10} EID₅₀/ml). These results suggest that CaH5N1/H9N2backbone is a cold-adapted and temperature-sensitive virus. No amino acid changes in H5 or N1 were observed after passaging in eggs (data not shown).

We also determined whether the CaH5N1/H9N2backbone vaccine virus was attenuated in chickens (Table 1).

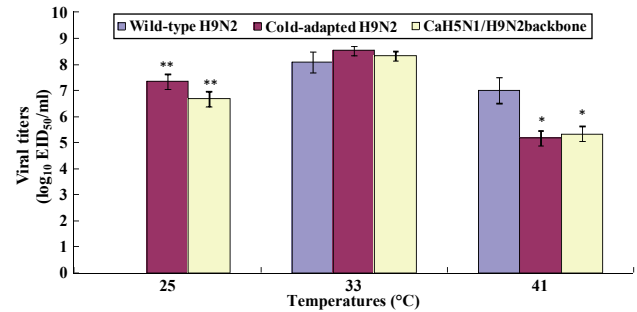


Figure 1. Cold adaptation and temperature sensitivity of the CaH5N1/H9N2backbone vaccine virus. Wild-type H9N2 virus (A/Chicken/Korea/S21/04), cold-adapted H9N2 virus (A/Chicken/Korea/S1/03), and rescued CaH5N1/H9N2backbone virus (2×10^5 EID₅₀/ml) were inoculated into five eggs per virus and incubated at 25, 33, or 41°C for 72 h. The viral titers in the allantoic fluids of each egg were determined in 10-day-old chicken eggs at 33°C by \log_{10} EID₅₀/ml. The data are the mean of five independent experiments \pm SEM. Statistical analysis was performed by comparing the viral titers of vaccine virus with those of wild-type H9N2 virus using the data obtained from five experiments. **P* < 0.05, ***P* < 0.001.

Table 1. Virus replication of CaH5N1/H9N2backbone vaccine virus in chickens

| Days post-infection | Viral titer (\log_{10} EID ₅₀ /ml) | | | |
|---------------------|--|--------------|-------|------------|
| | Nasal turbinate | Tracheas | Lungs | Intestines |
| 1 | – | – | – | – |
| 2 | – | 1.25 (2/10)* | – | – |
| 3 | – | – | – | – |
| 1 boost** | – | – | – | – |
| 3 boost | – | – | – | – |
| 5 boost | – | – | – | – |

–, Under detection limit of viruses (1 EID₅₀/ml).

*Number of positive chickens out of 10 chickens.

**Boosted 3 weeks after the first vaccination.

The replication of CaH5N1/H9N2backbone virus in chickens was reduced. Viral titers were under the detection limit (<1 \log_{10} EID₅₀/ml) in the nasal turbinate, lung, and intestinal tissues of chickens inoculated with 1 dose of vaccine; however, a low mean viral titer (1.25 \log_{10} EID₅₀/ml) was detected in the tracheas of 2 of 10 chickens inoculated with 1 dose of vaccine. In chickens inoculated with two doses, virus titers were under the detection limit in all tissues analyzed. No CaH5N1/H9N2backbone vaccine virus was transmitted from inoculated to contact chickens (data not shown).

Immunogenicity and antibody subtypes in chickens inoculated with the CaH5N1/H9N2backbone vaccine virus

Chickens were i.n. inoculated with 1×10^6 EID₅₀/ml (0.5 ml) of CaH5N1/H9N2backbone vaccine virus and subsequently boosted with the same amount of virus 3 weeks later. Antibody titers were determined in chickens by the HI assay using an H5N1 influenza virus (A/Vietnam/1203/04) (Fig. 2A). Antibody titers peaked 4 weeks post-vaccination (mean HI titer, 1186) and then gradually declined, reaching a mean HI titer of 296 at 11 weeks post-vaccination. HI antibody titers against the heterologous virus, A/Vietnam/HN31244/2007 (clade 2), were <20, indicating very limited cross-reactivity between clade 1 and clade 2 H5N1 viruses.

Subtypes of antibodies were measured in the sera and tissues (nasal turbinate, tracheas, lungs, and intestines) of chickens inoculated with one or two doses of CaH5N1/H9N2backbone vaccine virus. IgA antibody was strongly induced in the nasal turbinate, trachea, and lung tissues of chickens inoculated with two doses of vaccine (Fig. 2B).

IgG antibody was similarly induced in the sera and tissues (nasal turbinate, tracheas, lungs, and intestines) of chickens immunized with the two-dose vaccine regimen (Fig. 2C). IgM antibody was predominantly detected in the nasal turbinate, trachea, and lung tissues of immunized chickens inoculated with the two-dose vaccine regimen (Fig. 2D). We subsequently measured the subtypes of antibodies against the heterologous (clade 2) H5N1 or H9N2 antigens in the sera and tissues of chickens immunized with two doses of CaH5N1/H9N2backbone vaccine virus. IgA, IgG, and IgM subtypes were detected against heterologous (clade 2) H5N1 antigens, while antibodies against H9N2 antigens were not detected (Fig. 3).

Protective efficacy of the CaH5N1/H9N2backbone vaccine virus against HP H5N1 virus

Chickens inoculated with one or two doses of CaH5N1/H9N2backbone vaccine virus were challenged with homologous or heterologous HP H5N1 or H9N2 viruses. The survival rate and virus shedding in the tracheas and cloacae of challenged chickens were determined. The

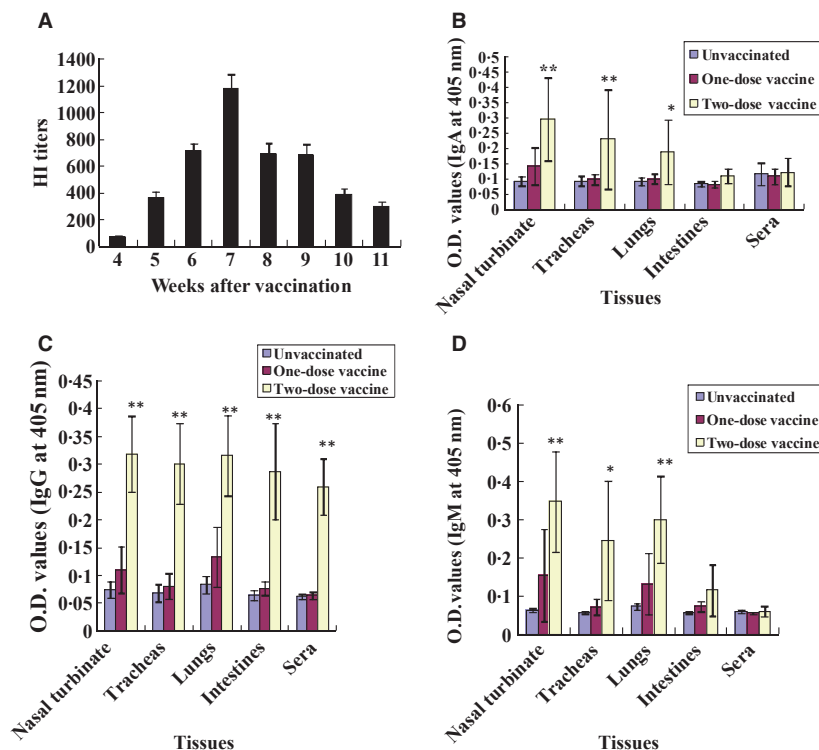


Figure 2. Immunogenicity and antibody subtypes in chickens immunized with CaH5N1/H9N2backbone vaccine virus. Groups of 3-week-old SPF chickens ($n = 10$ per group) were intranasally inoculated with 0.5 ml of 10^6 EID₅₀/ml of the CaH5N1/H9N2backbone vaccine virus. Chickens were boosted using the same dose and route 3 weeks after the first inoculation. Sera were collected at 3, 5, 6, 7, 8, 9, 10, and 11 weeks post-vaccination, and antibody titers were determined by an HI assay. Nasal turbinate, trachea, lung, and intestinal tissues and/or sera from chickens ($n = 10$ per group) immunized with 1 or two doses of vaccine were collected in PBS, and antibody subtypes were determined by ELISA using chicken-specific IgA, IgG, and IgM antibodies and H5N1 virus antigens (clade 1). Statistical analysis was performed by comparing the OD values of vaccinated chickens with those of unvaccinated chickens. Panels A, B, C, and D denote HI titers, IgA antibody, IgG antibody, and IgM antibody, respectively. * $P < 0.05$, ** $P < 0.001$. HI, hemagglutination inhibition; OD, optical density.

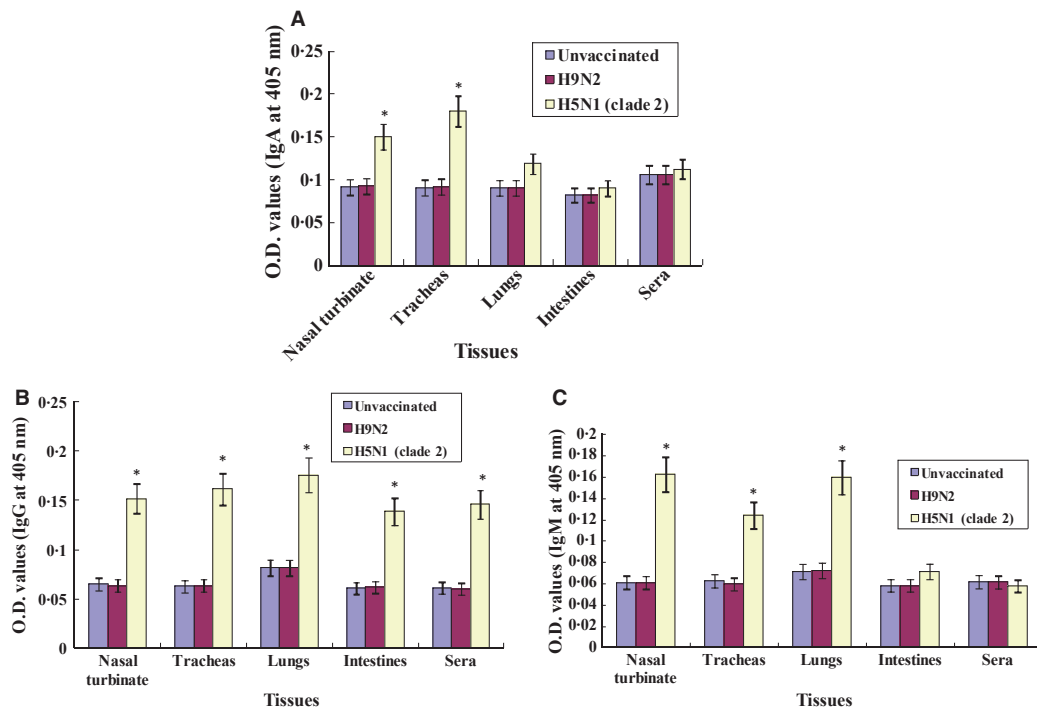


Figure 3. Subtypes of antibodies against heterologous H5N1 or H9N2 antigens in chickens immunized with two doses. The nasal turbinates, trachea, lung, and intestinal tissues and/or sera from chickens ($n = 10$ per group) immunized with two doses of CaH5N1/H9N2backbone vaccine virus were collected in PBS. Antibody subtypes were determined by ELISA using chicken-specific IgA, IgG, and IgM antibodies and H5N1 (clade 2) or H9N2 virus antigens. Statistical analysis was performed by comparing the OD values of vaccinated chickens with those of unvaccinated chickens. Panels A, B, and C denote IgA antibody, IgG antibody, and IgM antibody, respectively. $*P < 0.05$.

survival rate of chickens inoculated with 1 dose of vaccine and challenged with homologous or heterologous HP H5N1 virus was 80% and 20%, respectively (Fig. 4A). The survival rate of chickens inoculated with two doses of vaccine and challenged with homologous or heterologous HP H5N1 virus was 100% and 80%, respectively, which is better than that of chickens inoculated with just 1 dose (Fig. 4B).

We studied the survival rate of chickens inoculated with two doses of CaH5N1/H9N2backbone vaccine virus 11 weeks before challenge with HP H5N1 virus (Fig. 4C). The survival rate of chickens challenged with homologous and heterologous HP H5N1 viruses was 100% and 60%, respectively. While chickens that survived did not display clinical symptoms, chickens that did not survive showed severe clinical signs before death, including hemorrhage in skins, combs, and wattles.

We analyzed virus shedding in the tracheas and cloacae of immunized chickens following challenge with HP H5N1 or H9N2 viruses. Only 1 of the 10 surviving chickens immunized with 1 dose shed virus following infection with homologous H5N1 virus, displaying a mean titer of $1.5 \log_{10} \text{EID}_{50}/\text{ml}$ in the tracheal swabs 7 days after challenge. The quantity of virus was below the detection limit ($<1 \log_{10} \text{EID}_{50}/\text{ml}$) in the tracheas and cloacae of surviving

chickens immunized with two doses or two doses 11 weeks prior to infection with homologous HP H5N1 virus (Table 2). The immunized chickens that were infected with heterologous HP H5N1 virus shed more virus than those infected with homologous HP H5N1 virus (Table 3). Five days after challenge with heterologous HP H5N1, 2 of the 10 surviving chickens immunized with 1 dose shed virus in the tracheas and cloacae (mean viral titers of 3.5 and 4.0 $\log_{10} \text{EID}_{50}/\text{ml}$, respectively), and 2 of the 10 surviving chickens immunized with two doses shed virus in the tracheas and cloacae (mean viral titers of 3.5 and 3.5, $\log_{10} \text{EID}_{50}/\text{ml}$, respectively). Five days after challenge with heterologous HP H5N1 virus, 4 of 10 surviving chickens immunized with two doses 11 weeks before challenge shed virus in the tracheas and cloacae (mean titers of 3.25 and 4.0 $\log_{10} \text{EID}_{50}/\text{ml}$, respectively). Thus, a live attenuated H5N1 vaccine virus containing cold-adapted internal genes of H9N2 influenza virus can provide chickens with protective immunity against HP H5N1 influenza virus.

Protective efficacy of the CaH5N1/H9N2backbone vaccine virus against H9N2 influenza virus

We subsequently determined whether CaH5N1/H9N2backbone vaccine virus can protect chickens from H9N2 infection. Virus shedding was determined in the tracheas and

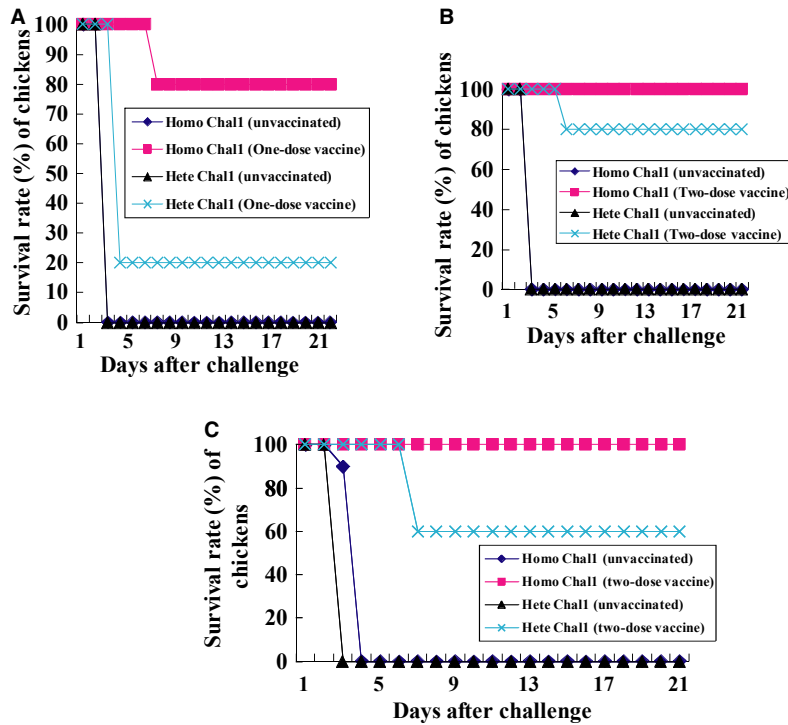


Figure 4. Mortality of immunized chickens infected with HP H5N1 influenza virus. Chickens ($n = 10$ per group) inoculated with one or two doses of CaH5N1/H9N2backbone vaccine virus were infected with 1×10^3 CLD₅₀/ml of homologous or heterologous HP H5N1 virus, and the survival rate was observed for 21 days after challenge. (A) Survival rate of chickens immunized with 1 dose and infected with homologous or heterologous HP H5N1 influenza virus. (B) Survival rate of chickens immunized with two doses and infected with homologous or heterologous HP H5N1 influenza virus. (C) Chickens challenged 11 weeks post-initial vaccination. Homo, homologous; Hete, heterologous; Chall, challenged.

Table 2. Viral shedding in the survived immunized chickens challenged by the homologous HP H5N1 influenza virus

| Days post-challenge | One-dose vaccine | | Two-dose vaccine | | 11 weeks after two-dose vaccines | |
|---------------------|------------------|---------|------------------|---------|----------------------------------|---------|
| | Tracheas | Cloacae | Tracheas | Cloacae | Tracheas | Cloacae |
| 3 | – | – | – | – | – | – |
| 5 | – | – | – | – | – | – |
| 7 | 1.5 (1/10)* | – | – | – | – | – |
| 14 | – | – | – | – | – | – |

–, Under detection limit of viruses ($1 \text{ EID}_{50}/\text{ml}$).

*Number of positive chickens out of 10 chickens.

We did not show the results of unvaccinated chickens because they died within 3 days after challenge.

cloacae only, because H9N2 virus does not cause any specific clinical signs in SPF chickens. The number of shed virus, mean viral shedding titer, and duration of viral shedding were reduced in immunized chickens (Table 4). Virus was detected in all 10 unimmunized chickens infected with H9N2 virus, with the titer in the tracheas and cloacae ranging from 2.5 to 3.9 $\log_{10}\text{EID}_{50}/\text{ml}$ up to 7 days after chal-

lenge. In contrast, just 1 of 10 chickens immunized with 1 dose and infected with H9N2 virus shed virus in the tracheas and cloacae (mean viral titer of 2.3 and 2.5 $\log_{10}\text{EID}_{50}/\text{ml}$, respectively). In chickens immunized with two doses or two doses 11 weeks before challenge, virus was detected only in the trachea (mean viral titers of 2.0 and 1.5 $\log_{10}\text{EID}_{50}/\text{ml}$, respectively). Thus, the

Table 3. Viral shedding in the survived immunized chickens challenged by the heterologous HP H5N1 influenza virus

| Days post-challenge | One-dose vaccine | | Two-dose vaccines | | 11 weeks after two-dose vaccines | |
|---------------------|------------------|------------|-------------------|-------------|----------------------------------|------------|
| | Trachea | Cloacae | Trachea | Cloacae | Trachea | Cloacae |
| 3 | 3.0 (8/10)* | 3.5 (8/10) | 3.0 (2/10) | 3.25 (2/10) | 3.0 (4/10) | 3.5 (4/10) |
| 5 | 3.5 (2/10) | 4.0 (2/10) | 3.5 (2/10) | 3.5 (2/10) | 3.25 (4/10) | 4.0 (4/10) |
| 7 | – | – | – | – | – | – |
| 14 | – | – | – | – | – | – |

–, Under detection limit of viruses (1 EID₅₀/ml).

*Number of positive chickens out of 10 chickens.

We did not show the results of unvaccinated chickens because they died within 3 days after challenge.

Table 4. Viral shedding in the survived immunized chickens challenged by H9N2 influenza virus

| Days post-challenge | Unvaccinated | | One-dose vaccine | | Two-dose vaccines | | 11 weeks after two-dose vaccines | |
|---------------------|--------------|-------------|------------------|------------|-------------------|---------|----------------------------------|---------|
| | Tracheas | Cloacae | Tracheas | Cloacae | Tracheas | Cloacae | Trachea | Cloacae |
| 3 | 2.6 (10/10)* | 1.5 (4/10) | 2.1 (10/10) | – | 2.0 (5/10) | – | 1.5 (7/10) | – |
| 5 | 3.4 (10/10) | 3.2 (10/10) | 2.3 (1/10) | 2.5 (1/10) | – | – | – | – |
| 7 | 3.1 (10/10) | 3.9 (10/10) | – | – | – | – | – | – |
| 14 | – | – | – | – | – | – | – | – |

–, Under detection limit of viruses (1 EID₅₀/ml).

*Number of positive chickens out of 10 chickens.

CaH5N1/H9N2backbone vaccine virus limits the replication of H9N2 influenza virus in immunized chickens.

Protection of immunized chickens from infections with different doses of heterologous HP H5N1 or H9N2 influenza viruses

Chickens immunized with two doses were infected with different doses of heterologous H5N1 or H9N2 viruses to confirm the protective efficacy of the developed vaccine in chickens. The survival rate of immunized chickens decreased as the dose of heterologous H5N1 influenza virus increased (Fig. 5). The survival rate of immunized chickens infected with 4, 5, and 6 log₁₀EID₅₀/ml of heterologous H5N1 influenza virus was 80%, 70%, and 60%, respectively (Fig. 5). All immunized chickens infected with different doses of H9N2 virus survived; however, the number of chickens that shed virus in tracheas and cloacae increased as the dose of H9N2 influenza virus increased (Table 5). The number of chickens infected with 4, 5, and 6 log₁₀EID₅₀/ml of H9N2 virus that shed virus in the tracheas and cloacae at 5 days post-infection was 0, 2, and 3, respectively (Table 5).

Role of cellular immunity in protecting chickens from infection with HP H5N1 and H9N2 viruses

Our results showed that immunized chickens were protected against infection with heterologous HP H5N1 or H9N2 viruses. Therefore, we analyzed CD4+ and CD8+ T lymphocytes expressing IFN- γ , which may inhibit virus replication in infected chickens. The proportion of CD4+ and CD8+ lymphocytes expressing IFN- γ was higher in chickens immunized with two doses (mean, 9.04%–9.15%) than that in chickens immunized with 1 dose (mean, 5.57–6.33%), while the mean in unimmunized chickens was 3.70–4.38% (Fig. 6). Therefore, cellular immunity contributes to protecting immunized chickens from infection with HP H5N1 or H9N2 influenza viruses.

Discussion

We developed a live attenuated H5N1 vaccine using cold-adapted H9N2 influenza virus as backbone. The resulting CaH5N1/H9N2backbone vaccine virus was temperature-sensitive and attenuated in chickens. Immunized chickens were protected from challenge with homologous and

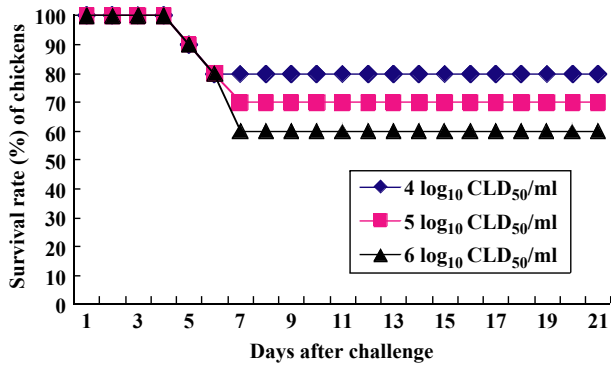


Figure 5. Mortality of two-dose immunized chickens infected with different doses of HP H5N1 influenza virus. Chickens ($n = 10$ per group) inoculated with two doses of the CaH5N1/H9N2backbone vaccine virus were infected with 10^4 – 10^6 CLD₅₀/ml of heterologous HP H5N1 influenza virus, and the survival rate was observed for 21 days after challenge.

heterologous HP H5N1 or H9N2 viruses. Moreover, CD4+ and CD8+ T lymphocytes expressing IFN- γ were induced in immunized chickens.

Mean HI titers exceeding 290 were detected in immunized chickens until 11 weeks post-vaccination—the last week that was observed. Thus, chickens immunized with two doses of live attenuated H5N1 vaccine maintain protective levels of antibodies for at least 11 weeks. Similarly, single-dose immunization of chickens with an oil-adjuvant inactivated H5N1 vaccine induced antibodies for 12 weeks following vaccination.³⁶ Furthermore, antibody specific for H5N1 influenza virus was shown to last up to 138 weeks post-vaccination in chickens immunized with oil-adjuvant non-pathogenic H5N1 (A/duck/Hokkaido/Vac-1/04).³⁸ The *in ovo* immunization of 18-day-old chicken embryos with live attenuated H5N1 vaccine elicited antibody for 12 weeks post-vaccination, before antibody titers started to decline.³⁹

In the present study, chickens immunized with CaH5N1/H9N2backbone vaccine virus induced both IgA and IgG subtypes in the nasal turbinate, trachea, lung, and intestinal tissues. However, in chickens intramuscularly immunized with inactivated H5N1 vaccine, the IgG subtype—rather than IgA antibody—was dominantly induced in the trachea, lung, and nasal cavity tissues.^{36,45} IgA antibody is the most abundantly induced species in humans or animals and plays an important role in protecting the mucosal surface in the respiratory and gastrointestinal tracts from the invasion of bacteria and viruses.⁴⁷

Chickens immunized with two doses of CaH5N1/H9N2backbone vaccine virus were protected from infection by homologous and heterologous HP H5N1 or H9N2 viruses. Thus, our strategy may help to control 2 viruses with a single vaccine, because both HP H5N1 and H9N2 are endemic in many Asian countries. Many efforts have been made to develop a vaccine to protect chickens from infection by HP H5N1, but not H9N2, influenza virus. An adenovirus-based live vaccine virus expressing H5N1-derived HA and NA protects chickens from lethal infections caused by HP H5N1 virus.^{35,48} Chickens immunized with infectious laryngotracheitis virus (ILT) recombinants expressing H5 HA are protected from infection by both HP H5N1 influenza virus and ILTV.^{34,48} The NS1 mutant vaccine of H7 or H5 influenza virus subtypes has also been used to protect chickens from infections caused by HP H7N3 or H5N1 influenza viruses,^{30,32} however, this vaccine virus can revert to virulence.³² A single dose of a Newcastle disease virus (NDV)-based live attenuated vaccine expressing H5N1-derived HA protected chickens from infection by both HP H5N1 influenza virus and NDV.²⁹ Another study showed that chickens immunized *in ovo* with NDV expressing H5 were protected from infection with HP H5N1 influenza virus, but immunized chicks were not protected.⁴⁰

CD4+ and CD8+ T lymphocytes expressing IFN- γ were induced in chickens immunized with the CaH5N1/

Table 5. Viral shedding in the survived immunized chickens challenged by the different doses of H9N2 influenza virus

| Days post-challenge | 4 Log ₁₀ EID ₅₀ /ml | | 5 Log ₁₀ EID ₅₀ /ml | | 6 Log ₁₀ EID ₅₀ /ml | |
|---------------------|---|---------|---|------------|---|------------|
| | Tracheas | Cloacae | Tracheas | Cloacae | Tracheas | Cloacae |
| 3 | 2.5 (6/10)* | – | 2.5 (7/10) | – | 3.0(8/10) | – |
| 5 | – | – | 2.0 (2/10) | 2.5 (2/10) | 2.5 (3/10) – | 3.0 (3/10) |
| 7 | – | – | – | – | – | 2.0 (1/10) |
| 14 | – | – | – | – | – | – |

–, Under detection limit of viruses (1 EID₅₀/ml).

*Number of positive chickens out of 10 chickens.

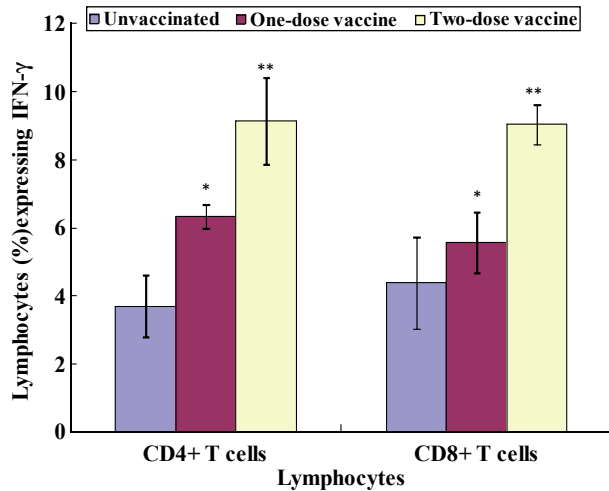


Figure 6. Determination of lymphocytes expressing IFN- γ in immunized chickens. Lymphocytes were isolated from the spleens of chickens ($n = 5$ per group) inoculated with one or two doses of CaH5N1/H9N2backbone at 3 or 4 weeks post-vaccination, respectively. Lymphocytes were stimulated overnight with an H9N2 influenza virus (MOI, 0.001) and subsequently double-labeled with FITC-labeled mouse anti-chicken CD4+ and CD8+ or rabbit anti-chicken IFN- γ and R-phycoerythrin-labeled sheep anti-rabbit IgG. The stained lymphocytes were counted by flow cytometry. Statistical analysis was performed by comparing the percentage of lymphocytes in vaccinated chickens with that in unvaccinated chickens. * $P < 0.05$, ** $P < 0.001$.

H9N2backbone vaccine virus, indicating that cellular immunity may be involved in protecting chickens from infection with heterologous HP H5N1 or H9N2 influenza viruses. Cross-reactive cellular immunity elicited by H9N2 infection in chickens was shown to protect from lethal infections caused by HP H5N1 virus.⁴⁹

While low-level replication of the CaH5N1/H9N2backbone vaccine virus was observed in the tracheas of chickens only, IgG and IgA were strongly induced in chickens immunized with two doses of vaccines. If the vaccine virus were widely used, this low-level replication could be beneficial for reducing the chance of reassortment between the vaccine virus and avian influenza viruses circulating in poultry. Before the developed live vaccine is commercially used for protecting chickens from H5N1 or H9N2 influenza virus in farms, it will be necessary to study the possibility of reassortment, to perform a dose-sparing study in chickens, and to develop a method to effectively deliver the vaccine to each chicken. In addition, if mass aerosol or intranasal vaccination is not feasible, we also need to study whether *in ovo* inoculation of the live vaccine can efficiently protect chickens from infection by HP H5N1 or H9N2 influenza viruses.

In conclusion, a live attenuated H5N1 vaccine—developed using cold-adapted H9N2 internal genes as a back-

bone—may protect chickens from infections with HP H5N1 and H9N2 influenza viruses by eliciting humoral and cellular immunity.

Acknowledgments

This study was supported, in part, by a grant (A084411) from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea. An English editor in Editage, Cactus Communications Pvt. Ltd. kindly edited this manuscript.

Authors' contributions

NTN drafted the manuscript and performed experiments on the development of the vaccine strain and its protective efficacy in chickens. BMS, YMK, HMK, and HSK assisted with the animal experiments. SHS conceived the study and participated in its design and coordination.

Conflict of interests

The authors declare that they have no competing interests.

References

- 1 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev* 1992; 56:152–179.
- 2 Fouchier RA, Munster V, Wallensten A *et al.* Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol* 2005; 79:2814–2822.
- 3 Homme PJ, Easterday BC. Avian influenza virus infections. IV. Response of pheasants, ducks, and geese to influenza A-turkey-Wisconsin-1966 virus. *Avian Dis* 1970; 14:285–290.
- 4 Xu KM, Li KS, Smith GJ *et al.* Evolution and molecular epidemiology of H9N2 influenza A virus from quail in southern China, 2000 to 2005. *J Virol* 2007; 81:2635–2645.
- 5 Kandeel A, Manoncourt S, Abd el Kareem E *et al.* Zoonotic transmission of avian influenza virus (H5N1), Egypt, 2006–2009. *Emerg Infect Dis* 2010; 16:1101–1107.
- 6 Nagarajan S, Rajukumar K, Tosh C *et al.* Isolation and pathotyping of H9N2 avian influenza virus in Indian poultry. *Vet Microbiol* 2009; 133:154–163.
- 7 Iqbal M, Yaqub T, Reddy K, McCauley JW. Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 virus. *PLoS ONE* 2009; 4:e5788.
- 8 Golender N, Panshin A, Banet-Noach C *et al.* Genetic characterization of avian influenza virus isolated in Israel during 2000–2006. *Virus Genes* 2008; 37:289–297.
- 9 Kim JA, Cho SH, Kim HS, Seo SH. H9N2 influenza virus isolated from poultry in Korean live bird markets continuously evolve and cause the severe clinical signs in layers. *Vet Microbiol* 2006; 118:169–176.
- 10 Negovetich NJ, Feeroz MM, Jones-Engel L *et al.* Live bird markets of Bangladesh: H9N2 viruses and the near absence of highly pathogenic H5N1 influenza. *PLoS ONE* 2011; 6:e19311.

- 11 Nang NT, Lee JS, Song BM, Kang YM, Kim HS, Seo SH. Induction of inflammatory cytokines and toll-like receptors in chickens infected with avian H9N2 influenza virus. *Vet Res* 2011; 42:64.
- 12 Lee CW, Song CS, Lee YJ *et al.* Sequence analysis of the hemagglutinin gene of H9N2 Korean avian influenza virus and assessment of the pathogenic potential of isolate MS96. *Avian Dis* 2000; 44:527–535.
- 13 Choi YK, Seo SH, Kim JA, Webby RJ, Webster RG. Avian influenza virus in Korean live poultry markets and their pathogenic potential. *Virology* 2005; 332:529–537.
- 14 Guan Y, Shortridge KF, Krauss S, Webster RG. Molecular characterization of H9N2 influenza virus: were they the donors of the “internal” genes of H5N1 viruses in Hong Kong? *Proc Natl Acad Sci USA* 1999; 96:9363–9367.
- 15 Xu KM, Smith GJ, Bahl J *et al.* The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. *J Virol* 2007; 81:10389–10401.
- 16 Dong G, Luo J, Zhang H *et al.* Phylogenetic diversity and genotypical complexity of H9N2 influenza A viruses revealed by genomic sequence analysis. *PLoS ONE* 2011; 6:e17212.
- 17 Peiris M, Yuen KY, Leung CW *et al.* Human infection with influenza H9N2. *Lancet* 1999; 354:916–917.
- 18 Butt KM, Smith GJ, Chen H *et al.* Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J Clin Microbiol* 2005; 43:5760–5767.
- 19 Xu X, Subbarao K, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 virus from the 1997 outbreaks in Hong Kong. *Virology* 1999; 261:15–19.
- 20 Claas EC, Osterhaus AD, van Beek R *et al.* Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* 1998; 351:472–477.
- 21 Subbarao K, Klimov A, Katz J *et al.* Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 1998; 279:393–396.
- 22 Takano R, Nidom CA, Kiso M *et al.* Phylogenetic characterization of H5N1 avian influenza viruses isolated in Indonesia from 2003–2007. *Virology* 2009; 390:13–21.
- 23 Mase M, Tsukamoto K, Imada T *et al.* Characterization of H5N1 influenza A viruses isolated during the 2003–2004 influenza outbreaks in Japan. *Virology* 2005; 332:167–176.
- 24 Mase M, Kim JH, Lee YJ *et al.* Genetic comparison of H5N1 influenza A virus isolated from chickens in Japan and Korea. *Microbiol Immunol* 2005; 49:871–874.
- 25 Viseshakul N, Thanawongnuwech R, Amonsin A *et al.* The genome sequence analysis of H5N1 avian influenza A virus isolated from the outbreak among poultry populations in Thailand. *Virology* 2004; 328:169–176.
- 26 Nguyen DC, Uyeki TM, Jadhao S *et al.* Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. *J Virol* 2005; 79:4201–4212.
- 27 Liu J, Xiao H, Lei F *et al.* Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science* 2005; 309:1206.
- 28 Liljebjelke KA, Petkov DI, Kapczynski DR. Mucosal vaccination with a codon-optimized hemagglutinin gene expressed by attenuated *Salmonella* elicits a protective immune response in chickens against highly pathogenic avian influenza. *Vaccine* 2010; 28:4430–4437.
- 29 Ge J, Deng G, Wen Z *et al.* Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. *J Virol* 2007; 81:150–158.
- 30 Wang L, Suarez DL, Pantin-Jackwood M *et al.* Characterization of influenza virus variants with different sizes of the non-structural (NS) genes and their potential as a live influenza vaccine in poultry. *Vaccine* 2008; 26:3580–3586.
- 31 Swayne DE, Garcia M, Beck JR, Kinney N, Suarez DL. Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. *Vaccine* 2000; 18:1088–1095.
- 32 Brahmakshatriya VR, Lupiani B, Reddy SM. Characterization and evaluation of avian influenza NS1 mutant virus as a potential live and killed DIVA (differentiating between infected and vaccinated animals) vaccine for chickens. *Vaccine* 2010; 28:2388–2396.
- 33 Pavlova SP, Veits J, Keil GM, Mettenleiter TC, Fuchs W. Protection of chickens against H5N1 highly pathogenic avian influenza virus infection by live vaccination with infectious laryngotracheitis virus recombinants expressing H5 hemagglutinin and N1 neuraminidase. *Vaccine* 2009; 27:773–785.
- 34 Lüschow D, Werner O, Mettenleiter TC, Fuchs W. Protection of chickens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (H5) gene. *Vaccine* 2001; 19:4249–4259.
- 35 Toro H, Tang DC, Suarez DL, Sylte MJ, Pfeiffer J, Van Kampen KR. Protective avian influenza in ovo vaccination with non-replicating human adenovirus vector. *Vaccine* 2007; 25:2886–2891.
- 36 Hwang SD, Kim HS, Cho SW, Seo SH. Single dose of oil-adjuvanted inactivated vaccine protects chickens from lethal infections of highly pathogenic H5N1 influenza virus. *Vaccine* 2011; 29:2178–2186.
- 37 Römer-Oberdörfer A, Veits J, Helferich D, Mettenleiter TC. Level of protection of chickens against highly pathogenic H5 avian influenza virus with Newcastle disease virus based live attenuated vector vaccine depends on homology of H5 sequence between vaccine and challenge virus. *Vaccine* 2008; 26:2307–2313.
- 38 Sasaki T, Kokumai N, Ohgihara T *et al.* Long lasting immunity in chickens induced by a single shot of influenza vaccine prepared from inactivated non-pathogenic H5N1 virus particles against challenge with a highly pathogenic avian influenza virus. *Vaccine* 2009; 27:5174–5177.
- 39 Song H, Nieto GR, Perez DR. A new generation of modified live-attenuated avian influenza virus using a two-strategy combination as potential vaccine candidates. *J Virol* 2007; 81:9238–9248.
- 40 Steel J, Burmakina SV, Thomas C *et al.* A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. *Vaccine* 2008; 26:522–531.
- 41 Lee JS, Kim HS, Seo SH. Genetic characterization and protective immunity of cold-adapted attenuated avian H9N2 influenza vaccine. *Vaccine* 2008; 26:6569–6576.
- 42 Zhou B, Li Y, Belser JA *et al.* NS-based live attenuated H1N1 pandemic vaccines protect mice and ferrets. *Vaccine* 2010; 28:8015–8025.
- 43 Cai Y, Song H, Ye J *et al.* Improved hatchability and efficient protection after in ovo vaccination with live-attenuated H7N2 and H9N2 avian influenza virus. *Virol J* 2011; 8:31.
- 44 Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* 2001; 146:2275–2289.
- 45 Hwang SD, Shin JS, Ku KB, Kim HS, Cho SW, Seo SH. Protection of pregnant mice, fetuses and neonates from lethality of H5N1

- influenza viruses by maternal vaccination. *Vaccine* 2010; 28:2957–2964.
- 46** Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* 1938; 27:493–497.
- 47** Fagarasan S, Honjo T. Regulation of IgA synthesis at mucosal surfaces. *Curr Opin Immunol* 2004; 16:277–283.
- 48** Gao W, Soloff AC, Lu X *et al.* Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. *J Virol* 2006; 80:1959–1964.
- 49** Seo SH, Webster RG. Cross-reactive, cell-mediated immunity and protection of chickens from lethal H5N1 influenza virus infection in Hong Kong poultry markets. *J Virol* 2001; 75:2516–2525.