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Original Research

Bivalent Hemagglutinin Cleavage-Site Peptide Vaccines Protect Chickens from Lethal Infections with Highly Pathogenic H5N1 and H5N6 Avian Influenza Viruses

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Abstract

Background: Outbreaks of highly pathogenic avian influenza viruses cause huge economic losses to the poultry industry worldwide. Vaccines that can protect chickens from infections caused by various variants of highly pathogenic H5Nx avian influenza viruses are needed owing to the continuous emergence of new variants. We previously showed that vaccines containing the H5 cleavage-site peptide from clade 2.3.4.4. H5N6 avian influenza virus protects chickens from infection with homologous clade 2.3.4.4. H5N6 avian influenza virus, but not from infection with the heterologous clade 1 H5N1 avian influenza virus. Therefore, we developed bivalent peptide vaccines containing H5 cleavage sites of viruses from both clades to protect chickens from both H5N1 and H5N6 avian influenza viruses. **Methods**: Chickens were vaccinated with two doses of a combined peptide vaccine containing cleavage-site peptides from clade 1 and clade 2.3.4.4. highly pathogenic H5N1 and H5N6 avian influenza viruses and then challenged with both viruses. The infected chickens were monitored for survival and their tracheae and cloacae were sampled to check for viral shedding based on the median tissue culture infectious dose of 50 ($log_{10}TCID_{50}$/mL) in Madin–Darby canine kidney cells. **Results**: Antibody production was induced at similar levels in the sera of chickens immunized with two doses of the combined peptide vaccines containing cleavage-site peptides from highly pathogenic H5N1 and H5N6 avian influenza viruses. The immunized chickens were protected from infection with both H5N1 and H5N6 avian influenza viruses without viral shedding in the tracheae and cloacae. **Conclusions**: Dual-peptide vaccines containing cleavage-site peptides of both clades can protect chickens from highly pathogenic avian influenza virus infections.

Keywords: dual-peptide vaccine; cleavage-site peptide; highly pathogenic avian influenza virus

1. Introduction

Avian influenza viruses infect wild birds and domestic poultry. Based on their pathogenicity, avian influenza viruses are classified as low or highly pathogenic (HP) avian influenza viruses. Avian influenza viruses contain a single-stranded negative-sense RNA genome and belong to the Orthomyxoviridae family [1]. Two glycoproteins, hemagglutinin (HA) and neuraminidase (NA) are present on the surface of avian influenza virus particles. These viruses can be classified into different subtypes based on their HA and NA proteins; birds have been found to be infected with viruses containing 15 types of HA and 9 types of NA [2]. HP avian influenza viruses contain polybasic amino acids in the HA cleavage site, which enables the replication of these viruses in cells of poultry birds. Among the subtypes of avian influenza viruses, few H5 and H7 viruses contain polybasic amino acids at the HA cleavage site [2].

Outbreaks of HP H5Nx avian influenza viruses have caused major economic losses to the poultry industry worldwide [3–10]. Additionally, HP H5Nx avian influenza viruses infect mammals, such as minks, red foxes, polecats, otters, and badgers [3,4]. Various methods have been used to develop vaccines against HP H5Nx avian influenza viruses. The major types of vaccines include inactivated recombinants, virus-like particles (VLPs), and DNA vaccines [11–13]. We previously showed that vaccines containing the H5 cleavage-site peptide from clade 2.3.4.4. H5N6 avian influenza virus protects chickens from infection with homologous clade 2.3.4.4. H5N6 avian influenza virus, but not from infection with the heterologous clade 1 H5N1 avian influenza virus [14]. Therefore, we developed bivalent peptide vaccines containing clades 1 and 2.3.4.4. HP H5 cleavage sites to protect chickens from HP H5N1 and H5N6 avian influenza viruses of both clades.

2. Materials and Methods

2.1 Cells and Viruses

The avian influenza virus A/waterfowl/Korea/S57/2016 (H5N6) (clade 2.3.4.4.), which was isolated in our laboratory, contains RRRK at the hemagglutinin (HA) cleavage site. The avian influenza virus, A/Hong Kong/213/2003 (H5N1) (clade 1) containing RRRKK at the HA cleavage site was kindly provided by Dr. Malik Peiris (University of

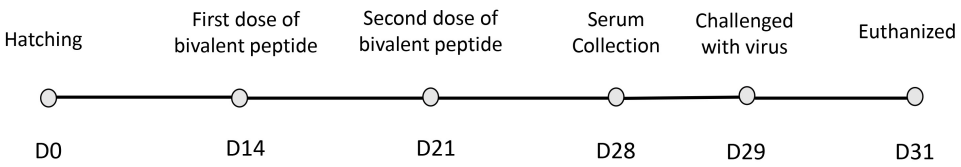

Fig. 1. Vaccination and challenge diagram of bivalent vaccines in chickens.

Hong Kong). Madin–Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA, USA) were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic–antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). MDCK cell lines were validated by STR profiling and tested negative for mycoplasma. Cells were all cultured in a humidified incubator at 37 °C and 5% CO_2.

All experiments on HP avian influenza viruses were conducted in a biosafety level 3 facility in the animal facility building at Chungnam National University (Daejeon, South Korea).

2.2 Peptide Vaccine Antigens

H5 cleavage-site peptides labeled with keyhole limpet hemocyanin (KLH) were synthesized by Peptron Co. (Daejeon, South Korea). The synthesized peptides were KLH -TGLRNSPLRERRRKR/GLFGAIAGFIEGGWQ (clade 2.3.4.4.) and KLH-CTGLRNSPQRERRRKKR/GLFGAI AGFIEGGW (clade 1).

2.3 Chicken Immunization

Two-week-old chickens (n = 13 per group) hatched in our laboratory were intramuscularly vaccinated with 300 µL of KLH-labeled bivalent peptide antigens (5 µg per antigen) diluted in phosphate-buffered saline (PBS; pH 7.4) and 30% oil (SEPPIC, Courbevoie, France); after three weeks, the immunized chickens were vaccinated with the second dose. The schedule of the vaccine study is shown in Fig. 1.

2.4 Measurement of Antibody Titers

Sera were collected from the immunized chickens 1 week after they were immunized with a second dose of peptide antigens. An enzyme-linked immunosorbent assay (ELISA) was used to measure the antibody titers. Immunoplate wells were coated with bovine serum albumin (BSA)-labeled peptides: BSA-TGLRNSPLRERRRKR/GLFGAI AGFIEGGWQ (clade 2.3.4.4.) and BSA-CTGLRNSPQR ERRRKKR/GLFGAIAGFIEGGW (clade 1).

The ELISA microplate was coated with 100 µL of peptide (4 µg/mL) diluted in coating buffer and then incubated at 4 °C for 12 h. The peptide-coated plates were washed three times with PBS containing Tween 20 (0.05%), and 1% BSA (100 µL) diluted in PBS (pH 7.4) was added to the wells of the plate to block the wells for 2 h at 25 °C. Subsequently, 100 µL of chicken sera diluted in PBS (1:100) was added to the blocked wells following incubation for 1 h at 25 °C. The wells were washed five times with PBS–Tween 20 (0.05%) following the addition of horseradish peroxidase-conjugated rabbit anti-chicken antibody (Sigma-Aldrich; 100 µL) diluted in PBS (1:5000) prior to incubation for 1 h at 25 °C. After the wells were washed five times with PBS–Tween 20 (0.05%), 100 µL of 3,3′,5,5′-tetramethylbenzidine substrate solution (Thermo Fisher Scientific, Waltham, MA, USA) was added prior to incubation for 30 min at 25 °C. Finally, 2.5 M sulfuric acid (50 µL) was added to stop the reaction. Optical density (OD) was measured at 450 nm using an ELISA spectrophotometer (Thermofisher, MA, USA).

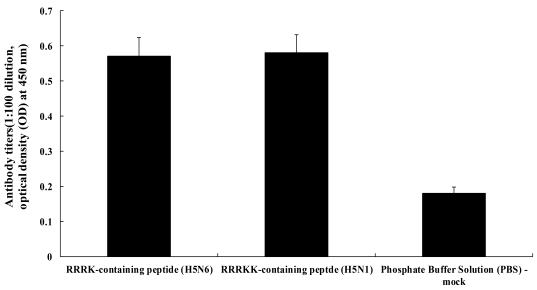

Fig. 2. Serum antibody titers from chickens vaccinated with dual H5 cleavage-site peptides. Chickens (n = 13 per group) were intramuscularly (i.m.) immunized with dual peptides of H5 cleavage sites (clade 1 and clade 2.3.4.4.) (5.0 µg per each peptide) and 4 weeks later, were boosted with the same dose. Sera were collected one week after boosting. Antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) using bovine serum albumin (BSA)-labeled each peptide.

2.5 Challenge Studies of Immunized Chickens with HP H5N1 or H5N6 Avian Influenza Viruses

The bivalent peptide-immunized chickens were intranasally infected with 1 mL (1×10^5 median tissue culture infectious dose [$TCID_{50}$]/mL) of H5N1 (A/Hong Kong/213/2003, clade 1) or H5N6 HP (A/waterfowl/Korea/S57/2016, clade 2.3.4.4.) avian influenza viruses. The tracheae and cloacae of challenged chickens were swabbed with PBS (pH 7.4) for 10 d. In

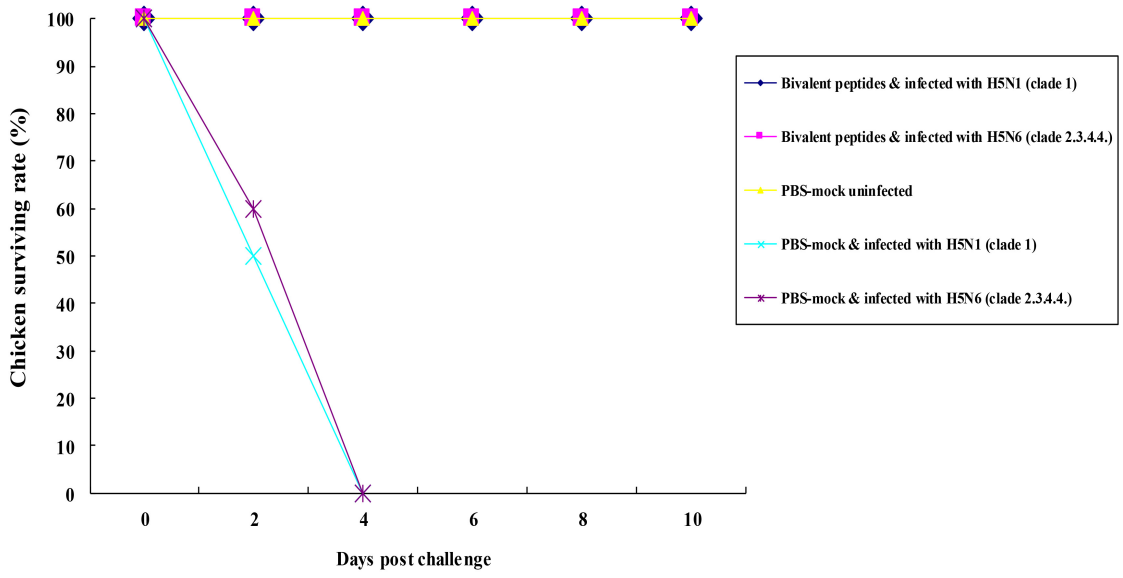

Fig. 3. Survival rate of the challenged chickens immunized with bivalent peptide antigens. The vaccinated chickens (Fig. 1) were intranasally (i.n.) infected with 1 mL (10^5TCID$_{50}$/mL) of A/Waterfowl/Korea/S57/2016 (H5N6) (clade 2.3.4.4.), or A/Waterfowl/Korea/S57/2016 (H5N6). The challenged chickens were monitored for survival rate for 10 days.

addition, three chickens per group were euthanized 2 d after the challenge to collect the lung and brain tissues for determining viral titers. The collected tissue samples were homogenized in PBS (0.1 g/1 mL PBS) using BeadBlaster 24 (Benchmark Scientific, Sayreville, NJ, USA). The swabbed samples were vortexed before they were used for viral titers.

2.6 Determination of Viral Titers

MDCK cells were cultured in MEM containing 10% FBS and 1× antibiotic–antimycotic solution (Sigma-Aldrich), and the cells were then detached using 1× trypsin–EDTA (Sigma-Aldrich) before seeding in 96-well culture plates. Confluent MDCK cells were washed with 400 µL of PBS (pH 7.4), and the wells were inoculated with swab and tissue samples that were 10-fold serially diluted in PBS containing 1.5% BSA. The inoculated wells were incubated for 5 d at 37 °C in a humidified incubator containing 5% CO_2. Cytopathic effects were observed to determine viral titers in $\log_{10}$TCID$_{50}$/mL as described by Muench and Reed [15].

2.7 Tissue Staining

Lung tissues from immunized chickens (n = 3 per group) challenged with H5N1 or H5N6 avian influenza viruses were collected at 2 days post-infection and fixed in 10% phosphate-buffered formalin (Triangle Biomedical Sciences, General Data Healthcare, Cincinnati, OH, USA) for 6 h before being embedded in paraffin. Tissue sections (5 µm) were prepared and stained with hematoxylin and eosin (H&E). Stained tissues were observed using an Olympus DP70 microscope (Olympus, Tokyo, Japan).

2.8 Statistical Analysis

Statistical analysis was performed via Student's *t*-test using IBM SPSS Statistics version 20 (IBM, Armonk, NY, USA). *p*-values < 0.05 were considered significant.

3. Results

3.1 Antibody Titers in Immunized Chickens

Sera were collected from chickens (n = 13 per group) immunized with bivalent peptide antigens comprising 5.0 µg of KLH-TGLRNSPLRERRRKR/GLFGAIAGFIEGG WQ (clade 2.3.4.4.) or 5.0 µg of KLH-CTGLRNSPQRE RRRKKR/GLFGAIAGFIEGGW (clade 1). The collected sera were used to determine antibody titers via ELISA. Strong antibody responses against each peptide were detected. The mean OD values for RRRK-containing (clade 2.3.4.4) and RRRKK-containing (clade 1) peptides were 0.57 and 0.58, respectively (Fig. 2).

3.2 Protective Efficacy of Bivalent Peptide Antigens

Chickens (n = 13 per group) vaccinated with two doses of bivalent peptide antigens were intranasally infected with A/Hong Kong/213/2003 (H5N1) (clade 1) or

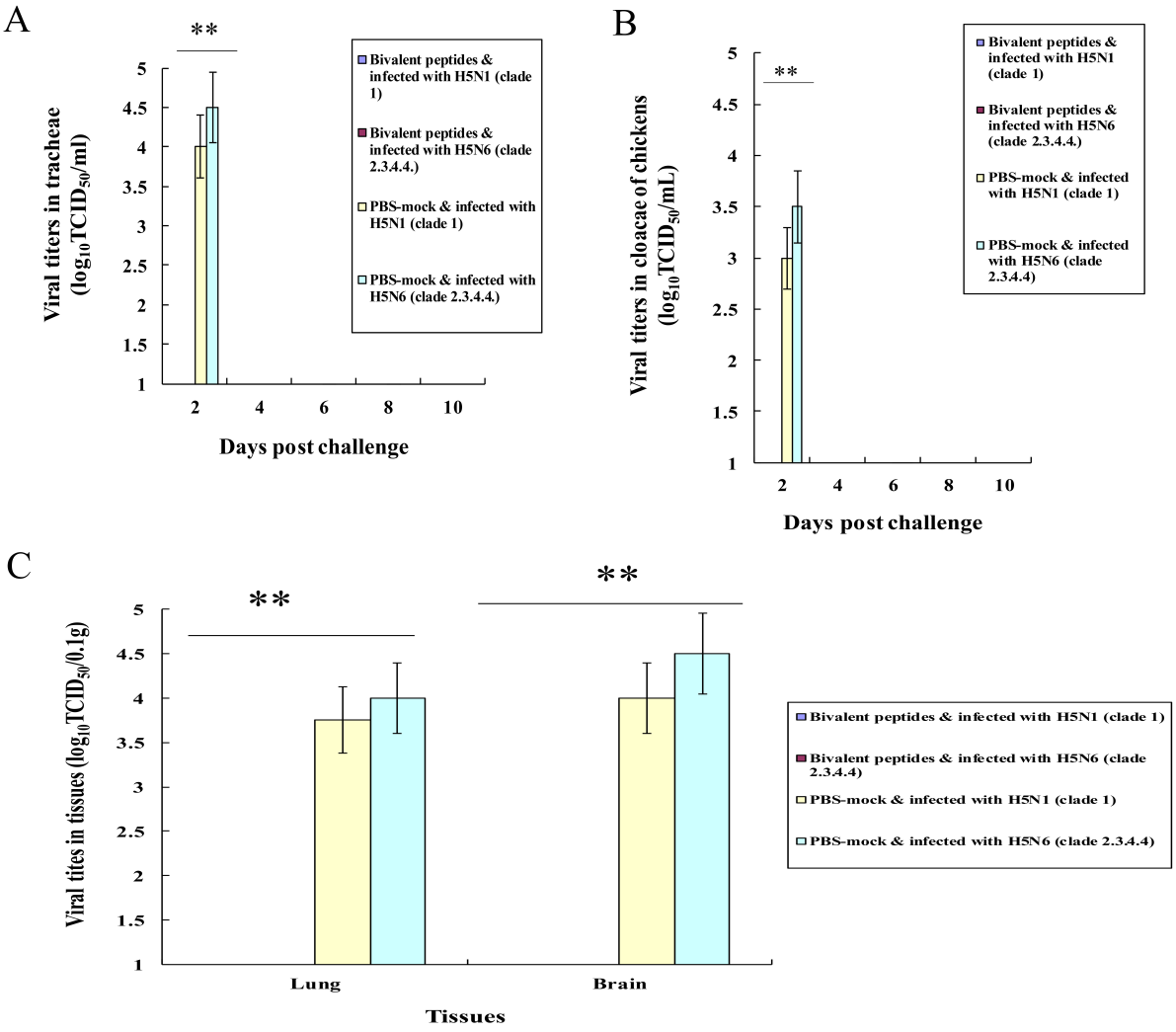

Fig. 4. Viral titers in swabbed samples and tissues in the challenged chickens. The bivalent-peptide immunized chickens were intranasally (i.n.) challenged with 1 mL (10^5 $TCID_{50}$/mL) of H5N1 (A/Hong Kong/213/2003, clade 1) or H5N6 HP (A/Waterfowl/Korea/S57/2016, clade 2.3.4.4). The surviving chickens were swabbed in the tracheas (A) and cloacae (B) with PBS (pH 7.4) for 10 days after the challenge. Three chickens per group were euthanized two days after the challenge to collect the tissues of lungs and brains (C) for viral titers. Viral titers in the samples were measured in MDCK cells by $log_{10}TCID_{50}$/mL. The limit of detection was 1 $TCID_{50}$/mL. $^{**}p < 0.001$.

A/waterfowl/Korea/S57/2016 (H5N6) (clade 2.3.4.4.), and mortality was monitored (Fig. 3). All chickens immunized with bivalent peptides survived, as did the uninfected control chickens. All PBS-inoculated chickens died at 4 days post-infection. On day 2 post-infection, the mortality rate increased up to 50–60% in PBS-mock immunized and challenged chickens.

The challenged chickens immunized with bivalent peptides did not show any clinical signs during the 10-day observation period, while the PBS-mock immunized and challenged chickens showed severe clinical signs such as hemorrhage in combs, wattles, and legs, and drooped heads and wings.

3.3 Viral Titers in Challenged Chickens

The tracheae and cloacae of challenged chickens were swabbed for 10 d, and viral titers in MDCK cells were measured based on $log_{10}TCID_{50}$/mL (Fig. 4A,B). No viruses were detected in the tracheae (Fig. 4A) and cloacae (Fig. 4B) of the challenged and bivalent peptide-immunized chickens. The mean viral titers in the tracheae of PBS-inoculated chickens on day 2 after challenge with HP H5N1 (clade 1) and HP H5N6 (clade 2.3.4.4.) were 4.0 and 4.5, respectively (Fig. 4A). The mean viral titers in cloacae of PBS-inoculated chickens on day 2 after challenge with HP H5N1 (clade 1) and HP H5N6 (clade 2.3.4.4.) were 3.0 and 3.5, respectively (Fig. 4A).

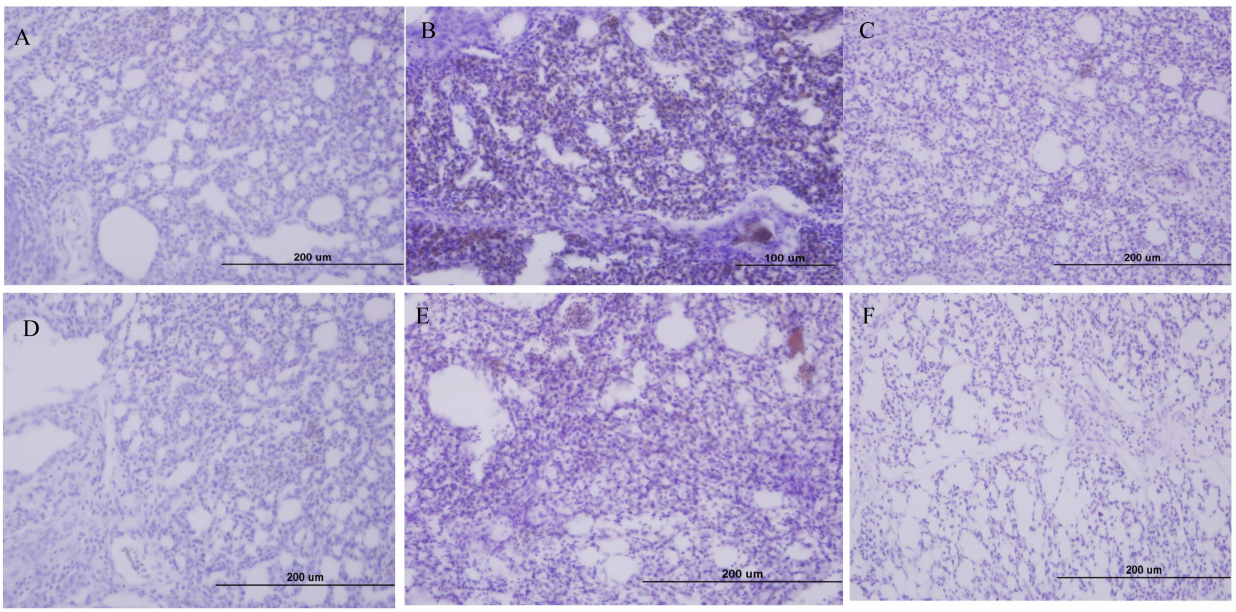

Fig. 5. Histopathology of lung tissues of the challenged immunized chickens. The lung tissues of challenged immunized chickens (n = 3 per group) with H5N1 or H5N6 avian influenza viruses were stained with hematoxylin and eosin. The stained tissues were observed under a microscope. (A,D) PBS-mock challenged naïve lung tissues of immunized chickens. (B) Lung tissue of PBS-mock immunized and challenged chickens with H5N1 avian influenza virus. (C) Lung tissue of bivalent-peptide immunized and challenged chickens with H5N1 avian influenza virus. (E) Lung tissue of PBS-mock immunized and challenged chickens with H5N6 avian influenza virus. (F) Lung tissue of bivalent-peptide immunized and challenged chickens with H5N6 avian influenza virus.

To evaluate viral infections in the tissues of challenged chickens, the chickens (n = 3 per group) were euthanized at 2 days post-infection for the collection of tissues (lungs and brains). Viral titers in tissues were measured in MDCK cells based on $\log_{10}TCID_{50}$/0.1 g of tissue. No viruses were detected in the lungs and brains of bivalent peptide-immunized and challenged chickens (Fig. 4C). The mean viral titers in the lungs and brains of PBS-inoculated chickens infected with HP H5N1 (clade 1) viruses were 3.75 and 4.0 $TCID_{50}$/0.1 g, respectively (Fig. 4C). Mean viral titers in the lungs and brains of PBS-inoculated chickens infected with HP H5N6 (clade 2.3.4.4.) viruses were 4.0 and 4.5 $TCID_{50}$/0.1 g, respectively (Fig. 4C).

3.4 Pathology of Infection in Lung Tissues of Challenged Chickens

Portions of lung tissues used for estimating viral titers were stained with H&E to evaluate pathological damage. The lung tissues (Fig. 5C,F) of bivalent peptide-immunized and challenged chickens did not show any signs of pneumonia and were similar to uninfected control chickens (Fig. 5A,D), whereas the lung tissues of PBS-inoculated and challenged chickens showed severe pneumonia with inflammatory cell infiltration (Fig. 5B,E).

4. Discussion

HP H5Nx avian influenza viruses cause major economic losses to the poultry industry worldwide. HP H5Nx viruses continue to evolve and create new variants. Therefore, the development of vaccines to protect poultry from various variants of HP H5Nx avian influenza virus is necessary. We used a dual-antigen vaccine containing clade 1 and clade 2.3.4.4. H5 cleavage-site peptides that contain polybasic amino acids to protect chickens against HP H5Nx avian influenza viruses of both clades.

Antibody response was similarly induced in chickens immunized with the bivalent peptide antigens containing HA-cleavage site RRRK and RRRKK. The results suggest that KLH-labeled H5 cleavage site peptides of clade 1 and clade 2.3.4.4. are immunogenic in chickens.

Chickens immunized with dual antigens containing H5 cleavage-site peptides of both clades 1 and 2.3.4.4. were completely protected against H5N1 and H5N6 avian influenza viruses. Chickens immunized with the bivalent peptide antigens did not show any clinical signs such as hemorrhage in comb, wattle, and legs, which appeared in the non-immunized infected chickens. We previously found that chickens immunized with clade 2.3.4.4. The previous vaccine studies did not provide complete protection for chickens against highly pathogenic avian influenza viruses, in contrast to our current study. H5 cleavage-site peptides are protected from lethal H5N6 infections of the H5N6 (clade 2.3.4.4.) avian influenza virus, but not protected from lethal infection of the H5N1 (clade 1) avian influenza virus [14]. The clade 1 H5 cleavage site contains RRRKK as polybasic amino acids, whereas the clade

2.3.4.4. H5 cleavage site contains RRRK as polybasic amino acids. The efficacy of immunization using antigens based on various ectodomains of influenza matrix protein 2 (M2e) expressed in *Escherichia coli* in chickens has been tested against HP H5N1 (A/chicken/Guangdong/04) avian influenza virus. Vaccinated chickens are partially protected from infection with HP H5N1 avian influenza virus [16]. The HA stalk regions (HA2 domain) were previously used to create a universal vaccine against avian influenza viruses. Sf9 insect cells have been used to prepare triple H5N1/NA-HA-M1 influenza VLPs by coexpressing NA, HA, and matrix proteins. Purified VLPs have been used to immunize broiler hens to produce universal antibodies. The generated universal antibodies recognize mammalian-expressed HA-stalk recombinant proteins from homologous H5N1 and heterologous H7N9 avian influenza viruses [17]. A chimeric vaccine (HA2-AtCYN) that contains subunit 2 of HA2 and *Arabidopsis thaliana* cyanase protein (AtCYN) induces HA2-specific IgA production in tears and serum IgG production, and it partially protected chickens against low-pathogenic H5N2 infections.

5. Conclusions

Antigens of bivalent H5-cleavage site peptides protected the immunized chickens from the lethal infections of HP H5N1 or H5N6 avian influenza viruses without showing clinical signs such as hemorrhage in comb, wattle, and legs. For future studies, the protective efficacy of the bivalent vaccines against the diverse strains of HP H5Nx avian influenza viruses in chickens needs to be investigated to examine the possibility of the vaccine as a universal vaccine.

Availability of Data and Materials

The data are available from the corresponding author upon reasonable request.

Author Contributions

DC & XHL performed animal experiments. SHS conceived the works and wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Animal experiments were performed according to the protocol (CNU-01191) approved by the Internal Animal Use Committee of Chungnam National University (CNU). The relevant guidelines and regulations of Chungnam National University, Republic of Korea were applied to all animal experiments.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest. Given Sang Heui Seo as Guest Editor, had no involvement in the peer-review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Dr. Giuseppe Murdaca.

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