Original Research

Differential Protection of Chickens against Highly Pathogenic H5 Avian Influenza Virus Using Polybasic Amino Acids with H5 Cleavage Peptide

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Abstract

Background: Highly pathogenic H5Nx viruses cause avian influenza, a zoonotic disease that can infect humans. The vaccine can facilitate the prevention of human infections from infected poultry. Our previous study showed that an H5 cleavage-site peptide vaccine containing the polybasic amino acid RRRK could protect chickens from lethal infections of the highly pathogenic H5N6 avian influenza virus. **Methods**: Chickens immunized with the various polybasic amino combinations (RRRK, RRR, RR, R, RK, and K) of H5 cleavage-site peptides were challenged with highly pathogenic H5N6 avian influenza viruses. The challenged chickens were monitored for survival rate, and viral titers in swabs and tissue samples were measured in Madin-Darby canine kidney (MDCK) cells using the median tissue culture infectious dose 50 ($\log_{10}$ TCID$_{50}$/mL). **Results**: Most H5 cleavage-site vaccines containing various combinations of polybasic amino acids protected chickens from lethal infection. Chickens immunized with the RK-containing peptide combination of the H5 cleavage site were not protected. **Conclusions**: The polybasic amino acids (RRRK) of H5 cleavage cleavage-site peptide vaccines are important for protecting chickens against HP H5N6 avian influenza virus. The H5 cleavage cleavage-site peptide containing RK did not protect chickens against the virus.

Keywords: vaccine; peptide; highly pathogenic avian influenza virus

1. Introduction

The highly pathogenic (HP) H5Nx viruses belong to the influenza A virus group of the family Orthomyxoviridae and contain enveloped RNA genomes [1]. Their genomes consist of eight segments that encode 10 proteins: polymerases—polymerase basic proteins 1 and 2, and polymerase acidic protein; two major surface proteins—hemagglutinin (HA) and neuraminidase; matrix proteins M2 and M1; non-structural proteins NS1 and NS2; and the nucleocapsid protein [1–3].

The influenza A virus has caused previous human pandemics: the H1N1, H2N2, H3N2, and H1N1 pandemics in 1918, 1957, 1968, and 2009, respectively [1]. HP H5Nx avian influenza viruses are considered potential pandemic viruses because they have infected numerous poultry flocks, including chickens and ducks in many countries, and are endemic to wild birds [4–11].

To control the outbreak of HP H5Nx viruses, conventional inactivated vaccines were developed to protect poultry [12,13]. Vaccine efficacy is related to neutralizing antibodies against the HA protein on the surface of avian influenza viruses.

Various methods were used to develop vaccines against HP H5Nx avian influenza viruses for poultry. Plant-derived H5 hemagglutinin antigens protected chickens from the lethal infections of HP H5N1 avian influenza viruses [14]. Newcastle disease virus expressing H5 hemagglu-tinin protein protected chickens from the HP H5N2 avian influenza viruses [15]. The H5/H7 trivalent inactivated vaccines protected chickens, ducks, and geese from the infections of HP H5N1, H5N8, and H7N9 viruses [16].

Our previous study showed that an H5 cleavage-site polybasic peptide vaccine (RRRK-containing cleavage peptide) protected chickens against the HP H5 viruses [17]. In this study, we investigated the role of each amino acid of RRRK in its action against the HP H5 avian influenza virus.

2. Materials and Methods

2.1 Viruses and Cells

HP avian influenza virus, A/waterfowl/Korea/S57/2016 (H5N6) (clade 2.3.4.4.), containing the polybasic amino acids (RRRK) at the HA cleavage site, was employed in this study. Madin–Darby canine kidney (MDCK) cells (American Type Culture Collection, VA, USA) were used for viral amplification. The MDCK cell line was validated by STR profiling and tested negative for mycoplasma. Cells were all cultured in a humidified incubator at 37 °C and 5% CO$_2$. MDCK cells were maintained in a minimal essential medium (MEM) (Sigma, MO, USA) supplemented with 10% fetal bovine serum and antibiotic–antimycotic solution (Sigma-Aldrich, MO, USA). All studies on HP avian influenza viruses were performed within a Korean government-certified biosafety level 3 laboratory facility.

Table 1. Cleavage-site peptides used for vaccine study.

Peptide sequences	Peptide names
KLH-TGLRNSPLRE**RRRK**R/GLFGAIAGFIEGGWQ	RRRK-containing peptide
KLH-TGLRNSPLRE**RRR**R/GLFGAIAGFIEGGWQ	RRR-containing peptide
KLH-TGLRNSPLRE**RR**R/GLFGAIAGFIEGGQ	RR-containing peptide
KLH-TGLRNSPLRE**RR**/GLFGAIAGFIEGGWQ	R-containing peptide
KLH-TGLRNSPLRE**RK**R/GLFGAIAGFIEGGWQ	RK-containing peptide
KLH-TGLRNSPLRE**K**R/GLFGAIAGFIEGGWQ	K-containing peptide
KLH-TGLRNSPLRER/GLFGAIAGFIEGGWQ	RRRK(-) peptide

KLH, keyhole limpet hemocyanin.

2.2 Synthesis of H5 Cleavage-Site Peptide Antigen

Synthesized H5 cleavage-site peptides were obtained from Peptron Co. (Daejeon, South Korea) and labeled with keyhole limpet hemocyanin. The synthesized peptides are listed in Table 1.

2.3 Chicken Vaccination with Peptides Containing Polybasic Amino Acids and Sera Collection from Immunized Chickens

Fertilized eggs (from white leghorns) were obtained from local farms and hatched in the laboratory. The hatched chicks were grown for 2 weeks before utilization for the vaccine study. Chickens (n = 13 per group) were intramuscularly inoculated with 300 μL containing 5.0 μg of keyhole limpet hemocyanin-labeled H5 cleavage-site peptide and 30% oil (SEPPIC, Courbevoie, France). Booster doses were administered to the immunized chickens 3 weeks after the first dose.

2.4 Antibody Titer Measurement of the Collected Chicken Sera

Immunized chicken sera were collected a week after administration of the second dose to measure antibody induction.

Enzyme-linked immunosorbent assay (ELISA) was performed to determine antibody titers with bovine serum albumin (BSA)-labeled peptides: BSA-TGLRNSPLRERRRKR/GLFGAIAGFIEGGWQ, BSA-TGLRNSPLRERRRR/GLFGAIAGFIEGGWQ, BSA-TGLRNSPLRERRR/GLFGAIAGFIEGGQ, BSA-TGLRNSPLRERR/GLFGAIAGFIEGGWQ, BSA-TGLRNSPLRERKR/GLFGAIAGFIEGGWQ, BSA-TGLRNSPLREKR/GLFGAIAGFIEGGWQ, and BSA-TGLRNSPLRER/GLFGAIAGFIEGGWQ.

The wells in the immunoplate were coated with 100 μL of peptide (4 μg/mL) in coating buffer, incubated overnight at 4 °C, and then washed three times with PBS–tween 20 (0.05%). The wells were blocked with 100 μL of 1% BSA in PBS (pH 7.4) for 2 h at room temperature and then 100 μL of diluted chicken sera in PBS (1:100) was added. The plates were incubated for 1 h at room temperature. The wells were washed five times and 100 μL of horseradish peroxidase-conjugated rabbit anti-chicken antibody (Sigma-Aldrich) diluted in PBS (1:5000) was added. The plates were incubated for 1 h at room temperature. The wells were washed five times with PBS–Tween 20 (0.05%) and 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate solution (Thermo Fisher Scientific, Waltham, MA, USA) was added. The wells were incubated for 30 min at room temperature. The reaction was stopped with 50 μL of 2.5 M sulfuric acid. Optical density (OD) was measured at 450 nm using an ELISA spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

2.5 Challenging Peptide-Immunized Chickens with HP H5N6 Virus

The vaccinated chickens were intranasally infected with 1 mL (10^5 TCID$_{50}$/mL) of H5N6 virus. The tracheas and cloacae of infected chickens were swabbed with PBS (pH 7.4) for 10 days post-infection before the viral titers were determined via log$_{10}$ TCID$_{50}$/mL (log10 tissue culture infectious dose 50) using MDCK cells. Chickens (three per group) were euthanized 2 days after infection, and tissues (lungs and brains) were collected in PBS (pH 7.4) to measure viral titers.

2.6 Measuring Viral Titers Using log$_{10}$ TCID$_{50}$/mL

Confluent MDCK cells were treated with trypsin–EDTA (Sigma-Aldrich) and seeded in a tissue culture plate (96 wells) with MEM containing 10% fetal bovine serum and 1× Antibiotic–Antimycotic Solution (Sigma-Aldrich). The cells were washed with warm PBS (pH 7.4) before inoculation with collected samples (tracheal and cloacal swabs, and lung and brain tissues). Samples were 10-fold diluted in MEM containing 1.5% BSA prior to inoculation. Prepared samples were inoculated into cells (four wells per diluted sample) for 5 days in a humidified incubator (37 °C, 5% carbon dioxide). Cells were observed for cytopathic effects. The viral titer (log$_{10}$ TCID$_{50}$/mL) was determined using the method described by Muench and Reed [18].

2.7 Statistical Analysis

Student's t-test using IBM SPSS Statistics version 20 software (IBM Corp., Armonk, NY, USA) was performed to analyze statistical significance. Statistical significance was based on p-values less than 0.05.
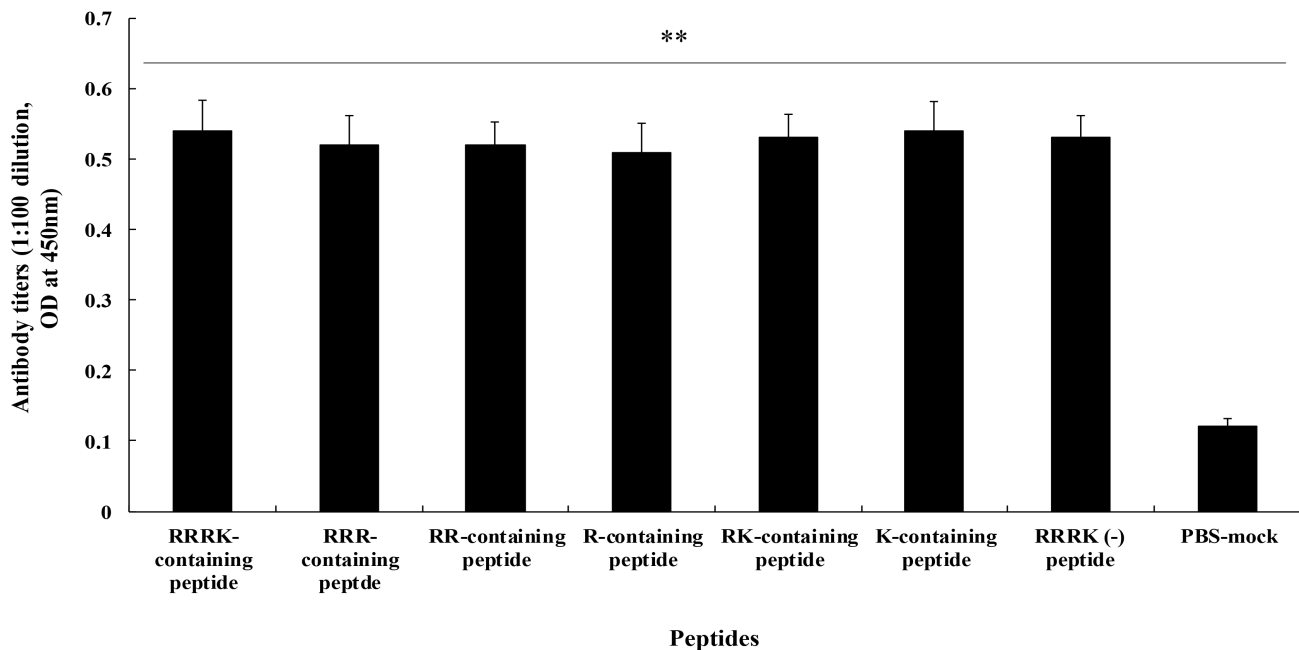
Fig. 1. Antibody titers in chickens immunized with H5 cleavage-site peptides containing different combinations of polybasic amino acids. Sera were collected from the intramuscularly immunized chickens (n = 13 per group)—with two doses (5.0 μg per dose) of oil-adjuvanted HLA-labelled peptides—4 weeks after the vaccination. Antibody titers were measured via ELISA using BSA-labelled peptides. **$p < 0.01$. ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

3. Results

3.1 Antibody Induction in Chickens Immunized with H5 Cleavage Peptide Antigens

Fig. 1 shows the antibody titers measured using ELISA in sera (n = 10 per group) collected from chickens vaccinated with two doses of H5 cleavage-site peptide containing RRRK. Antibodies were similarly induced in chickens immunized with H5 cleavage-site peptides containing diverse combinations of polybasic amino acids (Table 1), including peptides containing RRRK, RRR, RR, R, RK, K, and RRRK(-), with a mean OD range of 0.51–0.54. The mean OD value for the sera from PBS-mock vaccinated chickens was 0.12.

3.2 Survival Rate of Chickens Vaccinated with H5 Cleavage Peptide Antigens

We aimed to determine the role of individual amino acids of RRRK in protecting chickens from HP avian influenza viral infections. Chickens immunized with most combinations of polybasic amino acids survived when intranasally challenged with the HP H5N6 virus (Fig. 2). All the immunized chickens with H5 cleavage-site peptides, including RRRK-, RRR-, RR-, R-, and K-containing peptides survived, whereas all chickens immunized with RK-containing peptide died 6 days post-infection. All chickens immunized with RRRK(-) peptide and PBS-mock did not survive (Fig. 2).

3.3 Viral Titers of Immunized Chickens Infected with HP H5N6 Virus

No viruses were detected in the tracheal (Fig. 3A) and cloacal swabs (Fig. 3B) of chickens immunized with H5 cleavage-site peptides, including RRRK-, RRR-, RR-, R-, and K-containing peptides. In contrast, high viral titers were detected in tracheal and cloacal swabs in the chickens immunized with RK-containing peptide in the range of 2.0 to 4.5 $TCID_{50}$/mL (Fig. 3A,B). In addition, similar viral titers were detected in the chickens immunized with either RRRK(-) peptide or PBS-mock in the range of 1.5 to 5.0 $TCID_{50}$/mL (Fig. 3A,B).

The infected chickens (n = 3 per group) were euthanized to collect the lungs and brains 2 days post-infection to measure tissue viral titers. No virus was detected in either the lung or brain tissues of the chickens immunized with H5 cleavage-site peptides, including RRRK-, RRR-, RR-, R-, and K-containing peptides (Table 2), whereas high viral titers were detected in both the lung and brain tissues of chickens immunized with the RK-containing peptide, in a range of 3.0 to 3.5 $TCID_{50}$/mL (Table 2). Similar viral titers were detected in the lungs and brain tissues of chickens immunized with either RRRK(-) peptide or PBS-mock in the range of 3.5 to 4.5 $TCID_{50}$/mL (Table 2).

4. Discussion

HP H5Nx avian influenza viruses continue to infect poultry flocks, resulting in significant economic losses in the poultry industry. In addition, these viruses can infect
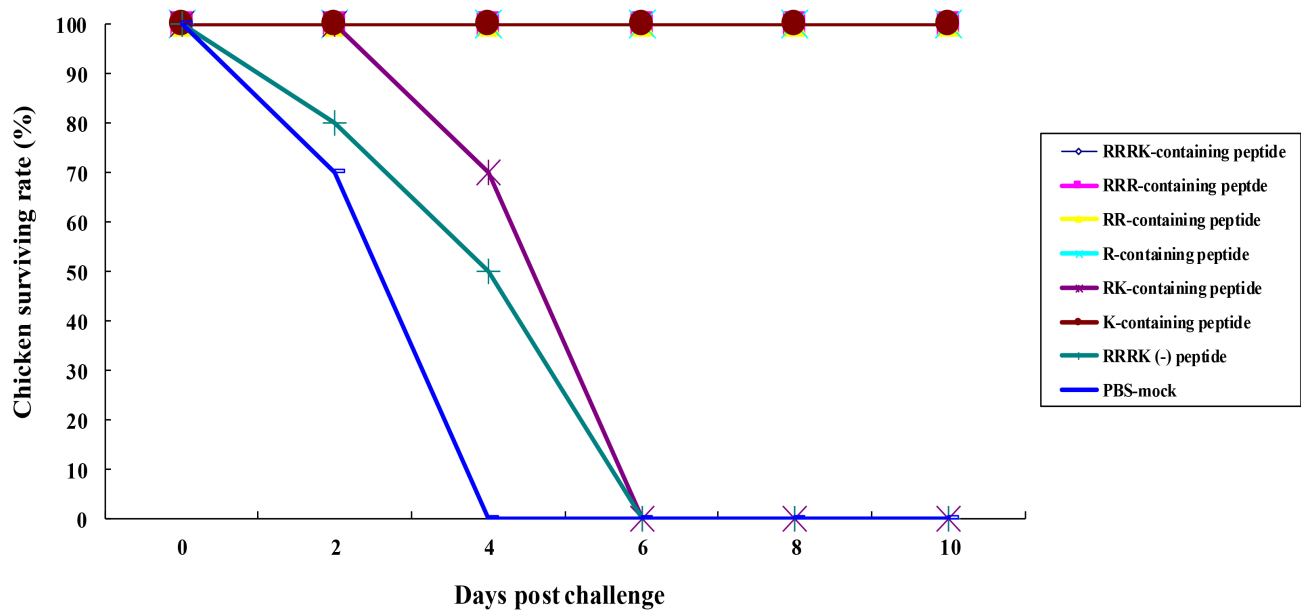
Fig. 2. Mortality of the immunized chickens with H5 cleavage-site peptides containing different combinations of polybasic amino acids. The immunized chickens (Fig. 1) were intranasally challenged with 1 mL (10^5 TCID$_{50}$/mL) of A/Waterfowl/Korea/S57/2016 (H5N6). The challenged chickens were monitored for survival rate.
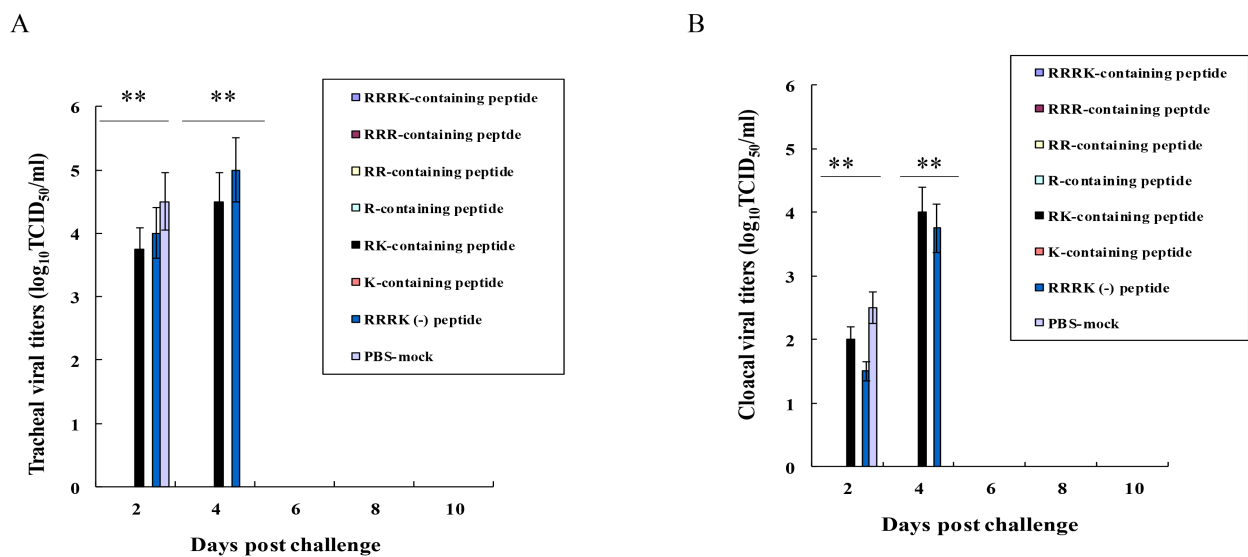

Fig. 3. Viral titers in swabbed samples in the challenged chickens. Swabs in tracheas (A) and cloacae (B) in the challenged chickens (Fig. 2) were performed in a 2-day interval until 10 days p.i. Viral titers in the swabbed samples were measured using Madin-Darby Canine Kidney (MDCK) cells by log$_{10}$ TCID$_{50}$/mL. The limit of detection was 1 TCID$_{50}$/mL. **$p < 0.01$.

humans and are considered potential pandemic pathogens. We developed a vaccine based on HA cleavage-site peptides containing polybasic amino acids to protect poultry from infections caused by the HP H5 virus.

This study was performed to determine the potential protective role of individual polybasic amino acids in chickens immunized against the HP H5N6 virus. Our results showed that most combinations of polybasic amino acids in the cleavage-site peptide provided complete protection against the HP H5N6 virus in immunized chickens. The

RK-containing H5 cleavage-site peptide vaccine did not protect immunized chickens against the virus. The development of broad-spectrum protection strategies against H5Nx infections has been attempted. The herpesvirus of turkeys was developed as a vector expressing the HA antigen of the HP H5N1 virus [19]. Layers vaccinated with this vector 2 weeks prior to infection with various HP H5N1 viruses were fully protected, with reduced shedding of the viruses. Newcastle disease virus vectors encoding H7 or H5 HA were shown to provide complete protection in chickens against

Table 2. Viral titers in lungs and brains of the challenged chickens.

Vaccine antigens	Mean viral titers in lungs ($\log_{10}$ TCID$_{50}$/0.1 g)	Mean viral titers in brains ($\log_{10}$ TCID$_{50}$/0.1 g)
RRRK-containing peptide	-	-
RRR-containing peptide	-	-
RR-containing peptide	-	-
R-containing peptide	-	-
RK-containing peptide	3.0 ± 0.5	3.5 ± 0.5
K-containing peptide	-	-
RRRK(-) peptide	3.5 ± 0.25	4.0 ± 0.25
PBS-mock	4.0 ± 0.5	4.5 ± 0.5

-: under detection limit, 1 TCID$_{50}$/0.1 g. Tissues (lungs and brains) were collected from the challenged chickens (n = 3 per group) (Fig. 2) 2 days after the challenge, and viral titers in them were measured using MDCK cells by $\log_{10}$ TCID$_{50}$/mL.

HP H7N9 or H5N1 viruses [20]. A chimeric QH/KJ recombinant virus vaccine encoding the HA-1 part of the HP virus A/chicken/China/QH/2017 (H5N6) (clade 2.3.4.4.), and HA-2 part of the HP virus A/chicken/China/KJ/2017 (H5N1) (clade 2.3.2.1.) completely protected immunized chickens against HP H5N6 and HP H5N1 viruses [21].

Our study showed that the H5 cleavage-site peptide containing the RK combination of polybasic amino acids (RRRK) did not protect immunized chickens from HP H5N6 virus infection. The lack of protection of chickens from the vaccine with RK-containing peptides may be due to the absence of antibody induction, which could bind to polybasic amino acids (RRRK) of the HP H5N6 virus. However, the underlying mechanism requires further investigation.

Keyhole limpet hemocyanin (KLH) was used for stimulating the immune response of H5 cleavage-site peptide vaccines. It is assumed that KLH could stimulate CD4$^+$ T lymphocytes, resulting in the production of various cytokines. The produced cytokines might help B lymphocytes to produce peptide-specific antibodies involved in protecting chickens from infections of the HP H5 avian influenza virus. If KLH alone is used for the vaccine, it might not protect chickens from the lethal infections of HP H5 avian influenza viruses since it may not induce the H5 cleavage-site-specific antibodies.

Polybasic amino acids are a determining factor for HP avian influenza viruses. The deletion of these amino acids for making infectious live attenuated vaccine is necessary to prevent reversion to HP avian influenza viruses. Our cleavage-site peptide containing polybasic amino acids could not be a source for HP reversion since the peptide could not contribute to the creation of a recombinant virus. The previous study to make fowlpox vaccine viruses co-expressing avian influenza H5 and chicken IL-15 deleted the polybasic amino acids in the H5 cleavage site [22].

We used homologous BSA-conjugated peptides to measure antibody titers. Further study may need to include heterologous BSA-conjugated peptides to find out the difference in antibody levels.

5. Conclusions

The polybasic amino acids (RRRK) of H5 cleavage cleavage-site peptide vaccines are important for protecting chickens against HP H5N6 avian influenza virus. The H5 cleavage cleavage-site peptide containing RK did not protect chickens against the virus.

Availability of Data and Materials

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

Author Contributions

SHS had the sole role in designing, data collecting, analyzing, and writing the manuscript.

Ethics Approval and Consent to Participate

The vaccine efficacy study in chickens was performed following the protocol (CNU-01191) approved by the Chungnam National University (CNU) Internal Animal Use Committee (202307A-CNU-115). All experiments were performed in accordance with the relevant guidelines and regulations of Chungnam National University, Republic of Korea.

Conflict of Interest

The author declares no conflict of interest. Given his role as Guest Editor, SHS 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 Giuseppe Murdaca.

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