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Recombinant NDV-vectored vaccine expressing mosaic HA and M2e confers broad and early protection against H9N2 avian influenza virus

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The development of a universal H9N2 avian influenza vaccine is essential to reduce the occurrence of disease and economic losses in the poultry industry. In this study, we developed a recombinant vaccine rTS-mH9-3M2e based on Newcastle disease virus (NDV) avirulent strain TS09-C, containing a chimeric mosaic H9 HA and three copies of H9N2 avian influenza virus (AIV) M2e. The 1-day-old chickens were vaccinated with rTS-mH9-3M2e, which induced broad neutralizing antibody activity against H9N2 AIV, effectively reduced viral load and virus shedding, and reduced clinical signs and tissue damage. Furthermore, rTS-mH9-3M2e can induce cross-neutralizing response to the heterologous subtype AIV H3N2 and conferred cross-protection against H3N2 AIV. When rTS-mH9-3M2e was used as *in ovo* vaccine, it conferred the earlier protection against NDV and H9N2 AIV compared with post-hatch vaccination. rTS-mH9-3M2e *in ovo* vaccination significantly increased the level of splenic $CD8^+$ T cells in chickens at 7 days of age and provided 100% protection against H9N2 AIV and 80% protection for NDV virulent strain. These results support that rTS-mH9-3M2e is a promising candidate for development as a broad H9N2 avian influenza vaccine and an *in ovo* vaccine.

KEYWORDS
$CD8^+$ T cell, cross-protection, early protection, H9N2 avian influenza virus, universal vaccine

Introduction

Avian influenza has posed threats worldwide, with significant effects on animal and public health, and cause heavy economic losses (1). The genome of AIV comprises eight negative-sense RNA segments and encodes polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein

(NP), NA, matrix protein (M), and nonstructural protein (NS). Influenza virus is categorized into different subtypes based on HA and NA. To date, 19 HA and 11 NA have been described (2). H9N2 AIV is classified as a low-pathogenic virus and considered to be the most epidemic AIV in poultry globally (3). It has been identified as genetic donors for several subtypes of influenza virus that infect humans, such as H3N8 (4), H5N1 (5), and H7N9 (6). The surveillance report of recent years on AIV showed that H9N2 AIV is predominant in domestic poultry and live poultry markets across China (7, 8).

Vaccination is the most effective strategy to control AIV in poultry (9). In China, whole-virus-inactivated vaccines have been used to control H9N2 AIV; however, viruses are still frequently isolated from immunized chickens (10). Inactivated vaccines are noninfectious, usually requiring multiple injections to ensure herd immunity, and mediate immune protection by eliciting neutralizing antibody response to epitopes on the head of HA (11). Under antibody pressure, H9N2 AIV undergoes antigenic drift of HA and causes the emergence of immune-escape mutations (12). The immune-escape mutations of HA enable the virus to escape recognition of neutralizing antibodies and result in the loss of vaccine protection and added new risks of epidemics (13, 14). To address the limitations of current vaccine strategies, the development of a universal H9N2 AIV vaccine is an urgent need (15).

The design of universal influenza vaccines can be achieved by optimizing antigenic epitopes or targeting functionally conserved epitopes. The Mosaic Vaccine Designer generates candidate vaccine protein cocktails by incorporating a small set of mosaic proteins, thereby optimizing the coverage of potential antigenic epitopes derived from a large and diverse set of homologous proteins (16–18). The influenza mosaic HA vaccines have been proven to provide cross-protection against multiple influenza virus infection, such as mosaic H1 HA (19), H3 HA (19, 20), H5 HA (21, 22), and influenza B HA vaccines (23, 24). In addition, cross-immunoreaction can be induced by conserved AIV protein as immunogens, such as the ectodomain of the M2 protein (M2e) (25). M2e includes highly conserved amino acids across different influenza virus subtypes and is an important target to circumvent antigenic mutations (26). The vaccine based on M2e can efficiently induce cellular immune responses and confer cross-protection against different influenza viruses (27, 28).

In addition to antigen design, the choice of vaccine delivery platform is equally critical for inducing immune protection. Newcastle disease virus (NDV) is a natural poultry vaccine vector and is often used to develop recombinant live vaccines. Compared with inactivated vaccines, live vaccines have more advantages; it not only can induce a strong humoral immune response but also can trigger a powerful cellular and mucosal immune response (29). Recombinant live vaccines expressing the HA of H9N2 AIV based on NDV vector can induce a favorable immune response and provide protection against H9N2 AIV (30, 31). Previously, we developed a new NDV strain by serial passaging of V4 strain in BHK-21 cells, named TS09-C (32). The TS09-C strain was a thermostable avirulent strain (33) and has been used for a vaccine vector expressing a heterologous gene (34).

In this study, we developed a recombinant vaccine rTS-mH9-3M2e expressing mosaic H9 HA and three copies of H9N2 AIV M2e (3M2e) using TS09-C as a vector. The rTS-mH9-3M2e vaccine candidate conferred broad protection against different H9N2 AIVs and cross-protection against H3N2 AIV. Compared with post-hatch vaccination, rTS-mH9-3M2e used as *in ovo* vaccine induced earlier $CD8^+$ T cell immune response and conferred early protection against NDV and H9N2 AIV. Our data supports rTS-mH9-3M2e as a promising candidate for a broad-spectrum H9N2 AIV vaccine and an *in ovo* vaccine.

Materials and methods

Ethics statement

All of the animal experiments strictly complied with the principles of animal welfare and scientific ethics and were approved and supervised by the Institutional Animal Care and Use Committee of the Hubei Academy of Agriculture Sciences, Wuhan, China (permit number: 2024SC022).

Chicken embryos, chickens, cells, and viruses

SPF Leghorn chicken embryos were purchased from Merial-Vital, Beijing, China. The chickens were obtained by hatching SPF Leghorn chicken embryos, and the chicken embryos were hatched at 37.5°C and 60% humidity. Baby Hamster Kidney (BHK-21) cells and Madin-Darby Canine Kidney (MDCK) cells originated from Institute of Animal Sciences and Veterinary Medicine, Hubei Academy of Agricultural Sciences. The cells were maintained in DMEM containing 10% FBS and incubated at 37°C under 5% CO_2.

NDV strain TS09-C (accession number: JX110635), LaSota (accession number: JF950510), F48E9 (accession number: MG456905), H3N2 AIV strain A/duck/China/Influenza A virus/2019(H3N2) (accession number: OK104799-OK104806, abbreviated as SD strain), H9N2 AIV strain A/chicken/Daye/DY0602/2017(H9N2) (accession number: MF794999-MF795006, abbreviated as DY0602 strain), A/chicken/JinShui/JS1002/2018(H9N2) (accession number: MH375875-MH375882, abbreviated as JS1002 strain), and A/chicken/Hubei/01/1999(H9N2) (accession number: CY077081-CY077088, abbreviated as HB-01 strain) were maintained at Hubei Academy of Agricultural Sciences, Institute of Animal Husbandry and Veterinary Medicine.

Acquisition and analysis of mosaic H9 HA

A total of 15,060 H9 HA sequences were downloaded from the GISAID database, and 10,785 sequences remained after eliminating the incomplete sequences. These sequences were submitted to Mosaic Vaccine Designer, and mosaic H9 HA sequence was obtained. pUC57-H9 mosaic plasmid was synthesized by Shanghai Sangon Biotech Co., Ltd.

The phylogenetic and homology analyses of mosaic H9 HA was performed with MEGA12 software. Briefly, the amino acid sequence of mosaic H9 HA was aligned with the different H9N2 AIV via ClustalW, and the phylogenetic tree was generated using the maximum likelihood method with 1,000 bootstrap replicates. Pairwise distance of mosaic H9 HA was calculated to generate a homologous matrix for subsequent clustering. The sequences (Supplementary Table S1) of the mosaic H9 HA and the representative HA strains from h9.4.2.5 and h9.4.2.6 branches were submitted to SWISS-MODEL website for structural prediction, and the results were visualized using PyMOL software. For antigen epitope analyses, the prediction of T-cell and B-cell epitopes of the mosaic H9 HA and representative strains HA was performed in the NetTepi 1.0 and BepiPred 2.0 website. T-cell epitopes with % Rank less than 1.0 and B-cell epitopes with a score higher than 0.5 were retained.

Plasmid construction and virus rescue

The insertion of the influenza virus HA gene into the NDV genome, replacing its transmembrane and cytoplasmic domains (TM/CT) with those of the NDV F gene, can enhance the incorporation of HA into virus particles and immune protection (35). The TM/CT of mosaic H9 HA was replaced with TM/CT of NDV TS09-C strain F protein to obtain mosaic H9 HA TM/CT, named mH9. 3M2e encodes three tandem copies of M2e from the H9N2 AIV strains DY0602, JS1002, and HB-01 (Supplementary Table S2), with M2e linked by flexible linkers (GGSSGGSS).

The infectious cDNA clone of TS09-C strain (pTS09-C) served as backbone. mH9 was inserted between P gene and M gene of pTS09-C to obtain pTS-mH9, and 3M2e (with His tag at its C-terminal) was fused to the C-terminal of HN gene in pTS-mH9 to obtain pTS-mH9-3M2e. The pTS-mH9 and pTS-mH9-3M2e cDNA clones were generated via multiple overlapping PCR and homologous recombination using In-Fusion® Snap Assembly (Takara, United States). rTS-mH9 and rTS-mH9-3M2e were rescued by co-transfecting MAV-T7-infected BHK-21 cells with pTS-mH9 or pTS-mH9-3M2e cDNA clone and helper plasmids NP, P, and L. Recombinant viruses were passaged and purified in SPF chicken embryos for 10 generations. The P10 viruses were used for the subsequent experiments.

Identification of exogenous protein expression

BHK-21 cells were infected with TS09-C, rTS-mH9, or rTS-mH9-3M2e at 0.01 MOI; the expression of mH9 and 3M2e was assessed by indirect immunofluorescence staining at 48 h post-infection (hpi). Briefly, the cells were washed, fixed, permeabilized, and blocked in PBS containing 3% bovine serum albumin. Then, the cells were incubated with primary antibodies (chicken anti-NDV antibody, mouse anti-influenza A H9N2 HA polyclonal antibody, and rabbit anti-His tag antibody) and secondary antibodies (rabbit anti-chicken IgG H&L/FITC, Cy3-labeled goat anti-mouse IgG H&L, and AF647-labeled goat anti-rabbit IgG H&L). Finally, cell nuclei were stained with DAPI, and cells were imaged under a confocal microscope.

Biological characteristic analysis of recombinant virus

The virus titers of TS09-C, rTS-mH9, or rTS-mH9-3M2e were determined using HA assay, 50% tissue culture infectious dose ($TCID_{50}$) in BHK-21 cells, and median embryo infectious dose (EID_{50}) in SPF chicken embryos. The pathogenicity of the recombinant virus was determined as mean death time of minimal lethal dose (MDT/MLD) in 10-day-old SPF chicken embryos and with intracerebral pathogenicity index (ICPI) in 1-day-old SPF chickens. For growth kinetics, BHK-21 cells were infected with recombinant virus at 0.1 MOI, and the cell supernatant was collected at 12, 24, 36, 48, 60, and 72 hpi to assess viral replication efficiency by $TCID_{50}$ assay.

The thermostability of recombinant virus was assessed by calculating the HA titer and virus titer loss. For the HA titer loss, TS09-C, rTS-mH9, and rTS-mH9-3M2e were subjected to heat treatment at 56°C for 10, 20, 30, 40, 50, and 60 min. The HA titers were determined using HA assay. For virus titer loss, the viruses were subjected to heat treatment at 56°C for 2.5, 5, 7.5, 10, 12.5, and 15 min, and the virus titers were determined using $TCID_{50}$ assay. The thermolabile strain LaSota was used as a reference.

Vaccination and challenge test

Three independent vaccination and challenge experiments were performed. Firstly, 1-day-old SPF chickens were vaccinated with rTS-mH9-3M2e, rTS-mH9, and TS09-C at a dose of $10^{6.0}$ EID_{50}/bird or an equal volume of PBS via the intranasal and intraocular (IN/IO) routes. Serum was collected for antibody detection at 7, 14, and 28 days post-vaccination (dpv). The chickens from the PBS or vaccinated groups were respectively challenged with NDV F48E9 strain at a dose of $10^{5.0}$ 50% embryo lethal doses (ELD_{50})/bird via intramuscular route ($n = 10$) or H9N2 AIV DY0602 strain at a dose of 2×10^6 EID_{50}/bird ($n = 13$) or H9N2 AIV HB-01 strain at a dose of 2×10^6 EID_{50}/bird ($n = 13$) at 28 dpv.

Secondly, 26 1-day-old SPF chickens were vaccinated with rTS-mH9-3M2e at a dose of $10^{6.0}$ EID_{50}/bird via the IN/IO routes, while an equal volume of PBS was given to 26 chickens. Serum was collected for neutralizing antibody detection at 7, 14, and 28 dpv. The chickens from the PBS or vaccinated groups were challenged with A/duck/China/influenza A virus/2019(H3N2) (named H3N2/SD) strain at a dose of $10^{7.5}$ EID_{50}/bird ($n = 13$) at 28 dpv.

Thirdly, 18-day-old SPF chicken embryos were vaccinated with rTS-mH9-3M2e at a dose of $10^{3.0}$ EID_{50}/egg or an equal volume of PBS via the amniotic route (*in ovo* vaccination group). Moreover, 1-day-old SPF chickens were vaccinated with rTS-mH9-3M2e at a dose of $10^{6.0}$ EID_{50}/bird via the IN/IO routes (post-hatch vaccination group). When the chickens were 3, 7, and 14 days of age, serum was collected for antibody detection and challenged with NDV F48E9 strain ($n = 10$) or H9N2 AIV DY0602 strain ($n = 10$).

Antibody detection in serum

The serum samples from the first and third experiments were used for antibody detection. ND- and H9-specific hemagglutination inhibition (HI) antibodies were measured using the HI assay. The HI assays were performed in 96-well V-bottom microtiter plates using 1% chicken erythrocyte suspensions and 4 hemagglutinating units (HAU) of NDV or H9N2 antigen. Briefly, the serum was serially twofold diluted in PBS and mixed with an equal volume of 4 HAU antigen. After incubation at room temperature for 30 min, an equal volume of 1% chicken erythrocytes was added to each well, and the mixture was incubated at room temperature for 30 min. The HI titers were recorded as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

The M2e-specific antibodies of serum samples from the first experiment were detected by ELISA using the 96-well ELISA plate, and each well was coated with 200 ng of synthetic M2e peptide overnight at 4 °C. The plates were washed with 1× TBST and blocked in 5% skim milk powder for 1 h, and the serum samples (1:1,600 dilution) were incubated at 37°C for 1 h. Then, the plates were washed, incubated with HRP-labeled goat anti-chicken IgG (1:25,000 in 1× PBST) at 37°C for 1 h and with TMB substrate at room temperature for 10 min. The optical density (OD) values of the samples were read with a multimode reader at an absorbance of 630 nm.

The neutralizing antibody titers to H3N2 and H9N2 AIVs were measured by using the microneutralization assay; the serum samples were from 14 dpv in the first and second experiment. Briefly, 100 $TCID_{50}$ of each virus was mixed in equal volume with twofold serial dilutions of the serum, the mixture was incubated for 1 h at room temperature, subsequently added into MDCK cells, and the cells were cultured at 37°C with 5% CO_2 for 72 h. The neutralizing antibody titers were determined based on the inhibition of hemagglutination and cytopathic effect (CPE).

Clinical sign

The chickens infected with H9N2 AIV may cause mild respiratory distress, anorexia, weight loss, rales, runny nose, and mild coughing (36). In this study, only weight loss, respiratory distress, or mild coughing were observed throughout the entire challenge period: clinical score: 0—no clinical signs, 1—weight loss alone, and 2—weight loss combined with respiratory distress or mild coughing. For the H3N2 AIV challenge, anorexia and mild coughing were observed: clinical score: 0—no clinical signs, 1—anorexia or mild coughing alone, and 2—anorexia combined with mild coughing. Each chicken was scored daily, and the final score was calculated as the average score of all chickens in each group.

Histopathology, tissue viral load, and virus shedding

The lungs and tracheas (n = 3) of chickens at 3 days post-challenge (dpc) with H3N2 and H9N2 AIVs were collected for histopathological examination and viral load quantification. For histopathological examination, tissue samples were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned (4 μm), stained with hematoxylin–eosin (HE), analyzed, and imaged microscopically. For viral load detection, the tissue samples were crushed, repeatedly frozen and thawed, and centrifuged, and the supernatant was used for total RNA extraction. The NP gene was detected by RT-qPCR, and virus RNA copies were calculated based on the standard curve of the NP gene. Throat swabs (n = 10) and cloaca swabs (n = 10) were collected with H3N2 and H9N2 AIV at 3, 5, and 7 dpc; the virus NP gene copies were detected and analyzed using the same method as described above. The positivity threshold was defined as Ct value <35. The limit of detection was 10 RNA copies/μL. Samples with Ct values ≥35 or below the limit of detection were considered negative.

Flow cytometry

In the third experiment, the spleens (n = 3) of chickens at 3 or 7 days of age from the *in ovo* vaccination group, post-hatch vaccination group, and PBS group were collected for the analysis of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations. Single splenic lymphocyte populations were prepared according to the manufacturer's instruction as indicated in the chicken splenic lymphocyte separation kit (Solarbio, Beijing, China). The percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were analyzed by flow cytometry assay, and data were processed using FlowJo_v10.10.0 software.

Statistical analysis

GraphPad Prism 10.1.2 software was used for statistical analysis. Data were presented as mean ± SD. Group comparisons for HI antibody, neutralizing antibody, viral load, and level of $CD4^+$ and $CD8^+$ T cell test were performed by unpaired two-tailed Student's t-test. Viral shedding was compared between different groups at the same time point using unpaired two-tailed Student's t-tests. A P-value equal or less than 0.05 was considered significant (significant, $^*P < 0.05$; very significant, $^{**}P < 0.01$) and not significant (ns, $P > 0.05$).

Results

Characterization of mosaic H9 HA and acquisition of recombinant virus rTS-mH9-3M2e

The phylogenetic tree and homology of mosaic H9 HA were analyzed. Mosaic H9 HA belongs to the h9.4.2.5 branch (Figure 1A) and shows 97.1% homology with the representative strain A/Chicken/Guang Xi/55/2005 strain from this branch (Figure 1B). The h9.4.2.5 and h9.4.2.6 branch H9N2 AIVs co-circulated in China, and the h9.4.2.5 branch was dominant (37, 38). The conformation of mosaic H9 HA was similar to that of the representative HA strains of the h9.4.2.5 and h9.4.2.6 branches, A/Chicken/Guang Xi/55/2005 and A/Chicken/Guang Dong/FZH/2011 (Figure 1C), and contained more T cell and B cell epitope than them (Supplementary Figure S1).

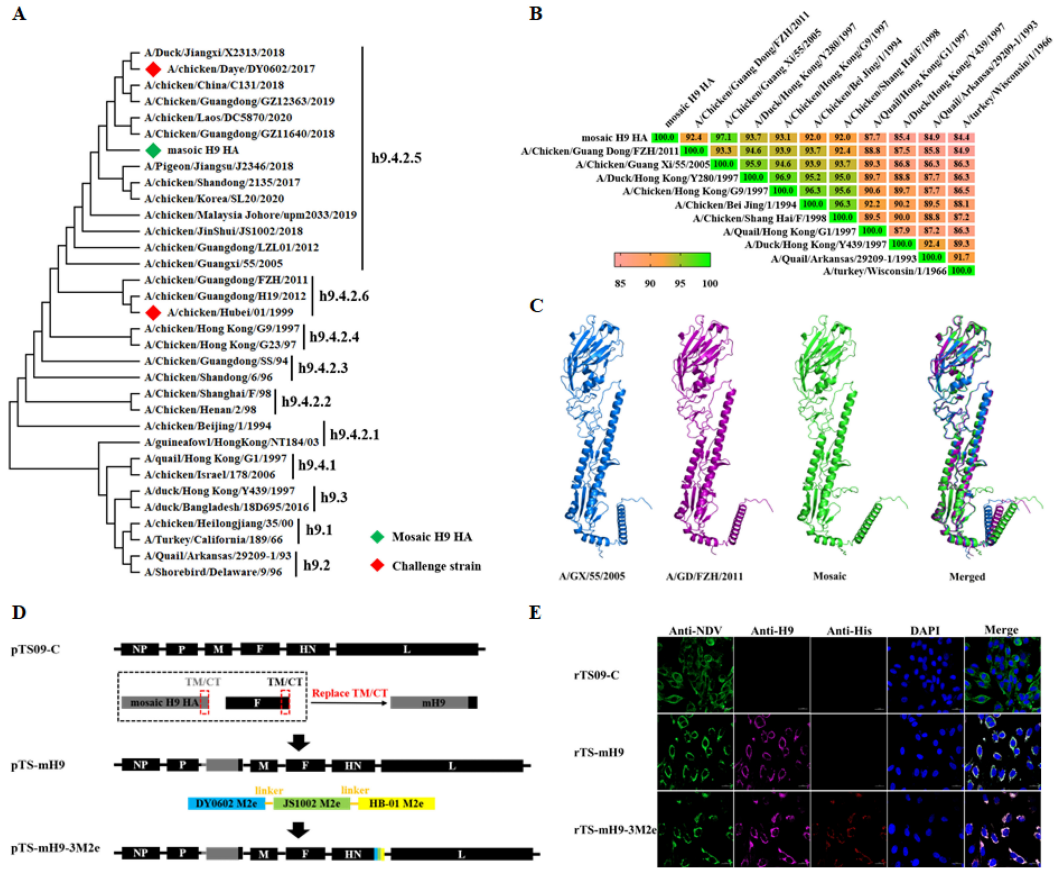

FIGURE 1
Characterization of mosaic H9 HA and construction of recombinant virus. **(A)** Phylogenetic tree of mosaic H9 HA. **(B)** Homology analysis of mosaic H9 HA. **(C)** Conformation of mosaic H9 HA. **(D)** Construction diagram of recombinant virus. **(E)** Expressing identification of mosaic H9 HA and 3M2e protein.

mH9 was inserted into the P and M genes of TS09-C strain for the construction of pTS-mH9, based on pTS-mH9, and 3M2e was fused into the C-terminal of the HN gene for the construction of pTS-mH9-3M2e (Figure 1D). The recombinant viruses rTS-mH9-3M2 and rTS-mH9 were rescued and found to exhibit biological characteristics similar to those of TS09-C (Supplementary Figure S2). The mH9 and 3M2e proteins were successfully expressed (Figure 1E).

Broad-immunoreaction induced with rTS-mH9-3M2e to different H9N2 AIVs

To evaluate the broad-immunoreaction and universal protection of rTS-mH9-3M2e vaccine candidate against H9N2 AIVs, we designed the first experiment according to the scheme shown in Figure 2A. The 1-day-old chickens were respectively vaccinated with rTS-mH9-3M2e, rTS-mH9, or rTS09-C, and the antibody levels of ND-specific and H9-specific HI, as well as M2e-specific antibody, were detected. rTS-mH9-3M2e, rTS-mH9, and rTS09-C all induced ND-specific HI antibody response, and no significant difference was observed among the three groups (Figure 2B). rTS-mH9-3M2e induced higher H9-specific HI antibody titers than rTS-mH9, but there was no significant difference between the two groups (Figure 2C). Only rTS-mH9-3M2e induced a M2e-specific antibody response (Figure 2D).

The neutralizing antibodies induced by vaccines against different H9N2 AIVs were evaluated. The DY0602 strain (Figure 2E) and JS1002 strain (Figure 2F) from h9.4.2.5 branch and the HB-01 strain from h9.4.2.6 branch were selected (Figure 2G). The rTS-mH9-3M2e and rTS-mH9 vaccines both induced neutralizing antibody responses against three H9N2 AIVs; the antibody titer of the rTS-mH9-3M2e group was higher than that of the rTS-mH9 group, but there was no significant difference between the two groups ($P > 0.05$).

Recombinant vaccine provided broad protection against different H9N2 AIVs

The challenge tests of NDV and H9N2 AIV were performed at 28 dpv. After challenge with NDV virulent strain F48E9, the survival rates were 100% in the rTS-mH9-3M2e, rTS-mH9, and TS09-C vaccination groups, and chickens from the PBS group were all dead at 6 dpc (Figure 3A). To evaluate the effect of broad protection against H9N2 AIV, we selected DY0602 and HB-01 strain to perform challenge tests. No clinical signs were observed in chickens from the rTS-mH9-3M2e and rTS-mH9 vaccination groups, whereas weight loss, respiratory distress, and mild coughing

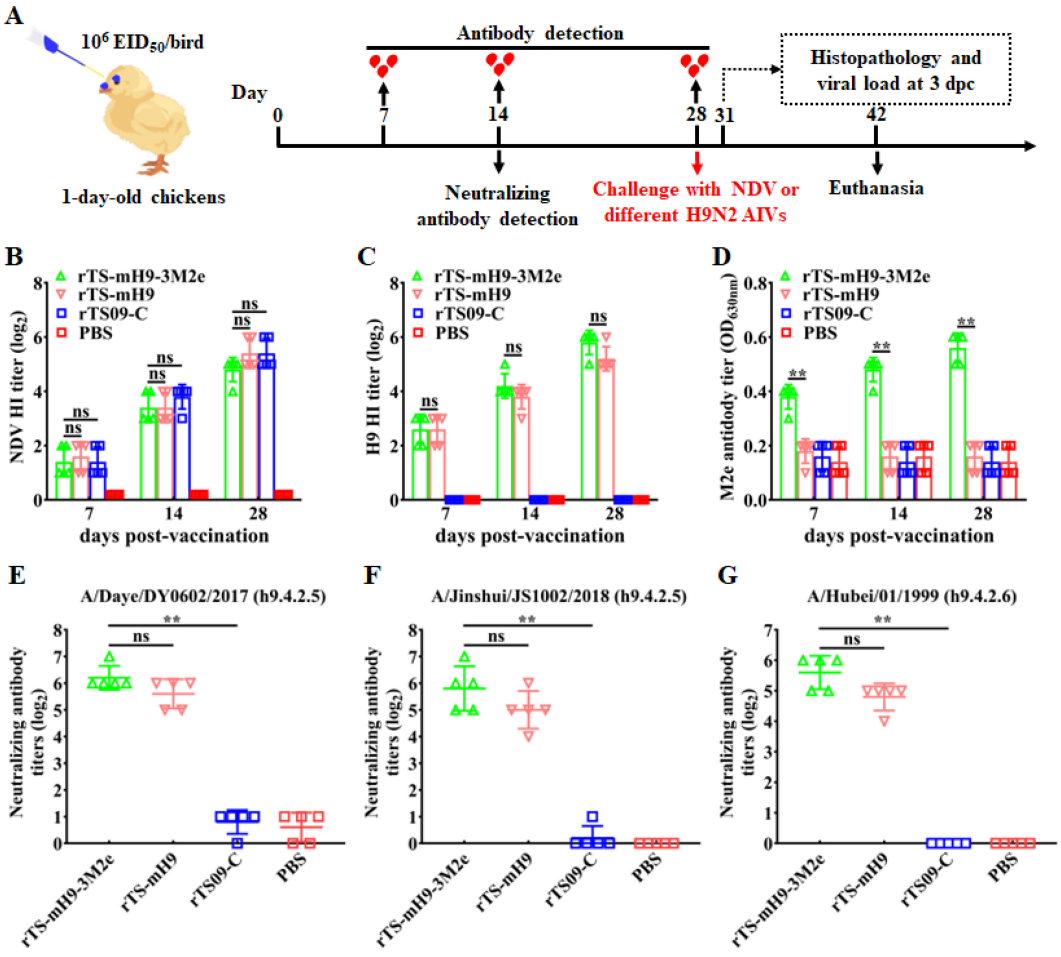

FIGURE 2
Immunogenicity of recombinant vaccines in 1-day-old chickens. **(A)** Vaccination and H9N2 AIVs challenge schedule of recombinant vaccines. **(B)** ND-specific HI antibody titers of serums. **(C)** H9-specific HI antibody titers of serums. **(D)** M2e antibody titers of serums. **(E–G)** Neutralizing antibody titers of serums to H9N2 AIVs DY0602, JS1002, and HB-01 strains. Serums for ND-, H9-specific HI antibody and M2e antibody detection from 7, 14, and 28 dpv. Serums for neutralizing antibody detection from 14 dpv. Statistical significance was determined with two-tailed *t*-tests: ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$.

were observed in chickens from the TS09-C and PBS groups after challenge with the DY0602 strain (Figure 3B). After challenge with the HB-01 strain, similar results were also observed (Figure 3C).

We further evaluated histopathological changes, viral load of the lung and trachea, and virus shedding in the rTS-mH9-3M2e and rTS-mH9 vaccination groups or the PBS group after challenge with the H9N2 AIV DY0602 or HB-01 strain. No histopathological lesions were observed in the lung and trachea of chickens from the rTS-mH9-3M2e and rTS-mH9 vaccination groups after challenge with the DY0602 or HB-01 strain. In the challenge tests of the DY0602 strain, severe lesions were observed in the lung (parenchymal lesions, disappearance of alveolar cavities, and hemorrhage) and trachea (cilia loss, mucosal epithelial cells necrosis, lumen containing mucus, and inflammatory cells) of chickens from the PBS group (Figure 3D). After challenge with the HB-01 strain, moderate lesions were observed in the lung (hemorrhage, congestion, and inflammatory cell infiltration) and trachea (cilia loss and mucosal epithelial cell necrosis) of chickens from the PBS group (Figure 3E). The viral loads of the lung and trachea of chickens from the rTS-mH9-3M2e and rTS-mH9 vaccination groups were significantly lower than those of the PBS group ($P < 0.05$) after challenge with the DY0602 strain (Figure 3F) or the HB-01 strain (Figure 3G).

Virus shedding is an important criterion to evaluate the effectiveness of the H9N2 vaccine (10). The respiratory and digestive tract virus shedding, respectively, was detected at 3, 5, and 7 dpc after challenge with the DY0602 strain or the HB-01 strain. For the DY0602 or HB-01 strain challenge, the amount of respiratory and digestive tract virus shedding in the rTS-mH9-3M2e and rTS-mH9 vaccination groups was lower than that of the PBS group at all test time points (Supplementary Table S3). After challenge with the DY0602 strain, the viral loads of the throat swab and cloaca swab in the rTS-mH9-3M2e and rTS-mH9 vaccination groups were both significantly lower than those of the PBS group ($P < 0.05$) at 3, 5, and 7 dpc. Furthermore, the viral loads of the throat swab and cloaca swab in rTS-mH9-3M2e were significantly lower than those of the rTS-mH9 vaccination group ($P<0.05$) at 5 dpc (Figure 3H). After challenge with the HB-01 strain, the viral load results of the throat swab and cloaca swab were similar with those of the DY0602 strain challenge (Figure 3I). Taken together, the rTS-mH9-3M2e

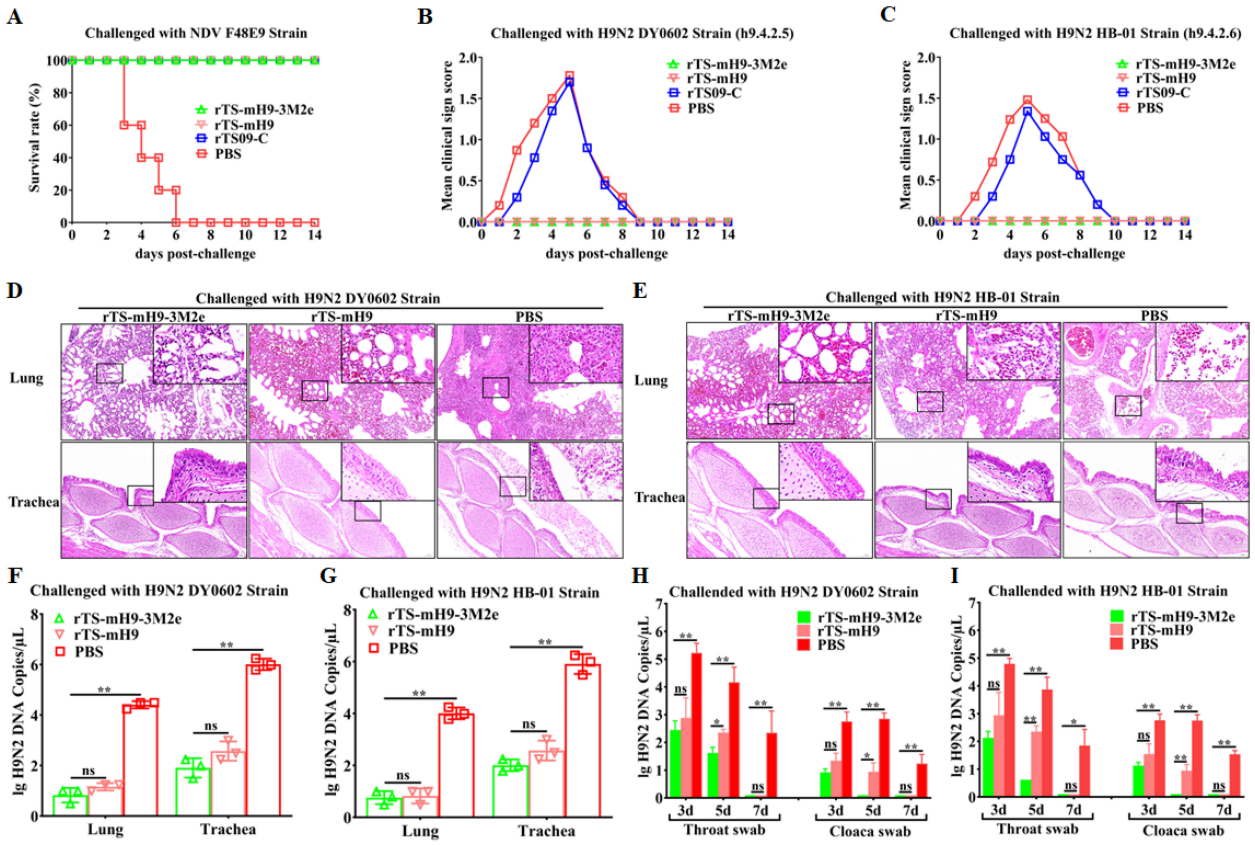

FIGURE 3
Broad protective evaluation of recombinant vaccines against different H9N2 AIVs. **(A)** Survival rate of chickens challenged with NDV virulent strain. **(B, C)** Clinical scoring of chickens challenged with H9N2 AIVs DY0602 and HB-01 strains. One clinical sign for one score; the final scores are the average value of all birds' scores. **(D, E)** Histopathological evaluation in the lung and trachea of chickens challenged with H9N2 AIVs DY0602 and HB-01 strains. **(F, G)** Viral load in the lung and trachea of chickens challenged with H9N2 AIVs DY0602 and HB-01 strains. **(H, I)** Viral titers of positive swabs after challenge with H9N2 AIVs DY0602 and HB-01 strains.

and rTS-mH9 vaccine candidates provide the broad protection against different H9N2 AIVs, and the protective effect of rTS-mH9-3M2e was superior than that of rTS-mH9.

rTS-mH9-3M2e provided heterologous cross-protection against H3N2 AIV

H9N2 AIV can recombine into H3N2 influenza viruses; the recombinant viruses showed high genetic compatibility, even enhancing the pathogenicity of the viruses (39). Therefore, we designed the second experiment, where 1-day-old chickens were vaccinated with rTS-mH9-3M2e, blood samples were collected at 14 dpv, and the chickens were challenged with the H3N2 SD strain at 28 dpv (Figure 4A). The neutralizing antibody titer of the serum from vaccinated chickens for the H3N2 SD strain was 3.4 ± 0.49 $\log_2$ and was significantly higher than that of the PBS group ($P < 0.05$) (Figure 4B).

In the challenge test, the mean clinical sign score in the rTS-mH9-3M2e vaccination group was lower, and the onset period was shorter than that of the PBS group (Figure 4C). At 3 dpc, histopathological changes and viral loads of the lung and trachea were evaluated. The histological lesions of the lung (lymphocyte infiltration) and trachea (cilia loss) of chickens from the rTS-mH9-3M2e group were milder than those of the lung (hemorrhage and lymphocyte infiltration) and trachea (cilia loss and submucosal edema) of chickens from the PBS group (Figure 4D). The viral loads of the lung and trachea in the rTS-mH9-3M2e group were significantly lower than those of the PBS group ($P < 0.05$) after challenge with the H3N2 SD strain (Figure 4E). At 3, 5, and 7 dpc, we collected throat swabs and cloaca swabs of chickens in both groups to detect virus shedding. The positive rate of virus shedding was not reduced after rTS-mH9-3M2e vaccination compared with the PBS group (Supplementary Table S4). However, the viral loads of throat swab and cloaca swab in the rTS-mH9-3M2e group were significantly lower than those of the PBS group ($P < 0.05$) (Figure 4F). These results demonstrate that the rTS-mH9-3M2e vaccine candidate can induce cross-reactive neutralizing antibody and confer cross-protection against H3N2 AIV.

rTS-mH9-3M2e as *in ovo* vaccine provides early protection against NDV and H9N2 AIV

In ovo vaccination is an attractive immunization approach for poultry industry, and it can induce earlier immune responses (Peebles ED 2018). Our previous studies have shown that the TS09-C strain used as an *in ovo* vaccine was safe to SPF and commercial chicken embryos (Wen et al., 2017; Fan et al., 2020).

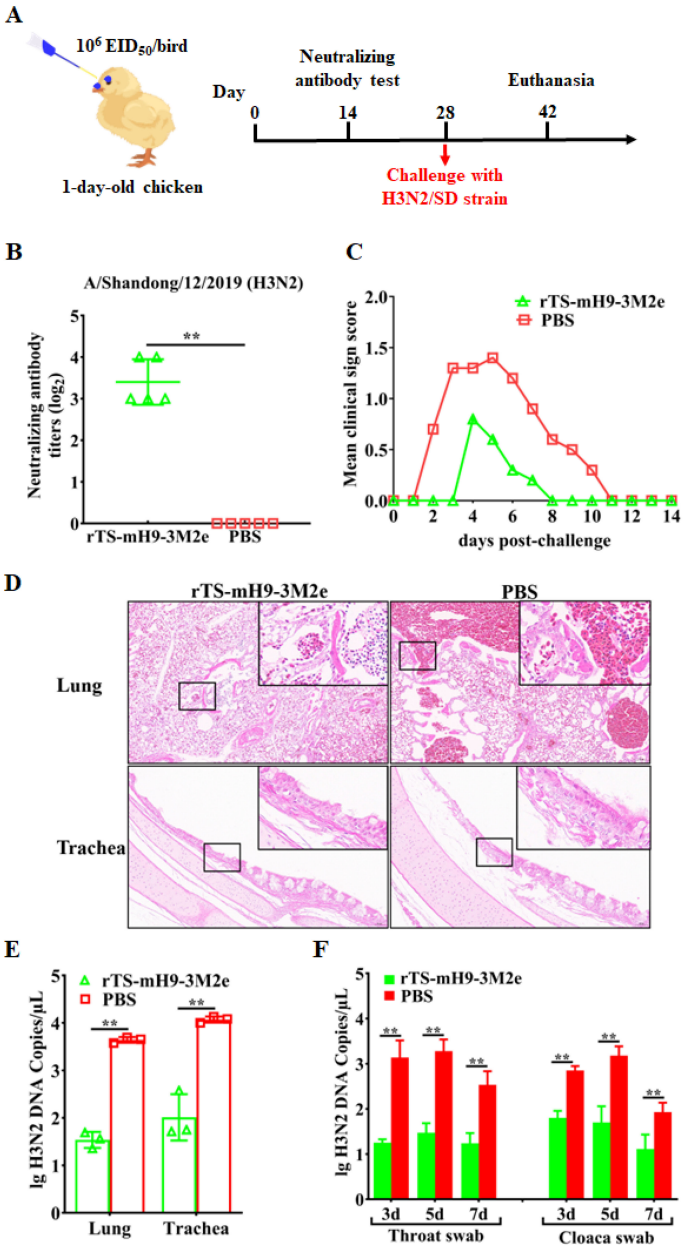

FIGURE 4
Cross-protection of recombinant vaccine against H3N2 AIV. **(A)** Vaccination and H3N2 AIV challenge schedule of recombinant vaccines. **(B)** Neutralizing antibody titers of serums to H3N2 AIV. Serums from 14 dpv. **(C)** Clinical scoring after challenge with H3N2 AIV. **(D, E)** Histopathological evaluation and viral load in the lung and trachea of chickens challenged with H3N2 AIV. **(F)** Viral titers of positive swabs after challenge with H3N2 AIV.

Here we designed the third experiment to evaluate the early protection against NDV and H9N2 AIV (DY0602 strain). Thus, 18-day-old chicken embryos (*in ovo* vaccination group) and 1-day-old chickens (post-hatch vaccination group) were vaccinated with rTS-mH9-3M2e, and challenge was performed in chickens at 3, 7, and 14 days of age (Figure 5A). The hatchability rates of the *in ovo* vaccination group and the PBS group were both 93.3% (Figure 5B) and indicate that rTS-mH9-3M2e used as *in ovo* vaccine is safe.

In the NDV challenge tests, the survival rates of the chickens were respectively 40%, 0%, and 0% in the *in ovo* vaccination group, post-hatch vaccination group, and PBS group at 3 days of age challenge (Figure 5C), 80%, 20%, and 0% at 7 days of age challenge (Figure 5D), and 100%, 40%, and 0% at 14 days of age challenge (Figure 5E). For the H9N2 AIV challenge, 60% of the chickens in the *in ovo* vaccination group showed clinical signs at 3 days of age challenge as well as 100% in the post-hatch vaccination group and the PBS group (Figure 5F). No clinical signs were observed in the *in ovo* vaccination group at 7 days of age challenge, whereas clinical signs were observed in 70% and 100% of chickens in the post-hatch vaccination group and the PBS group (Figure 5G). At 14 days of age challenge, no clinical signs were observed in the *in ovo* vaccination group, whereas clinical signs were observed in 40% and 100% of chickens in the post-hatch vaccination group and the PBS group (Figure 5H). The detailed clinical scoring records after challenge with H9N2 AIV are shown in Supplementary Figure S3.

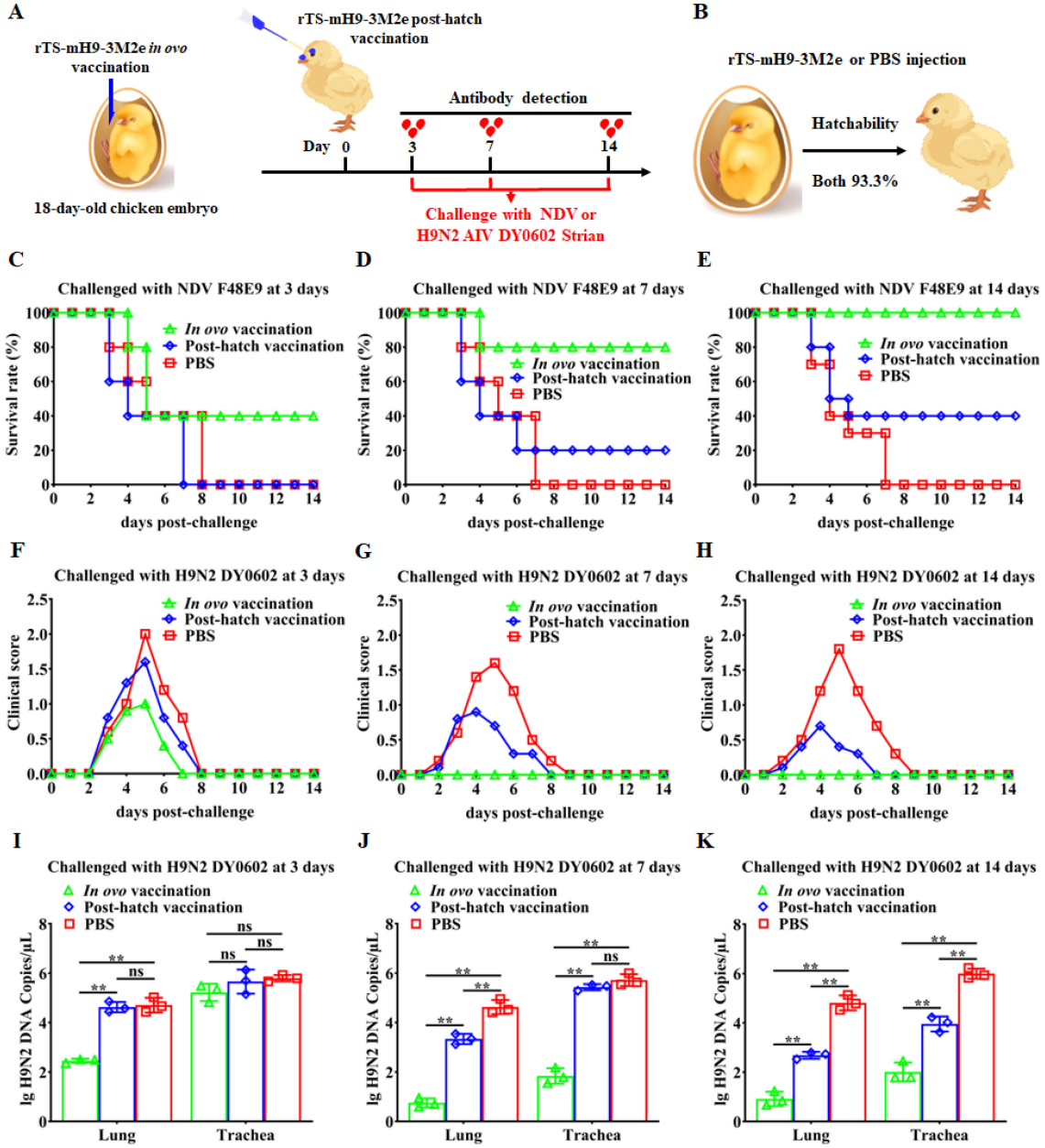

FIGURE 5
The early protective effect of recombinant virus used as *in ovo* vaccine. **(A)** Vaccination and challenge schedule of recombinant virus as *in ovo* vaccines. **(B)** Hatchability after *in ovo* vaccination. **(C–E)** Survival rate of chickens challenged with NDV virulent strain at 3, 7, and 14 days of age. **(F–H)** Clinical scoring of chickens challenged with H9N2 AIV DY0602 strain at 3, 7, and 14 days of age. **(I–K)** Viral load in the lung and trachea of chickens challenged with H9N2 AIV DY0602 strain at 3, 7, and 14 days of age.

Furthermore, we evaluated the viral load of the lung and trachea after challenge with H9N2 AIV. At 3 days of age challenge, the viral load of the lung from chickens in the *in ovo* vaccination group was significantly lower than that in the post-hatch vaccination group and the PBS group ($P < 0.05$), and no significant difference was observed for the trachea among the three groups ($P > 0.05$) (Figure 5I). The viral loads of both the lung and trachea from chickens in the *in ovo* vaccination group were significantly lower than those of the post-hatch vaccination group and PBS group ($P < 0.05$) at 7 (Figure 5J) and 14 (Figure 5K) days of age challenge. These results demonstrate that rTS-mH9-3M2e as *in ovo* vaccines can provide earlier protection than post-hatch vaccination against NDV and H9N2 AIV.

rTS-mH9-3M2e as *in ovo* vaccine induced earlier cellular immune response

In order to explore the reasons for the earlier protection against NDV and H9N2 AIV, we analyzed the humoral and cellular immune response induced by rTS-mH9-3M2e. The ND-specific HI and H9-specific HI antibody levels of the *in ovo* vaccination group had no significant difference versus those of the post-hatch

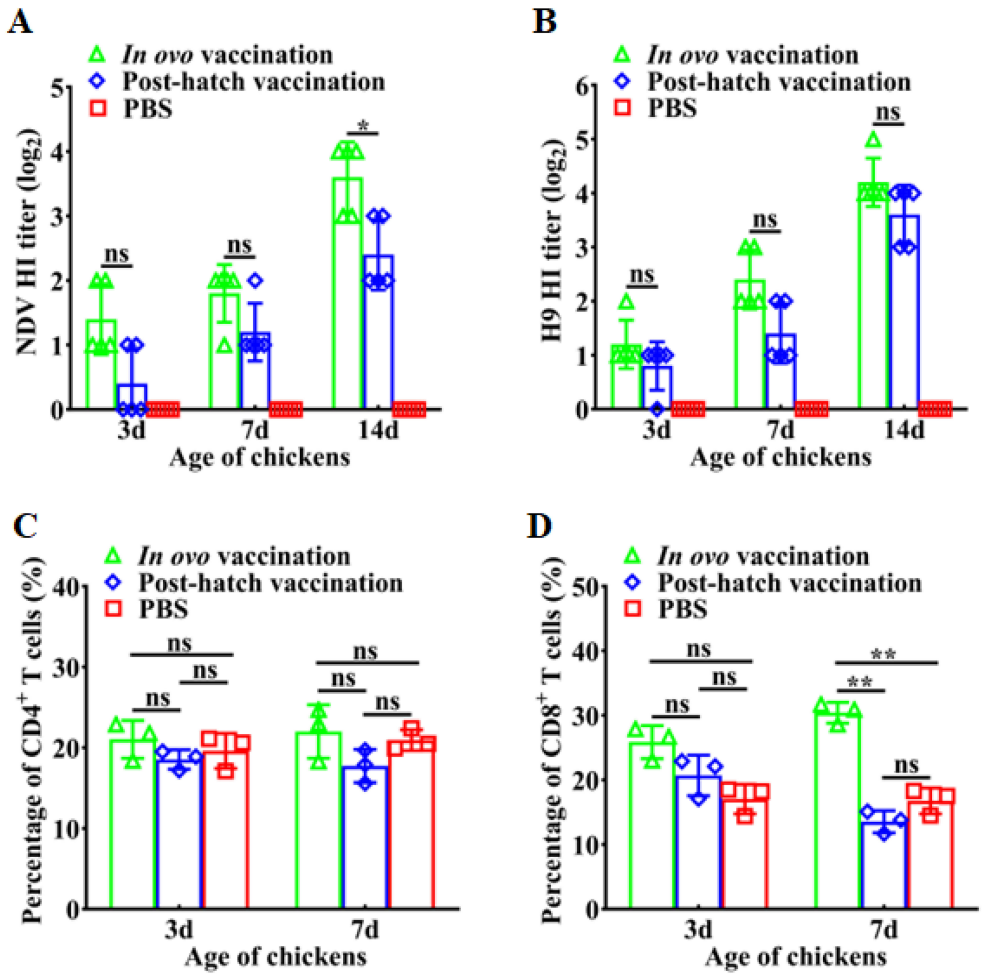

FIGURE 6
Evaluation of humoral and cellular immunity induced by recombinant viruses used as *in ovo* vaccines. **(A, B)** ND- and H9 HI titer of serums from 3 and 7 days of age chickens. **(C, D)** Percentage of $CD4^+$ and $CD8^+$ T cells in the spleens of chickens at 3 and 7 days of age.

vaccination group ($P > 0.05$) at 3 and 7 days of age (Figures 6A, B). For cellular immune response, the $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cell levels of spleens were detected. The $CD3^+$ $CD4^+$ T cells levels of the *in ovo* vaccination group had no significant difference versus those of the post-hatch vaccination group ($P > 0.05$) at 3 and 7 days of age (Figure 6C). The $CD3^+$ $CD8^+$ T cell levels in the *in ovo* vaccination group were significantly higher than those in the post-hatch vaccination group ($P < 0.05$) at 7 days of age, with no significant difference at 3 days of age ($P > 0.05$) (Figure 6D). The results demonstrate that rTS-mH9-3M2e as *in ovo* vaccine induced earlier $CD8^+$ T cellular immune response than post-hatch vaccination.

Discussion

Avian influenza vaccines must be updated regularly to counter the antigenic drift of AIV. Vaccines primarily target the immunodominant head domain of HA and induce narrow, strain-specific immune responses (40). WHO annually monitors the global avian influenza and predicts the possible prevalent virus strains. However, it takes 6 to 8 months from the prediction of influenza strains to the production of vaccines, and the virus strains may undergo antigenic drift, causing them to be not fully compatible with the vaccine strains during this period (15). Therefore, there is an urgent need to develop universal avian influenza vaccines, and these vaccines can induce cross-reactive immunity to deal with drift variants of influenza virus. In this study, we developed and evaluated the efficacy of recombinant vaccines rTS-mH9-3M2e expressing mosaic H9 HA and 3M2e and rTS-mH9 expressing mosaic H9 HA against different H9N2 AIVs and heterogeneous subtype AIVs.

The mosaic HA vaccines can elicit cross-reactive antibodies against AIV and robust virus-specific cellular immune responses (41). The recombinant vaccines rTS-mH9-3M2e and rTS-mH9 induced broad and effective neutralization activity to different H9N2 AIVs and provided broad protection against the tested H9N2 AIVs from h9.4.2.5 (DY0602 strain) and h9.4.2.6 (HB-01 strain) branches. The rTS-mH9-3M2e and rTS-mH9 vaccines can effectively prevent clinical morbidity and lesions of respiratory tissues caused by the DY0602 and HB-01 strain challenge. The ideal H9N2 vaccines should not only prevent the clinical morbidity of chickens but also should reduce virus shedding, which may help limit virus spread within a flock (42). The rTS-mH9-3M2e and rTS-

mH9 vaccines effectively reduced the amount of virus shedding, shortened the period of virus shedding, and decreased the viral load of positive throat and cloaca swabs after different H9N2 AIVs. However, the protection of rTS-mH9-3M2e vaccine was superior than that of the rTS-mH9 vaccine; it can further inhibit virus shedding after H9N2 AIV challenge. Therefore, we focused on evaluating the effectiveness of the rTS-mH9-3M2e vaccine in our subsequent experiments.

In this study, we have confirmed that the rTS-mH9-3M2e vaccine can confer cross-protection against H3N2 AIV. The rTS-mH9-3M2e vaccine alleviated the lesion and decreased the viral load in the lung and trachea. The mosaic H3 HA live attenuated influenza vaccine can confer cross-protection for H3N2 and H1N1 influenza virus by inducing robust cellular immune responses (20). Subbiah et al. developed a chimeric M2e-H3 stalk vaccine using the H3 stalk domain conjugated with M2e; the vaccine confers protection against H1N1, H3N2, H5N1, H7N9, and H9N2 in mice (43). The A/H1H3 virus-like particle vaccine expressing M2e can induce IgG and IgA antibody response as well as B cell and T cell response and provide cross-protection against influenza B virus (44). Mosaic antigens designed to maximize T-cell epitope coverage induced cross-reactive antibody response and T cell immune response (23). M2e is highly conserved in influenza virus; the vaccine based on M2e can induce a robust cellular immune response against a broad range of influenza virus infections (45). The T cell response of M2e-based vaccines was shown to contribute to cross-protection against H1N1 and H3N2 influenza virus in mice (46). Cellular immune response can efficiently select immune escape mutants after influenza virus infection and recognize epitopes derived from immunogenic or conserved internal proteins to provide universal protection against influenza virus (11).

Newborn chickens are particularly vulnerable to H9N2 AIV infection due to the time required to establish adaptive immunity (47). *In ovo* vaccination could fill the immune window period and induce immune responses earlier (48). The live cell-associated HVT vaccine provided a protection rate of 67.4% in 10-day-old chickens via *in ovo* vaccination after challenge (49). The recombinant turkey herpesvirus vaccine expressing conserved B and T cell epitopes from H9N2 AIV can effectively reduce viral load of the tissues and virus shedding in chickens at 14 days of age challenged with H9N2 AIV (42). Here we confirmed that rTS-mH9-3M2e as *in ovo* vaccine (*in ovo* vaccination group) could provide 100% protection for chickens challenged with H9N2 AIV at 7 days of age but provided protection for only some chickens when administered to 1-day-old chickens (post-hatch vaccination group). The viral load of the lung and trachea in chickens from the *in ovo* vaccination group was significantly lower than those from the post-hatch vaccination group. In addition, rTS-mH9-3M2e as *in ovo* vaccine provides 80% protection for chickens challenged with virulent NDV at 7 days of age. Therefore, we speculate that the early protection may be related with the *in ovo* vaccination method.

Commercialized ND and influenza vaccines are designed to induce robust humoral immune responses because HI titers are the primary criterion for vaccine licensing (16, 50). However, the ND and H9 HI antibody titers of the *in ovo* vaccination group were not significantly different from those of the post-hatch vaccination group when the chickens were 7 days old. Hence, the earlier immune protection of rTS-mH9-3M2e as *in ovo* vaccine operates independently of humoral immunity. *In ovo* inoculation of turkey herpesvirus (HVT) vaccine hastened the maturation of the chicken embryo immune system, resulting in a higher percentage of $CD8^+$ T cells than in 1-day-old chickens inoculated with the vaccine (51–53). The increasing amount of evidence highlights that T lymphocyte-mediated cellular immunity plays a critical role in vaccine protection because they can directly target infected cells to clear the virus (54). T cells are considered to be fully immunocompetent in 18-day-old chicken embryos (55). In this study, the level of $CD8^+$ T cells in the *in ovo* vaccination group was significantly higher than that of the post-hatch vaccination group in 7-day-old chickens. The viral titers in the lung and trachea of chickens from the *in ovo* vaccination group were significantly lower than those from the post-hatch vaccination group. A limitation of our study is the lack of further exploration of the mechanism of early $CD8^+$ T cell proliferation induced by rTS-mH9-3M2e as an *in ovo* vaccine.

In conclusion, we developed a recombinant vaccine, rTS-mH9-3M2e, based on NDV vector, and vaccine provided cross-protection against homologous and heterologous subtype AIVs. Furthermore, rTS-mH9-3M2e as *in ovo* vaccine could induce significant $CD8^+$ T cell immune responses and provide early immune protection against the virulent NDV and H9N2 AIV. In the future, we will study further how rTS-mH9-3M2e as an *in ovo* vaccine induces early $CD8^+$ T cellular immunity.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of the Hubei Academy of Agriculture Sciences. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LJ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. GW: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. XS: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – review & editing. ZZ: Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Writing – review & editing. ZW:

Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. YZ: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. YS: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. HW: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. LY: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. RW: Data curation, Formal analysis, Software, Writing – review & editing. SW: Data curation, Formal analysis, Software, Writing – review & editing. MG: Data curation, Formal analysis, Software, Writing – review & editing. QL: Conceptualization, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing. HF: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2026.1985015/full#supplementary-material

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