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Efficacy of a Recombinant Newcastle Disease Virus Expressing H5N8 HA Against H5N1 Avian Influenza Virus (Clade 2.3.4.4b) in Broiler Chickens

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Abstract

Background: Since November 2022, the highly pathogenic avian influenza (HPAI) virus subtype H5N1 clade 2.3.4.4b has caused high mortality among wild birds and poultry in Peru. Vaccines, such as recombinant Newcastle disease virus (rNDV)-vectored vaccines expressing the hemagglutinin (HA) antigen of avian influenza virus (AIV), are considered effective for preventing AI; however, the HA protein in the vaccine must be antigenically matched to the field virus to prevent mortality and virus shedding caused by AI. In this study, we developed a live rNDV-vectored vaccine expressing an HA protein with antigenic similarity to the HA of field-circulating AIV. **Methods**: The rNDV rLS1 strain (the parental virus) was used as a viral vector to develop the rLS1-HA_{Opti} vaccine (an rNDV expressing the HA protein of H5N8 clade 2.3.4.4b) through reverse genetics. The immunogenicity and efficacy of the live vaccine against an HPAI H5N1 (clade 2.3.4.4b) challenge were evaluated in broiler chickens. **Results**: The vaccine induced a significant serum humoral response. After the H5N1 challenge, the vaccine provided protection and prevented virus shedding in most of the chickens. **Conclusions**: The rNDV vaccine candidate provided complete protection against mortality and clinical disease in broiler chickens challenged with H5N1 under the tested experimental conditions.

Keywords: Newcastle disease virus; highly pathogenic avian influenza virus; H5N1

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1. Introduction

Avian influenza virus (AIV), a member of the genus *Alphainfluenzavirus* and the family *Orthomyxoviridae* [1], has a negative-sense, single-stranded ribonucleic acid (RNA) genome [2]. The highly pathogenic avian influenza (HPAI) H5N1 subtype continues to cause high mortality and financial losses in the poultry sector worldwide [3]. The H5N1 genome can undergo mutations and re-assortment events, resulting in the emergence of other strains or subtypes with different pathogenicities, which increases the potential for the virus to persist within birds and cross species barriers [2,4,5]. For this reason, it is considered a threat to public health [3,4]. Therefore, there is an urgent need to develop vaccines with an antigenic profile that matches the circulating field strain, in order to prevent and control AI [6,7]. Although conventional inactivated vaccines against AIV are

widely used to control this disease [8,9], their efficacy is limited due to insufficient mucosal protection, incompatibility with the "Differentiating Infected from Vaccinated Animals" (DIVA) strategy, a suboptimal cellular immune response, and an inability to prevent viral shedding [10]. Consequently, live vector-based vaccines expressing H5 hemagglutinin (HA) have become a key alternative for addressing these unresolved limitations [8].

Newcastle disease virus (NDV) belongs to the genus *Orthoavulavirus* and the family *Paramyxoviridae* [11]. It has a negative-sense, single-stranded RNA genome [12]. Lentogenic strains (which cause mild or asymptomatic infections in chickens) of NDV, such as LaSota, are widely used as backbones for the construction of recombinant Newcastle disease virus (rNDV) live vaccines using reverse genetics [13]. rNDVs have been used as vectors to express human interleukins [14] or major protective antigens from human pathogens [15] and other avian pathogens [16,17]. Specifically, several rNDV-based influenza vaccines that express the HA proteins from the H5N1 subtype (clade 2.3.4.4b) [18], H5N2 [19], and H5N6 (clade 2.3.4.4e) [20] have demonstrated effective protection against an HPAI virus (HPAIV) challenge. These vaccines have various advantages, as they can be administered by the ocular/nasal route in poultry on a large scale and induce mucosal, humoral, and cellular immune responses [8]. Furthermore, they can be rapidly modified to target specific AIV strains circulating in the field [8].

Peru was considered an HPAIV-free country; however, in November 2022, the presence of HPAI H5N1 virus was confirmed in wild waterfowl (pelicans) in different regions of the country [21]. Therefore, a health alert was declared in Peru for the risk of the spread of HPAI H5N1 to domestic poultry [21]. Sequencing and phylogenetic analyses of the viral genome confirmed that the H5N1 strain belonged to clade 2.3.4.4b [22]. In 2023, a human case with HPAI H5N1 (clade 2.3.4.4b) infection was reported in Chile, which was considered a potentially significant public health event [23].

Therefore, in this study, we developed a live rNDV-vectored vaccine expressing the HA of H5N8 clade 2.3.4.4b (rLS1-HA_{Opti}) and evaluated its efficacy against an HPAI (A/Pelecanus/Peru/VFAR-140/2022) H5N1 clade 2.3.4.4b challenge in broiler chickens.

2. Materials and Methods

2.1. Chickens and Ethics

In this study, one-day-old broiler chickens from commercial sources were used to evaluate the efficacy of the vaccine. The broiler chickens were housed in cages in Biosafety Level 2 (BSL-2) facilities. Water and feed were provided ad libitum throughout the experimental period. At the time of the challenge, the chickens were identified with number/colored tags and placed in a Biosafety Level 3 (BSL-3) laboratory.

All experiments conducted in this study were performed in accordance with protocol No. 03-2023, approved on 5 March 2023, by the Ethics Committee of the Faculty of Veterinary Medicine and Zootechnics (FVMZ) of Hermilio Valdizan National University.

2.2. Cell Culture, Conventional Control Vaccine, and Challenge Virus

Chicken embryo fibroblasts (DF1) and Vero E6 cells (ATCC® CRL-1586™) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). DF1 and Vero cell cultures were maintained in 1× modified Dulbecco's medium (DMEM) (catalog No. SH30004.04, HyClone™, Logan, UT, USA) supplemented with 5% heat-inactivated bovine serum with iron (catalog No. SH30072.04, Hyclone™, Logan, UT, USA) and 1 × antibiotic–antimycotic (catalog No. 15240062, Thermo Fisher Scientific, Waltham, MA, USA). Both cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

An inactivated conventional control vaccine against H5N1 was manufactured by FARVET S.A.C. utilizing a commercial Montanide adjuvant formulation. This inactivated

vaccine contained an antigen fully homologous to the circulating field strain used for the experimental challenge, specifically the HPAI H5N1 strain A/Pelecanus/Peru/VFAR-140/2022.

The HPAIV A/Pelecanus/Peru/VFAR-140/2022 (H5N1) strain was pre-identified and sequenced by FARVET S.A.C., and its genotype was confirmed to belong to clade 2.3.4.4b. The eight genome sequences/segments were deposited in GenBank under accession numbers OQ565625–OQ565632 [22]. This HPAI H5N1 strain was used as both the challenge virus and as an antigen in the hemagglutination inhibition (HI) test.

2.3. Construction of Plasmids

The rLS1-HA$_{Opti}$ recombinant virus was generated using the pFLC-LS1 plasmid, which contains the full-length complementary deoxyribonucleic acid (cDNA) of the genome of a derivative of the LaSota strain (rLS1), as previously described in [13]. The open reading frame (ORF) encoding the HA of the HPAIV strain Broiler chicken/Giza/VRLCU/2019 (H5N8), which belongs to clade 2.3.4.4b (GenBank accession No. MT256068.1), was codon-optimized to *Gallus gallus* using the GenSmart™ Codon Optimization tool to enhance gene expression. The deoxyribonucleic acid (DNA) fragment containing BbvCI digestion sites was added upstream and downstream of the DNA sequence; after synthesis, it was cloned into plasmid pUC57 by GenScript (Piscataway, NJ, USA), resulting in the plasmid HA$_{Opti}$–pUC57.

The pFLC-LS1 plasmid, which contains a single BbvCI restriction site located between the phosphoprotein (P) and matrix (M) genes, was linearized by digestion with the BbvCI restriction enzyme; this was verified by agarose gel electrophoresis. Subsequently, the HA$_{Opti}$–pUC57 plasmid, which carries the HA$_{Opti}$ expression cassette, was digested with the same enzyme, and the resulting DNA fragment was purified and ligated into the linearized pFLC-LS1 backbone, allowing for transcription as a separate unit. The resulting recombinant plasmid was designated as pFLC-LS1-HA$_{Opti}$ (Figure 1A). The integrity of the construct was verified by restriction analysis and sequencing.

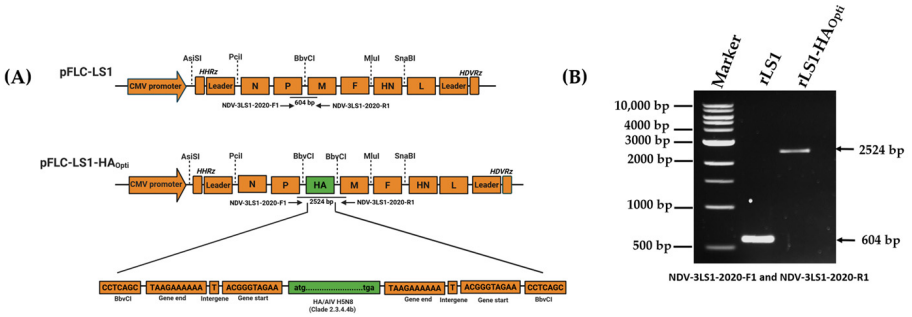


Figure 1. Generation of rNDV expressing the HA gene from H5N8. (**A**) Schematic of the strategy used to construct the rNDV expressing the HA protein. The full-length anti-genome derived from an NDV strain LaSota clone (pFLC-LS1 plasmid) was used as the backbone; the pFLC-LS1-HA$_{Opti}$ plasmid was generated using a cassette that contained the complete ORF of the HA from H5N8 (clade 2.3.4.4b). The primer names, positions, and directions are shown with arrows (black) indicating the size of the PCR products. This drawing was created with BioRender.com. (**B**) The insertion of the HA$_{Opti}$ expression cassette in the rNDV genome was verified by RT-PCR using the primers NDV-3LS1-2020-F1 and NDV-3LS1-2020-R1 (as shown in (**A**)) for both rLS1-HA$_{Opti}$ (~2524 bp, base pairs = bp) and rLS1 (~604 bp) viruses.

2.4. Generation and Rescue of the Recombinant rLS1-HA$_{Opti}$ Virus

In brief, the rLS1-HA$_{Opti}$ virus was recovered by co-transfection of the full-length plasmid pFLC-LS1-HA$_{Opti}$ together with the support plasmids pCI-N, pCI-P, and pCI-L, as previously described in [13]. The recovered virus was then propagated in 9-day-old

specific pathogen-free (SPF) chicken embryonated eggs (AVS Bio, Norwich, CT, USA). The allantoic fluid (AF) was collected after 4 days of incubation, clarified, and then stored at −80 °C. The rescued virus stock was verified using the hemagglutination (HA) assay and reverse transcription polymerase chain reaction (RT-PCR) with specific primers that amplified the intergenic region between the P and M genes. The integrity of the HA_{Opti} expression cassette in the rLS1-HA_{Opti} virus genome was confirmed by sequencing. The vaccine was evaluated using the intracerebral pathogenicity index (ICPI) test, following standard procedures [24].

2.5. Detection of the HA Gene of the rLS1-HA_{Opti} Virus by RT-PCR

To detect the presence of the HA protein expression cassette in the rLS1-HA_{Opti} recombinant virus genome, the RNA was extracted from AF stocks using the QIAamp MinElute Virus Spin kit (catalog No. 57704, Qiagen, Valencia, CA, USA). cDNA was generated from this RNA using the ProtoScript II cDNA Synthesis kit (catalog No. M0368, NEB, New England BioLabs, Ipswich, MA, USA) according to the manufacturer's instructions. The cDNA was amplified using the Q5® high-fidelity DNA polymerase Master Mix (catalog No. M0492S, NEB, New England BioLabs, Ipswich, MA, USA). The primer sequences to amplify the non-coding region between the P and M genes were as follows: 5′-GATCATGTCACGCCCAATGC-3′ (NDV-3LS1-2020-F1) and 5′-GCATCGCAGCGGAAAGTAAC-3′ (NDV-3LS1-2020-R1). The amplification was carried out with an initial denaturation step at 98 °C for 30 s, followed by 35 cycles of amplification: 98 °C for 10 s, annealing at 65 °C for 15 s, and extension at 72 °C for 1 min and 20 s. The polymerase chain reaction (PCR) was performed on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

2.6. Western Blotting

To detect the HA protein expression in the lysate of cells infected with the recombinant virus, DF1 cells grown in 6-well plates were infected with the rNDV expressing HA (rLS1-HA_{Opti}) or parental virus (rLS1) at a multiplicity of infection (MOI) of 0.01. At 48 h post-infection (hpi), the infected cells were detached from the surface of the wells and centrifuged at 7000 rpm (revolutions per minute) for 10 min at 4 °C using a ROTINA 380R centrifuge (Hettich, Tuttlingen, Germany). The supernatant was discarded, the cells were washed with 1× Dulbecco's phosphate-buffered saline (1× DPBS), and lysed with lysis buffer. To verify whether the AIV HA protein had been incorporated into the structure of the rLS1-HA_{Opti} recombinant virus, viral particles from the AF of SPF chicken embryonated eggs infected with the HA-expressing virus or parental virus were harvested, concentrated at 18,000 rpm at 4 °C overnight using a Type 19 rotor, and then purified through a 30% sucrose cushion for 6 h at 24,000 rpm, and at 4 °C using an SW-28 rotor in an Optima L-100K Ultracentrifuge (Beckman Coulter Inc., Brea, CA, USA). Western blot analysis was carried out on the infected cell lysates and purified viral particles using a polyclonal rabbit antibody specific to Influenza A H5N8 HA (diluted 2:5000; catalog No. 11717-RP02, Sino Biological, Beijing, China) as the primary antibody and anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (diluted 2:5000; catalog No. A01827, GenScript Laboratories, Piscataway, NJ, USA) as the secondary antibody. The detection of the NDV fusion (F) surface glycoprotein was used as an internal and positive control for infection in treated cells; the F protein was detected using a specific rabbit polyclonal antibody against NDV F protein (diluted 10:5000; GenScript Laboratories, Piscataway, NJ, USA) as the primary antibody and a mouse anti-rabbit IgG antibody conjugated to HRP (diluted 2:5000; catalog No. A01827, GenScript Laboratories, Piscataway, NJ, USA) as the secondary antibody. Beta-actin was used as a loading control for the cell lysates; it was

detected using a mouse monoclonal antibody against beta-actin (diluted 5:5000; catalog No. ab8226, Abcam, Cambridge, MA, USA) as the primary antibody and goat anti-mouse IgG conjugated to HRP (diluted 3:5000; catalog No. A00160, GenScript Laboratories, Piscataway, NJ, USA) as the secondary antibody. The detection of hemagglutinin–neuraminidase (HN) protein expression was used to indicate the presence of NDV in purified viruses; it was detected using a mouse monoclonal [9F7] IgG1 antibody to NDV HN (diluted 2:5000; catalog No. ab38750, Abcam, Cambridge, MA, USA) as the primary antibody and goat anti-mouse IgG conjugated to HRP (diluted 3:5000; catalog No. A00160, GenScript Laboratories, Piscataway, NJ, USA) as the secondary antibody. The proteins were detected using a CCD camera and the Azure c600 imaging system (Azure Biosystems, Inc., Dublin, OH, USA). The HA (uncleaved HA0 precursor protein and cleaved subunits HA1 and HA2) and F (inactive precursor (F0) and the cleaved subunit (F1)) protein bands were quantified by densitometry using ImageJ software version 1.54g, as described previously [25]. For cell lysates, HA and F protein levels were normalized to beta-actin. For the purified viral particles, expression levels were calculated and expressed as the HA/HN densitometric ratio. All experiments were performed in triplicate.

2.7. Indirect Immunofluorescence Assay (IFA)

DF1 cell monolayers were grown in 24-well plates. After 24 h, the cell monolayers were infected with the rLS1-HA_{Opti} (5th passage) or rLS1 virus at an MOI of 0.01. At 48 hpi, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature (RT). The monolayer was then washed three times with 1× DPBS for 5 min per wash, with gentle agitation at RT, and permeabilized with 0.3% Triton X-100 for 15 min at RT. The permeabilizing agent was then removed by rinsing three times with 1× DPBS. The monolayer was then incubated with the blocking buffer (5% Bovine Serum Albumin (BSA) (catalog No. A7030-500G, Sigma-Aldrich, St. Louis, MO, USA) in 1× DPBS) for 2 h, followed by incubation with a rabbit polyclonal antibody specific to influenza A H5N8 HA (diluted 1:200 in blocking buffer; catalog No. 11717-RP02, Sino Biological, Beijing, China) as the primary antibody for 1 h 30 min at RT, and donkey F(ab')2 anti-rabbit IgG H&L-Alexa Fluor® 594 (diluted 1:250; catalog No. ab150072, Abcam, Cambridge, MA, USA) as the secondary antibody for 60 min at RT. The NDV infection process was detected using a mouse monoclonal antibody against NDV ribonucleoprotein (RNP) (diluted 1:200; catalog No. ab138719, Abcam, Cambridge, MA, USA) as the primary antibody for 1 h 30 min, followed by incubation with goat anti-mouse IgY H&L-Alexa Fluor® 488 (diluted 1:1000; catalog No. ab150105, Abcam, Cambridge, MA, USA) as the secondary antibody for 60 min at RT. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (catalog No. ab104139, Abcam, Cambridge, MA, USA) for 5 min. Digital images were taken at 630 × magnification, and fluorescence images of the treated cells were taken in 3 channels simultaneously using an AxioCam MRc5 camera (Carl Zeiss, Jena, Germany) connected to an Axio Observer A1 fluorescence microscope (Carl Zeiss, Jena, Germany).

2.8. Replication and Expression Kinetics

The rLS1 and rLS1-HA_{Opti} viruses were assessed using a multi-cycle replication kinetics assay. DF1 cells were cultured in monolayers in 12-well plates in 1× DMEM supplemented with 5% bovine serum for 24 h. The culture medium was then replaced with fresh 1 × DMEM supplemented with 2% bovine serum and 5% AF. Subsequently, the cells were infected with an MOI of 0.05 and were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The supernatant was collected at 12, 24, 36, 48, 60, 72, and 84 hpi, clarified by centrifugation at 500× *g* for 5 min at 4 °C using a ROTINA 380R centrifuge (Hettich, Tuttlingen, Germany), and stored at -80 °C until analysis. Finally, as described previously,

the virus titration was determined using the plaque assay [13]. At the same time, the cytopathic effect (CPE) on the cells was observed using brightfield microscopy with an Axio Observer A1 fluorescence microscope (Carl Zeiss, Jena, Germany); digital images were captured at 100× magnification with an AxioCam MRc5 camera (Carl Zeiss, Jena, Germany). This experiment was performed in triplicate.

To determine the kinetics of HA protein expression. Monolayers of DF1 cells were grown in 6-well plates in 1× DMEM containing 5% bovine serum. Afterward, the cells were infected with rLS1 or rLS1-HA_{Opti} at an MOI of 0.05 in DMEM containing 2% bovine serum and 5% AF and incubated at 37 °C in a humidified atmosphere with 5% CO_2. To determine the changes in HA protein expression over time, the supernatant was removed at 12, 24, 36, 48, 60, 72, and 84 hpi, and the cells were then lysed with a lysis buffer and stored at −20 °C. The lysates were then analyzed by Western blotting to detect AIV HA, NDV F, and beta-actin (loading control), following the procedure described above. The densitometric analysis of Western blotting at each time point was performed as previously described. All experiments were performed in triplicate.

2.9. Genetic Stability of the HA Protein Expression Cassette in rLS1-HA_{Opti}

The genetic stability of rLS1-HA_{Opti} was evaluated up to passage 5, which represents the maximum number of passages required for industrial vaccine scale-up.

To evaluate the genetic stability of the AIV HA protein expression cassette, the rLS1-HA_{Opti} virus was passaged in 9-day-old SPF chicken embryonated eggs up to passage 5. For each passage, the AF of the infected eggs was harvested, clarified by centrifugation at 5000× *g* for 15 min at 4 °C using a ROTINA 380R centrifuge (Hettich, Tuttlingen, Germany), and the AF was stored at −80 °C. Afterward, the presence of the HA protein expression cassette in the viral genome was verified via RT-PCR using the primers NDV-3LS1-2020-F1 and NDV-3LS1-2020-R1 at passages 1 and 5, following the procedure described above. To evaluate HA protein expression, DF1 cell monolayers were infected with rLS1-HA_{Opti} virus from passages 1 and 5; at 48 hpi, the infected cells were evaluated via Western blotting and IFA, as previously described, and compared with cells infected with rLS1.

2.10. Experimental Design

To evaluate the immunogenicity and efficacy of the rLS1-HA_{Opti} vaccine against HPAIV H5N1, twenty-one 1-day-old, unsexed broiler chickens were systematically allocated into three experimental groups ($n = 7$ birds per group): group #1 (non-vaccinated controls), group #2 (vaccinated with rLS1-HA_{Opti} vaccine), and group #3 (vaccinated with inactivated AIV vaccine). All groups were vaccinated at one day of age. Each group was housed in a separate cage. Birds were selected based on strict criteria of physical homogeneity and the absence of apparent clinical signs, with individuals sequentially assigned to each group at the onset of the study to achieve maximum baseline uniformity across groups.

Group #2 was vaccinated with the live rLS1-HA_{Opti} vaccine at a dose of 5×10^6 Plaque-Forming Units (PFU)/chicken in a total volume of 200 µL through the oculo-naso-oral route (50 µL ocularly, 50 µL intranasally, and 100 µL orally). Fifteen days after the primary vaccination, a booster immunization was administered utilizing the same dose of 5×10^6 PFU/chicken in 200 µL via the identical oculo-naso-oral route (50 µL ocularly, 50 µL intranasally, and 100 µL orally).

Group #3 was immunized via subcutaneous injection with a 0.1 mL dose of the conventional inactivated H5N1 vaccine, according to the manufacturer's recommendations.

Blood samples were collected from all experimental groups at designated time points to evaluate the humoral immune response against HPAIV.

To assess vaccine efficacy, all animals were transferred to the BSL-3 facility and assigned numbered/colored tags for identification. At 39 days post-vaccination (dpv), groups #1, #2, and #3 were challenged with HPAI A/Pelecanus/Peru/VFAR-140/2022 (H5N1) virus at a dose of 10^6 50% embryo infective dose (EID_{50})/chicken (HA titer 3 Log_2) in 200 µL (100 µL ocularly and 100 µL intranasally). Clinical signs and mortality were monitored daily for 14 days post-challenge (dpc). Swabs from the palatal cleft and cloaca were collected at 6 and 9 dpc to evaluate viral shedding using real-time RT-PCR.

Animal allocation and initial cage labeling (group #1, group #2, and group #3) were performed by independent technical staff who were not involved in the authorship or design of the study. Due to the facility's strict biosafety protocols, a single designated technician was responsible for daily animal care, vaccine administration, sample collection, virus challenge, and clinical monitoring.

2.11. Serological Analysis

Before the distribution and vaccination of groups, sera were collected from ten chickens to assess the presence of maternally derived antibodies (MDAs) against AIV and NDV using enzyme-linked immunosorbent assays (ELISAs). Serum samples were also collected from groups #1, #2, and #3 at 14, 27, and 33 dpv to measure the humoral immunogenicity of the vaccine against AIV and NDV using ELISAs and HI tests.

The MDAs and humoral immunogenicity of the rLS1-HA_{Opti} vaccine against AIV were measured using an in-house ELISA that detects specific antibodies against AIV HA protein. For this purpose, Nunc MaxiSorp™ 96-well flat-bottom plates (catalog No. M9410, Sigma-Aldrich, St. Louis, MO, USA) were coated with recombinant influenza A H5N8 (A/duck/NY/191255-59/2002) HA protein with a histidine tag (catalog No. 11717-V08H, Sino Biological, Beijing, China) (0.25 µg/mL in carbonate–bicarbonate buffer (pH 9.6), 100 µL/well), and incubated overnight at 4 °C. The plates were then washed six times with 1× phosphate-buffered saline (1× PBS) containing 0.05% Tween-20 (PBS-T) for 15 min at RT and blocked with 3% Difco™ Skim Milk (catalog No. 232100, BD Biosciences, Sparks, MD, USA) diluted in PBS-T (0.05%) for 2 h at RT, and then washed six times with PBS-T (0.05%). Next, 100 µL of serum sample from immunized birds, diluted 1:50 in 3% skim milk, was added to the wells and incubated for 1 h at 37 °C. After six washes with PBS-T (0.05%), 100 µL of HRP-conjugated goat anti-chicken IgG (H&L) secondary antibody (diluted 1:5000 in 3% skim milk–PBS-T; catalog No. 60320, Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) was added to each well and incubated for 1 h at 37 °C. Following a final wash (six times with PBS-T), 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well, and the plate was incubated for 15 min at RT in the dark. The enzymatic reaction was terminated by adding 50 µL of 2 N H_2SO_4, and the optical density (OD) was measured at 450 nm using an EPOCH 2 plate reader (BioTek, Winooski, VT, USA). The cut-off value was set at 0.14 (mean OD of the negative control sera plus three standard deviations (mean + 3 SDs)).

Anti-NDV MDA titers were detected and quantified using a commercial anti-NDV ELISA kit (Newcastle Disease Virus Antibody Test Kit, catalog No. 99-09263, IDEXX, Westbrook, ME, USA), according to the manufacturer's instructions. Briefly, 100 µL of a serum sample (diluted 1:500) was added to an NDV-coated well and incubated for 30 min at RT. Next, HRP-conjugated anti-IgY antibody was added to the sample and incubated for 30 min at RT; then, TMB was added and incubated for 15 min at RT. Finally, a stop solution was added, and readings were taken using an EPOCH2 plate reader (BioTek, Winooski, VT, USA) at 650 nm. The optical density results were used to calculate the antibody titer following the manufacturer's protocol.

To measure HI titers, 50 µL of 1× DPBS plus 50 µL of serum sample were deposited in the first well of a 96-well U-bottom plate, and serial 2-fold dilutions were performed across the plate. Next, 50 µL of inactivated HPAIV H5N1 (A/Pelecanus/Peru/VFAR-140/2022 strain in 0.1% formalin; dose: 4 hemagglutinating units (HAU)) was added to each well and incubated for 30 min at RT. Then, 50 µL of 1% (*v*/*v*) chicken red blood cells were deposited into each well, mixed carefully, and incubated for 30 min at RT. The HI titer was defined as the highest serum dilution that completely inhibited hemagglutination and was expressed as the reciprocal of that dilution on a Log_2 scale.

Laboratory investigators conducting the ELISA and HI assays were blinded to the specific vaccine treatments and study design. Serum samples were identified and processed using only the generic cage codes (control group, group #2, and group #3). Decoding was performed exclusively after data acquisition was completed.

2.12. Evaluation of Challenge Virus Shedding Using Real-Time RT-PCR

Based on the initial study design and to optimize diagnostic resource allocation, a subgroup of five representative birds per cage ($n = 5$ out of 7) was systematically selected for viral shedding analysis. Given that all seven individuals within each group exhibited complete physical homogeneity and clinical uniformity, the subgroup selection was performed sequentially without clinical preference. A sample size of $n = 5$ per group was determined to retain sufficient statistical power to detect significant differences in viral loads without compromising study validity.

To evaluate virus shedding, five chickens were selected from group #2 ($n = 5$) (rLS1-HA_{Opti} vaccine), and five from group #3 (inactivated AIV vaccine) for palatal cleft and cloacal swab collection at 6 and 9 dpc. Regarding group #1 (non-vaccinated control), the chickens died at 3 dpc; thus, no samples were collected at 6 and 9 dpc in this group. The collected swabs were placed in 1.5 mL of 1× DPBS with 1× antibiotic–antimycotic (catalog No. 15240062, Thermo Fisher Scientific, Waltham, MA, USA). These samples were prepared for real-time RT-PCR analysis as follows. The viral RNA was extracted from 200 µL of swab samples using an automated QIAcube Connect instrument (Qiagen, Hilden, Germany) and the QI-Aamp MinElute Virus Spin Kit (catalog No. 57704, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Real-time RT-PCR was used in conjunction with reference primers to detect the presence of the AIV M gene, following the protocol described in [26]. Amplification was performed using the Luna® Universal One-Step RT-qPCR Kit (catalog No. E3005E, New England Biolabs, Ipswich, MA, USA) on a Rotor-Gene Q 5Plex HRM (Qiagen, Hilden, Germany), following the manufacturer's instructions. Reverse transcription was performed at 55 °C for 10 min, followed by an initial denaturation at 95 °C for 1 min, and then 40 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. The fluorescence signal for each amplified product was recorded continuously as the temperature increased from 70 to 95 °C, with fluorescence data acquired every 1.0 °C. This process amplified a 101 base pair (bp) segment with a melting temperature (Tm) of 84.3–84.8 °C. Both the amplification curve (C_t) and melting curve (Tm) data were used to determine whether the result was positive or negative. The molecular biologist conducting the real-time RT-PCR assay was fully blinded to the treatment groups, receiving and analyzing the samples using only the pre-assigned blinded codes.

2.13. Statistical Analysis

Statistical analyses were conducted using GraphPad Prism version 11.0.0 for Windows (GraphPad Software, Boston, MA, USA; www.graphpad.com). The viral replication kinetics were evaluated using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Data from the densitometric analysis of Western blotting for cell lysates

were evaluated using two-way ANOVA followed by Sidak's multiple comparisons test. Data for the purified viral particles were analyzed using an unpaired t-test with Welch's correction. Antibody levels measured by the ELISAs and HI tests were compared using two-way ANOVA with a mixed-effects model (REML) and Geisser–Greenhouse correction followed by Tukey's multiple comparisons test. Kaplan–Meier survival curves were compared using the Log-Rank (Mantel–Cox) test, with $p < 0.0001$ considered statistically significant. The clinical signs scores were analyzed using a two-way repeated measures ANOVA (matched by individual animal), followed by Tukey's multiple comparisons test. Data are reported as mean ± standard error of the mean (SEM) for replication kinetics and clinical signs scores; mean ± standard deviation (SD) for the other assays. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ were considered statistically significant.

3. Results

3.1. Generation of Recombinant rLS1-HA$_{Opti}$ Virus

rLS1-HA$_{Opti}$ was successfully recovered and confirmed by RT-PCR and sequencing. The PCR product from rLS1-HA$_{Opti}$ (~2524 bp) (Figure 1B) showed the insertion of the HA$_{Opti}$ expression cassette between the P and M genes. The rLS1-HA$_{Opti}$ vaccine was amplified in SPF chicken embryonated eggs and showed HA titers ranging from 9 to 10 Log$_2$. The vaccine had an ICPI value of 0.2 in 1-day-old SPF chicks. This result confirmed that the HA gene insertion did not affect the lentogenic properties of the vector.

3.2. Expression of AIV HA and Its Incorporation into Viral Particles

Western blot analysis of cell lysates revealed that the expression of the AIV HA protein showed three molecular patterns, with molecular masses of ~70, ~ 50, and ~ 25 kilodaltons (kDa), which correspond to the uncleaved HA0 precursor protein and cleaved subunits HA1 and HA2, respectively. The expression of the NDV F surface protein was detected in two molecular forms: the inactive precursor (F0) at ~59 kDa and the cleaved subunit (F1) at ~52 kDa. The results revealed that the expression of the F protein from the rLS1-HA$_{Opti}$ virus is similar to that of the parental virus. Beta-actin (~42 kDa) was also detected in the cell lysate as a loading control (Figure 2A). Subsequently, the incorporation of the AIV HA protein into rLS1-HA$_{Opti}$ virions was confirmed by Western blotting. The HA1 (~50 kDa) and HA2 (~25 kDa) subunits from HA were detected. Moreover, the presence of NDV HN (~70 kDa) was also confirmed in both viruses (Figure 2B), indicating that these proteins were successfully incorporated into the viral particles. Densitometric analysis of cell lysates confirmed that F protein expression levels did not differ significantly between the viruses. Furthermore, densitometry of both cell lysates and purified viral particles demonstrated that the rLS1- HA$_{Opti}$ virus exhibited a significantly higher level of HA protein expression compared to the parental virus, which showed negligible signal (Figure 2A,B).

3.3. Replication Characteristics

The growth characteristics of rLS1 and rLS1-HA$_{Opti}$ were evaluated using replication and expression kinetics. The replication kinetics of the two viruses did not show statistical differences ($p > 0.05$) (Figure 2C). Moreover, for the rLS1-HA$_{Opti}$ virus, the expression of the HA (HA0, HA1, and HA2) and F (F0 and F1) proteins increased progressively over time (from 12 to 84 hpi). Similarly, an increase in NDV F (F0 and F1) protein expression was detected for the parental virus, whereas, as anticipated, HA protein remained undetectable. Beta-actin protein expression was visualized at all time points as a loading control (Figure 2D). The densitometric analysis revealed a time-dependent increase in NDV F protein expression levels, with no significant differences between the viruses. In addition, rLS1-HA$_{Opti}$ demonstrated significantly higher HA protein expression levels compared

with rLS1 from 36 to 84 hpi. (Figure 2D). Furthermore, the plaque morphology and size in cells infected with rLS1 or rLS1-HA$_{Opti}$ were similar over time (Figure 2E). Therefore, these results indicate that the insertion of the foreign AIV HA gene did not adversely affect the biological properties of the viral vector.

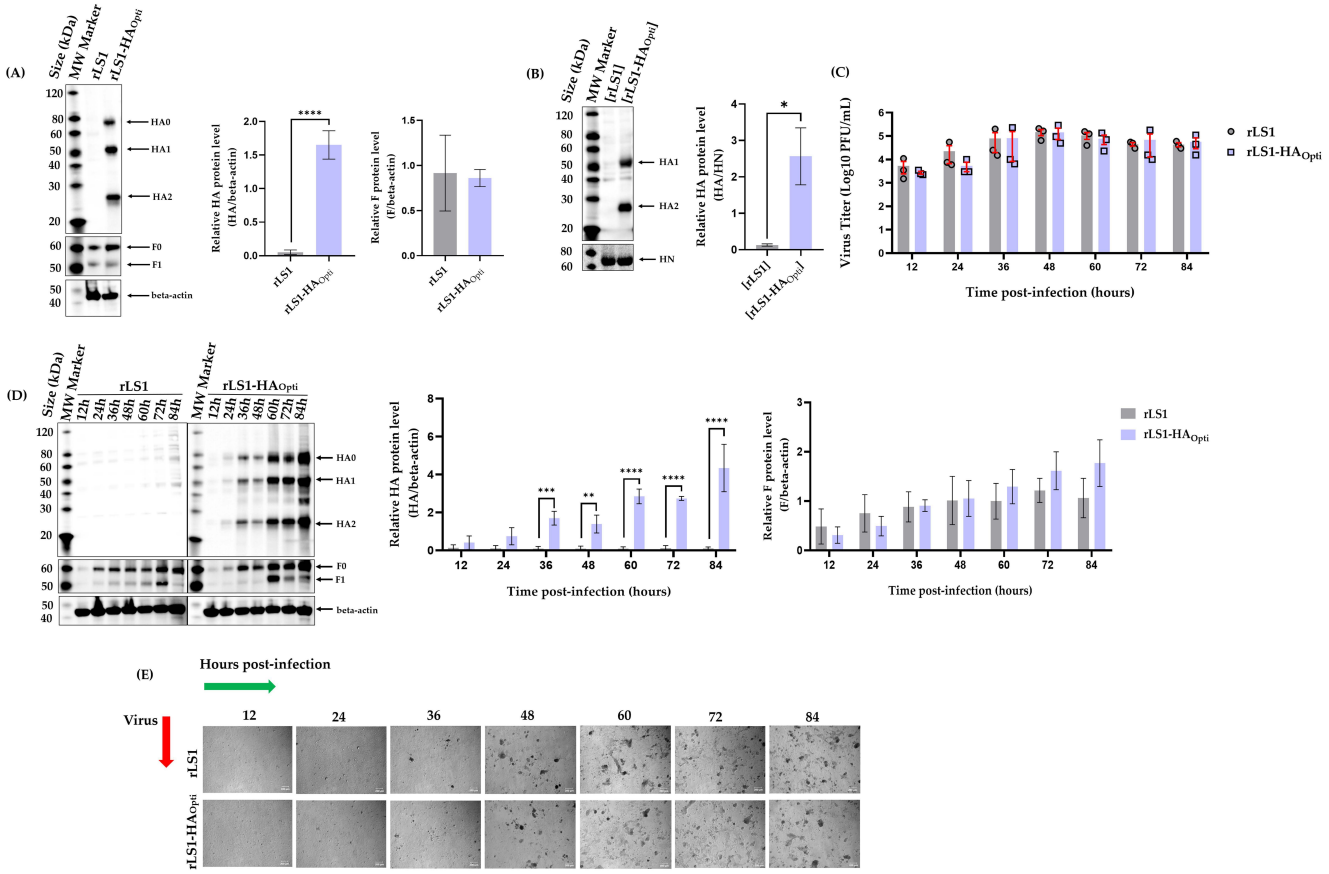


Figure 2. Biological characterization of rLS1-HA$_{Opti}$. (**A**) Western blot analysis of lysates from DF1 cells infected with rLS1 or rLS1-HA$_{Opti}$ virus. The AIV HA proteins (HA0 (~70 kDa), HA1 (~50 kDa), and HA2 (~ 25 kDa)), NDV F proteins (F0 and F1), and beta-actin (loading control) were visualized using specific antibodies. Densitometric analysis profiles are displayed as bar graphs representing protein expression levels in normalized arbitrary units (N.A.U.) relative to beta-actin. (**B**) Confirmation of the structural incorporation of the AIV HA proteins (HA1 and HA2) and NDV HN protein into the rLS1-HA$_{Opti}$ virus particles (concentrated and purified). Data are expressed as the HA/HN densitometric ratio. DF1 cells were infected with the viruses at an MOI of 0.05 to determine (**C**) the kinetics of replication (titrated using the plaque assay) and (**D**) expression, analyzed by Western blotting and densitometry as described in (**A**). (**E**) Cytopathic effect in DF1 cells infected with the viruses (from **C**), analyzed using brightfield microscopy at 100× magnification. Scale bars: 200 µm. Data are presented as mean ± standard deviation (SD) for densitometric analyses and mean ± standard error of the mean (SEM) for replication kinetics. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4. Genetic Stability and Expression of AIV HA Protein by Recombinant Virus

The IFA showed that the AIV HA and NDV RNP proteins were co-expressed in DF1 cells infected with rLS1-HA$_{Opti}$ virus from the 5th passage in chicken embryonated eggs (Figure 3A). As expected, rLS1-infected cells only expressed the RNP protein of the vector. These results show that replication during passages did not affect the expression of the HA and RNP proteins in rLS1-HA$_{Opti}$. The PCR analysis of the RNA from the HA-expressing virus at passages 1 and 5 revealed a ~2524 bp band, indicating the stability of the inserted

expression cassette (Figure 3B). The parental virus showed a ~604 bp band. Western blotting of cells infected with rLS1-HA$_{Opti}$ virus from the 1st and 5th passages showed three molecular forms of the HA protein: ~70 kDa (HA0), ~50 kDa (HA1), and ~25 kDa (HA2) forms. As expected, this protein was not detected in the cells infected with the parental virus. In addition, the NDV F protein was detected in cells infected with rLS1 or rLS1-HA$_{Opti}$ at ~59 kDa (F0) and ~52 kDa (F1). Beta-actin, the loading control, was detected as an ~42 kDa band (Figure 3C).

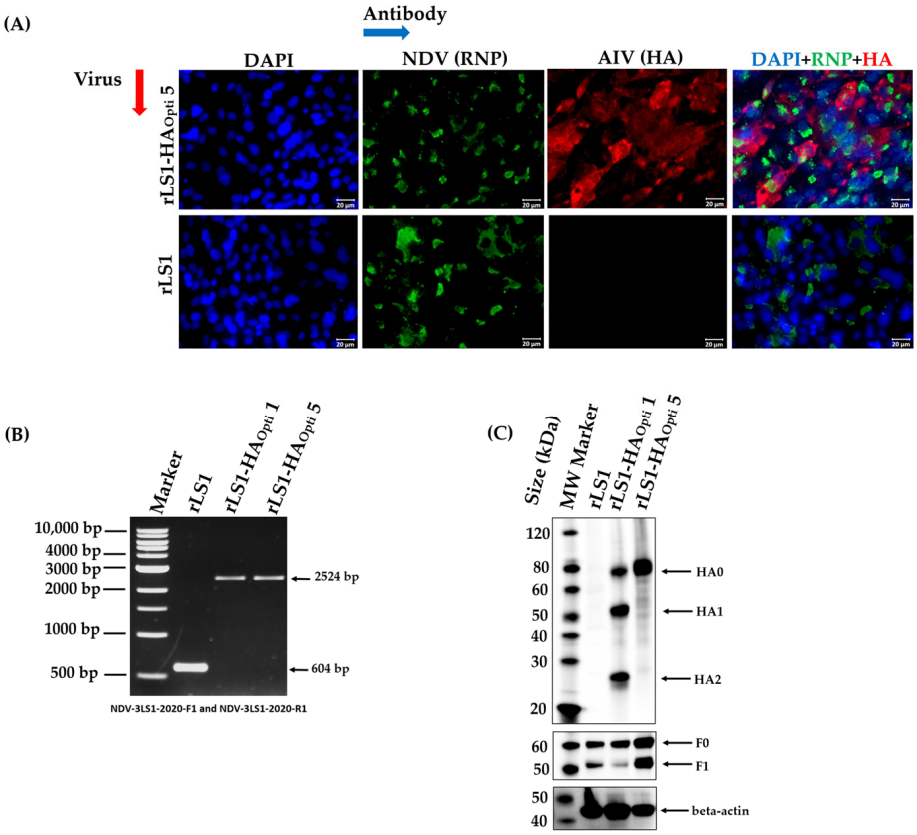


Figure 3. Genetic stability of the rLS1-HA$_{Opti}$ virus. (**A**) DF1 cells infected with rLS1 or rLS1-HA$_{Opti}$ (from the 5th passage in chicken embryonated eggs) at an MOI of 0.01 were analyzed by indirect immunofluorescence assay. The expression of NDV RNP (green) and AIV HA proteins (red) was revealed using specific antibodies. Cell nuclei were stained with DAPI (blue) and visualized by fluorescence microscopy at 630× magnification. Scale bars: 20 µm. (**B**) RT-PCR analysis demonstrating the presence of the HA$_{Opti}$ expression cassette in the rLS1-HA$_{Opti}$ virus at passages 1 and 5. The sizes of the RT-PCR products from rLS1-HA$_{Opti}$ (~2524 bp) and rLS1 (~604 bp) are indicated. (**C**) Western blot analysis showing the stable expression of the HA$_{Opti}$ cassette (HA0 (~70 kDa), HA1 (~ 50 kDa), and HA2 (~25 kDa)) in DF1 cells infected with rLS1-HA$_{Opti}$ from the 1st and 5th passages. The NDV F (F0 and F1) and beta-actin proteins were detected as controls.

3.5. Humoral Immune Response to Vaccination in Broiler Chickens

The vaccination and efficacy monitoring schedule is shown in Figure 4A. All serum samples collected (n = 10) before vaccination were positive for MDAs against NDV, with titers of 9849.10 ± 1897.41 (mean ± SD), as detected by a commercial ELISA (Figure 4B). These samples were negative for MDAs against the AIV H5 HA protein (0.11 ± 0.02; mean ± SD), based on an in-house ELISA.

The ELISA results demonstrated a progressive decline in NDV-specific MDA titers across all groups at 14, 27, and 33 dpv. Furthermore, the rLS1-HA$_{Opti}$ vaccine did not elicit a significant increase in antibody titers against NDV at 14 dpv (1081.29 ± 254.08), 27 dpv

(486.43 ± 626.93), and 33 dpv (331.29 ± 534.27) when compared to both the AIV inactivated vaccine and the non-vaccinated control groups (Figure 4B).

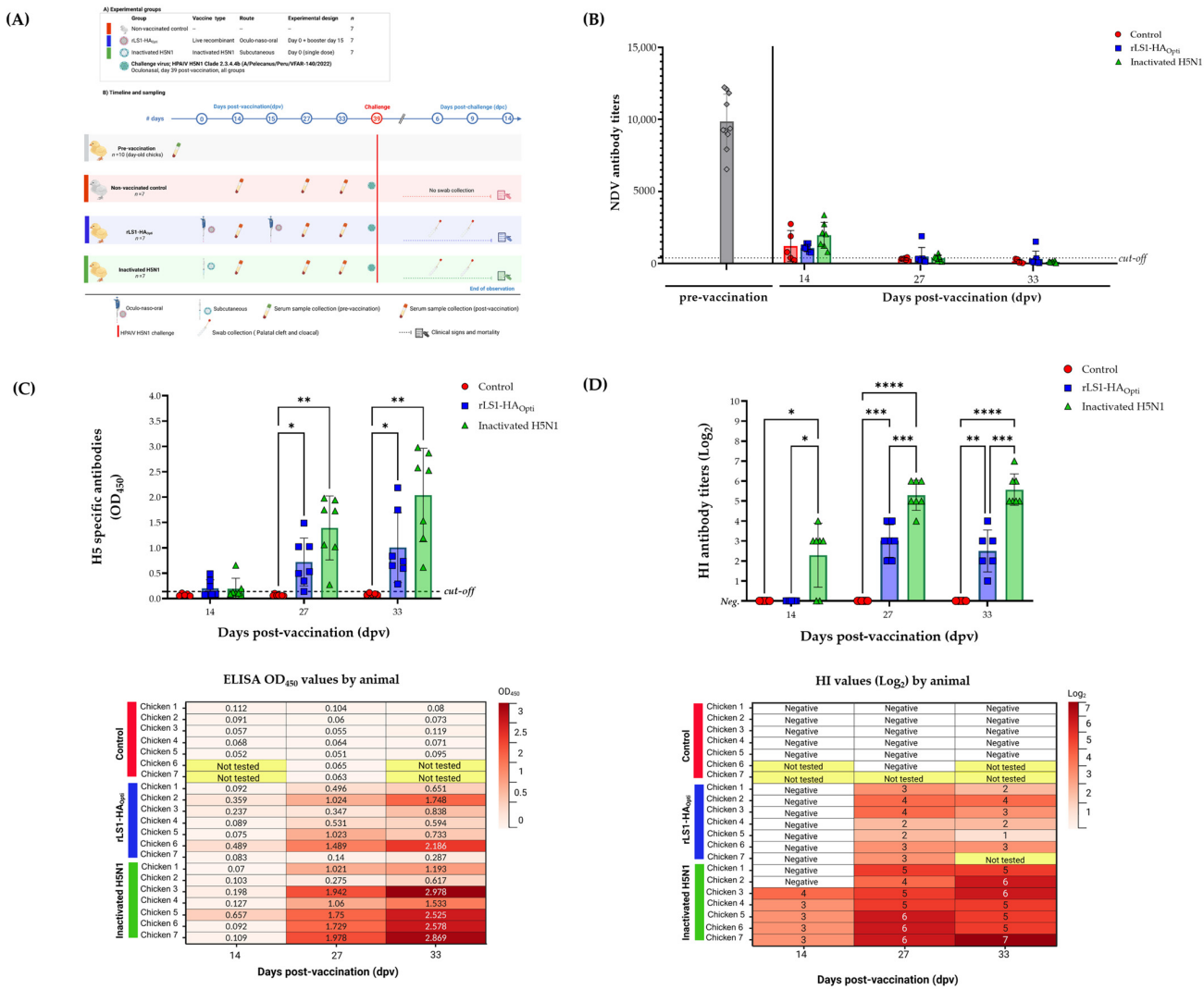


Figure 4. Immunogenicity of the rLS1-HA$_{Opti}$ vaccine in broiler chickens. (**A**) Experimental groups and timeline of vaccination and challenge in broiler chickens. (**B**) Evaluation of the NDV-specific humoral response by commercial ELISA at 14, 27, and 33 dpv. The dashed line represents the cut-off value (396). (**C**) H5-specific antibody response measured using an in-house ELISA at 14, 27, and 33 dpv. The dashed line indicates the cut-off threshold (0.14). (**D**) Hemagglutination inhibition (HI) antibody titers (Log$_2$) against HPAI H5N1 measured at 14, 27, and 33 dpv. Values < 1 Log$_2$ were considered negative (Neg). The heat maps display the individual broiler chicken results for panels (**C**,**D**). For panels (**C**,**D**), the initial sample size was $n = 7$ per group; the integrated tables display the specific cohort sizes resulting from insufficient sample volume or individual data omissions at designated time intervals. Data are presented as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The drawings of (**A**) and the heat maps in (**C**,**D**) were created using BioRender.com.

Serological analysis showed that the rLS1-HA$_{Opti}$ vaccine induced a specific antibody response against AIV H5 HA, as detected by the in-house ELISA. At 14 dpv, 42.86% of chickens were seropositive, with OD$_{450}$ values of 0.203 ± 0.165. Seropositivity reached 100% at 27 and 33 dpv, with OD$_{450}$ values increasing to 0.721 ± 0.472 and 1.005 ± 0.690, respectively. In the inactivated H5N1 vaccine group, 28.57% of chickens were seropositive at 14 dpv, with a value of 0.193 ± 0.208. Similarly, seropositivity in this group reached 100% at 27 and 33 dpv, with values of 1.393 ± 0.630 and 2.041 ± 0.921, respectively.

These results demonstrated that both vaccinated groups elicited significantly higher anti-HA H5 antibody levels than the control group at 27 dpv (rLS1-HA_{Opti}, $p = 0.0242$; inactivated H5N1, $p = 0.0034$) and 33 dpv (rLS1-HA_{Opti}, $p = 0.0291$; inactivated H5N1, $p = 0.0033$). Notably, no significant differences were observed between the two vaccines at any analyzed time point. In contrast, all non-vaccinated control chickens remained negative at 14, 27, and 33 dpv, with OD_{450} values of 0.076 ± 0.025, 0.066 ± 0.018, and 0.088 ± 0.020, respectively, remaining consistently below the positivity threshold of 0.14. Figure 4C shows the heat map with the individual chicken results.

The HI test for detecting specific antibodies against HPAIV H5N1 was performed at 14, 27, and 33 dpv. All serum samples from the non-vaccinated control group remained negative throughout the study.

In the rLS1-HA_{Opti} vaccinated group, all chickens were seronegative at 14 dpv, but reached 100% seropositivity at 27 and 33 dpv, displaying mean titers of 3.00 ± 0.82 Log_2 and 2.50 ± 1.05 Log_2 (mean ± SD), respectively (Figure 4D).

By contrast, the inactivated H5N1 vaccine group showed early detectable HI antibodies at 14 dpv, reaching 71.43% seropositivity with a mean titer of 2.29 ± 1.60 Log_2. Seropositivity in this group increased to 100% at both 27 and 33 dpv, with mean titers rising to 5.29 ± 0.76 Log_2 and 5.57 ± 0.79 Log_2, respectively (Figure 4D).

HI titers in the inactivated H5N1 vaccine group were significantly higher than those in the rLS1-HA_{Opti} group across all time points (14 dpv, $p = 0.0217$; 27 dpv, $p = 0.0004$; 33 dpv, $p = 0.0006$). When compared to the control group, the inactivated vaccine elicited significantly higher titers at all intervals (14 dpv, $p = 0.0217$; 27 and 33 dpv, $p < 0.0001$), whereas the rLS1-HA_{Opti} vaccine showed statistically significant differences relative to controls only at 27 dpv ($p = 0.0002$) and 33 dpv ($p = 0.0048$) (Figure 4D). The individual chicken results are displayed using a heat map in Figure 4D. These results demonstrate that both vaccines elicit a strong humoral immune response against HPAIV H5N1 HA.

3.6. Efficacy Against HPAIV H5N1 Challenge

To assess the protective efficacy of rLS1-HA_{Opti} or the inactivated H5N1 vaccine against the A/Pelecanus/Peru/VFAR-140/2022 HPAIV (H5N1) virus, all broiler chickens were challenged oculo-nasally with 10^6 EID_{50}/chicken at 39 dpv.

In the non-vaccinated control group, all chickens succumbed to the infection between 2 and 3 dpc, resulting in 100% mortality (7/7) by day 3 post-challenge. In contrast, all chickens in both rLS1-HA_{Opti} and inactivated H5N1 vaccinated groups survived until the end of the study, demonstrating 0% mortality (0/7) (Figure 5A). Survival rates differed significantly among the three groups during the 14 dpc observation period ($p < 0.0001$) (Figure 5A). Individual survival results for all groups are displayed using a Swimmer plot (Figure 5B).

Concurrently, all broiler chickens were monitored for clinical signs using a clinical scoring system (0 = healthy; 1 = sick; 2 = severely ill; 3 = dead). Vaccinated chickens were fully protected against clinical disease, showing no signs of illness throughout the observation period. Conversely, the non-vaccinated controls exhibited typical and severe signs of HPAIV infection. Statistical analysis of the clinical scores revealed significant differences among the experimental groups ($p < 0.0001$) (Figure 5C). Clinical sign scores per animal throughout the observation period are shown in Figure 5D.

Viral shedding was evaluated through the analysis of palatal cleft and cloacal swab samples collected from five selected chickens per group at 6 and 9 dpc, using real-time RT-PCR. Due to early mortality observed in the non-vaccinated control group, only samples from the vaccinated group could be tested (Figure 5E).

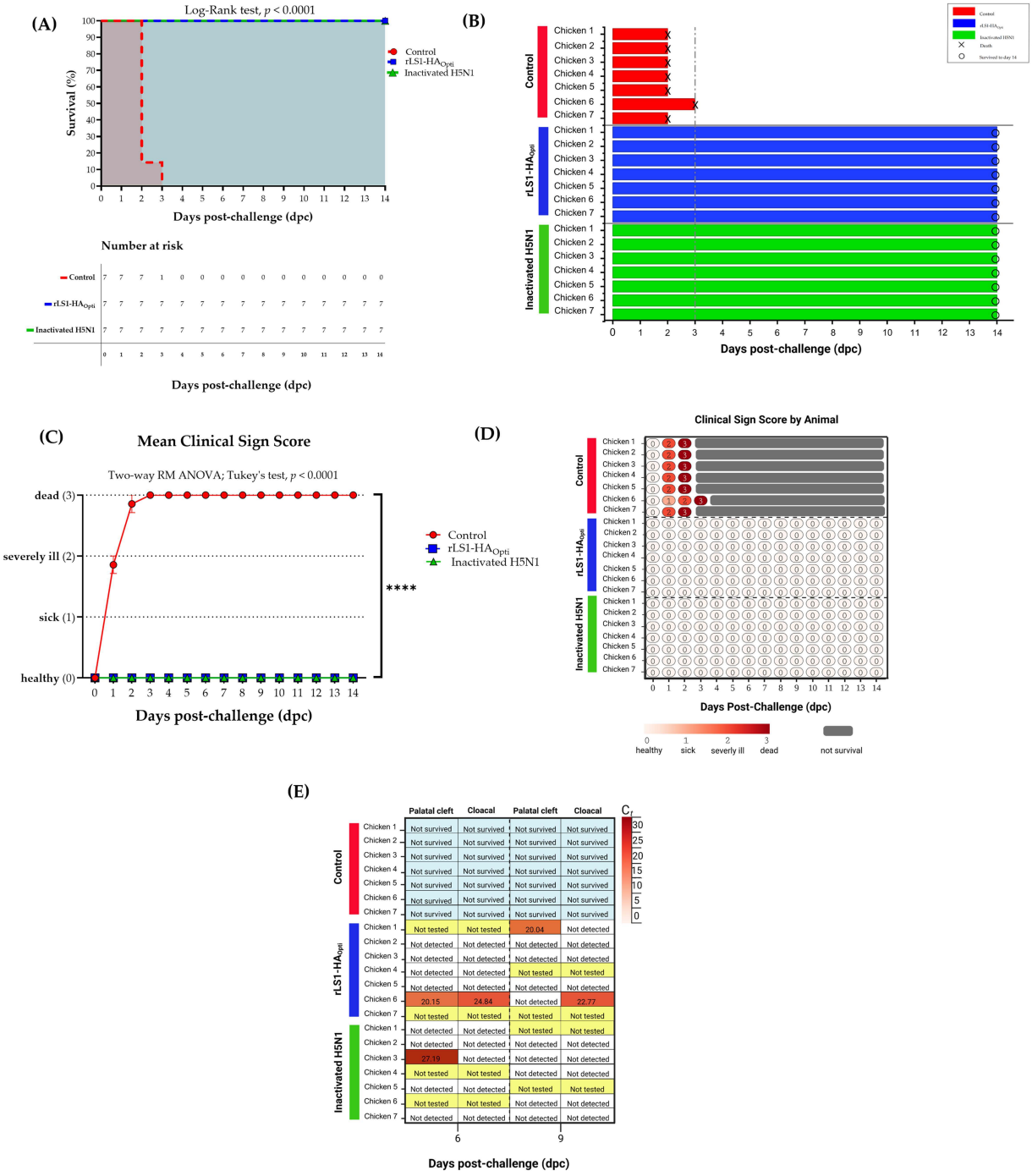


Figure 5. Protective efficacy of the rLS1-HA$_{Opti}$ vaccine against H5N1 highly pathogenic avian influenza (HPAIV) clade 2.3.4.4b challenge in broiler chickens. (**A**) Kaplan–Meier survival curves following the HPAIV H5N1 experimental challenge. Chickens were monitored for 14 dpc. The number of animals at risk at each time point is shown below the graph. (**B**) Swimmer plot depicting individual survival outcomes across all groups, complementary to the Kaplan–Meier analysis. Each horizontal bar represents an individual animal monitored for 14 dpc. The dashed vertical line highlights 3 dpc, when the control group reached 100% mortality. (**C**) Mean clinical sign scores of chickens post-challenge (0 = healthy; 1 = sick; 2 = severely ill; 3 = dead). Data are presented as the mean ± SEM. (**D**) Clinical sign scores by individual animal across the observation period. (**E**) The heat map of viral shedding displays individual broiler chicken results from real-time RT-PCR on palatal cleft and cloacal swabs, with data presented as C_t values. **** $p < 0.0001$. The drawings in (**B**,**D**,**E**) were created using BioRender.com.

In the rLS1-HA$_{Opti}$ group at 6 dpc, one out of five chickens tested positive in both palatal cleft (C_t = 20.15) and cloacal swabs (C_t = 24.84). At 9 dpc, two out of five chickens were positive: the same chicken that was positive at 6 dpc remained positive based on its cloacal swab (C_t = 22.70), while another chicken tested positive only on its palatal cleft swab (C_t = 20.04). In contrast, within the inactivated H5N1 vaccine group, only one out of five chickens tested positive on palatal cleft swabs (C_t = 27.19) at 6 dpc (Figure 5E).

4. Discussion

H5N1 AIV causes millions of deaths in poultry and wild birds worldwide [3]. Furthermore, the continuous evolution of H5N1 to genetically divergent HPAIV strains and their transmission between diverse animal species presents an ongoing global threat [2,4]. Another concern is the introduction of these novel strains into HPAI H5N1-free regions through migratory birds [21]. To address this issue, vaccines have been used for decades. Among these, live rNDV-vectored vaccines expressing the H5 HA protein are used both in research laboratories [27,28] and in commercial poultry vaccination programs [8].

Inactivated whole AIV vaccines are routinely used in immunization strategies for poultry because they induce a rapid protective humoral response [8]; however, these vaccines present some limitations, such as eliciting a limited cellular response, their application is costly and labor-intensive [29], and providing suboptimal protection in birds vaccinated before 2 weeks of age [10]. Live vectored AIV vaccines based on rNDV are valuable alternatives in vaccination programs [8] since they offer key advantages: they induce protective mucosal, humoral, and cellular immune responses; they can be administered through low-cost mass application methods; and they are inexpensive to produce [8]. Therefore, in this study, we developed a live rNDV-vectored vaccine carrying the H5N8 HA gene (rLS1-HA$_{Opti}$). Broiler chickens immunized with this vaccine via the oculo-naso-oral route elicited a humoral response (anti-HA) and achieved complete protection against mortality and observed clinical disease under this experimental H5N1 challenge; however, viral replication was not completely suppressed (non-sterilizing immunity). Although this individual route of vaccination is labor-intensive and not feasible for large commercial farms, it was strictly necessary here to ensure that each bird received an exact and controlled dose of the vaccine. Confirming that these routes of administration are highly effective in inducing protective immunity—even in the presence of high MDA titers—sets a vital precedent. These findings provide a solid foundation for future research testing less labor-intensive mass administration methods, such as vaccination via spray or drinking water, which target the same mucosal routes of entry.

A compelling finding of this study was the comparison between the live recombinant rLS1-HA$_{Opti}$ vaccine and the conventional homologous inactivated H5N1 vaccine. As expected, the inactivated vaccine elicited higher systemic humoral immune responses across all evaluated time points. This robust humoral profile is consistent with the high antigenic load and emulsion adjuvants typical of inactivated formulations, which strongly stimulate circulating antibodies. Remarkably, despite these differences in serum antibody levels, both vaccines provided 100% clinical protection following the lethal HPAIV H5N1 challenge. This pivotal outcome demonstrates that the systemic antibody titers induced by rLS1-HA$_{Opti}$ are nonetheless sufficient to prevent mortality, likely reinforced by local mucosal immunity and cell-mediated immune responses, which are inherently elicited by the live viral vaccine.

Antigenic similarity of the HA protein expressed by the vectored vaccines and the circulating field strains is a critical determinant for inducing complete protection against both clinical disease and viral shedding in poultry [29]. Consequently, the continuous updating of AIV vaccines is an undeniable necessity. In this context, robust epidemiological

surveillance is an indispensable tool, enabling the detection of emergent strains [22] and guiding the development of closely matched AIV vaccines. In the present study, we developed a live rNDV-vectored vaccine expressing the HA of an H5N8 strain belonging to clade 2.3.4.4b. This insert shares a high antigenic identity (97.71%) with the HA protein amino acid sequence of the HPAI H5N1 challenge virus (also clade 2.3.4.4b). This substantial antigen successfully mediated full clinical protection and suppressed viral shedding in the majority of vaccinated chickens following a lethal challenge.

Although conventional vaccines—widely used in poultry vaccination programs—consistently prevent mortality and morbidity against HPAI strains, a recurrent limitation is their inability to fully suppress viral shedding, which permits transmission to non-vaccinated flocks [30]. Our findings align with this paradigm, as neither formulation achieved sterile immunity post-challenge. Specifically, the inactivated homologous vaccine failed to completely block shedding in one bird at 6 dpc, whereas the rLS1-HA_{Opti} vaccine group exhibited residual viral shedding in two birds up to 9 dpc. This finding underscores that, while both vaccines successfully prevent lethal infection, the homologous inactivated vaccine produces a more rapid reduction in shedding compared to the more protracted clearance timeline required by the live viral vector. Addressing this problem is urgent, as the persistent viral replication and circulation within the vaccinated population exert selective pressures that could drive the emergence of genetic variants with greater pathogenicity and transmissibility. Therefore, it is crucial to evaluate alternative vaccination strategies [27] and develop updated vaccines to completely stop shedding [7]. Crucially, while the challenge virus genome was detected by real-time RT-PCR in the swabs of these positive chickens, our study did not confirm the viability and transmissibility of the shed viruses, warranting future transmission-contact studies to fully determine the epidemiological risk.

The MDAs against NDV or AIV may interfere with live rNDV-vectored AIV vaccine replication and compromise protective efficacy in chickens. For instance, an rNDV LaSota vector vaccine expressing the H5 ectodomain from H5N2 did not induce optimal seroconversion and clinical protection in commercial broilers challenged with homologous virus due to the presence of MDAs to NDV LaSota and/or H5N1 virus [31]. Similarly, passively transferred antibodies (PTAs) (anti-H5N9) in SPF chicks affected both the humoral response to H5N1 and the protection against homologous challenge in chickens vaccinated with an rNDV-vectored vaccine expressing H5N1 HA; however, the humoral response against the NDV vector remained unaffected [32]. Nevertheless, despite the presence of MDAs against NDV in broiler chickens, our rNDV vaccine expressing H5 HA (rLS1-HA_{Opti}) showed seroconversion to H5 HA and conferred 100% survival after HPAI H5N1 challenge, which was also observed in another study [18]. However, the ELISA results revealed a progressive decline in NDV-specific MDA titers over time, with no post-vaccination anamnestic response developing against the vector. This finding could be partially attributed to the absence of H5 MDAs. Therefore, future research must evaluate the immunogenicity and efficacy of rLS1-HA_{Opti} in the presence of both NDV and H5 MDAs. Furthermore, although rLS1-HA_{Opti} replication was not monitored following vaccination, and limited viral replication with de novo HA expression cannot be entirely discounted, our serological data strongly suggest that high pre-existing maternal immunity succeeded in limiting vector replication. Alternatively, the robust immunogenicity and protective efficacy observed could be due, to some extent, to the considerable amount of HA antigen already integrated into the structural architecture of the vaccine virus. This dual mechanism would explain why the presence of high NDV-specific MDAs did not compromise the vaccine's protective properties.

We acknowledge as a limitation of this study the absence of a control group immunized with the parental rNDV vector (rLS1 lacking the HA insert) in the in vivo challenge experiment. While previous research indicates that parental NDV vectors generally do not

confer cross-protection or reduce viral shedding against lethal avian influenza challenge in chickens [20,33], partial nonspecific protection mediated by the NDV backbone has been reported in other avian species, such as ducks [20]. Consequently, although rLS1-HA_{Opti} elicited robust anti-HA humoral responses and complete clinical protection in chickens, the lack of a vector-only control prevents us from definitively determining whether this protection is mediated exclusively by HA-specific adaptive immunity or also involves potential nonspecific innate immune effects induced by the viral vector.

Other limitations of this study include the lack of evaluation of the vaccine-induced mucosal/cellular immune response, as well as the omission of histopathology and the assessment of viral loads in internal organs. Providing deeper insights into systemic protection remains a critical indicator of vaccine efficacy and justifies further research on rLS1-HA_{Opti}.

5. Conclusions

We developed a live rNDV-vectored vaccine expressing the HA protein of H5N8 (clade 2.3.4.4b) that elicited a significant HA-specific humoral response and conferred complete protection against mortality and clinical disease in broiler chickens following experimental challenge with HPAIV H5N1 strain A/Pelecanus/Peru/VFAR-140/2022 (clade 2.3.4.4b). Although sterilizing immunity was not achieved—as viral shedding was still detected—and further studies incorporating a parental rNDV vector control group are needed to definitively resolve the relative contributions of HA-specific immunity versus potential nonspecific vector-mediated effects, rLS1-HA_{Opti} demonstrates strong potential as an effective vaccine candidate.

Author Contributions: Conceptualization, K.C., A.R.-N., M.F.-S., and M.F.-D.; methodology, K.C., A.R.-N., B.C.-L., G.G.-F., A.M.-A., and D.V.-P.; validation, K.C., A.R.-N., B.C.-L., and G.G.-F.; formal analysis, K.C., A.R.-N., B.C.-L., and G.G.-F.; investigation, K.C., A.R.-N., B.C.-L., and G.G.-F.; resources, M.F.-S. and M.F.-D.; data curation, A.R.-N., K.C., B.C.-L., and G.G.-F.; writing—original draft preparation, A.R.-N., K.C., B.C.-L., and G.G.-F.; writing—review and editing, K.C., A.R.-N., and M.F.-D.; visualization, A.R.-N. and K.C.; supervision, A.R.-N., K.C., and M.F.-D.; project administration, A.R.-N., K.C., M.F.-S., and M.F.-D.; funding acquisition, M.F.-D. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

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