Efficacy of recombinant Newcastle disease virus expressing HA protein of H9N2 Avian influenza virus in respiratory and intestinal tract

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ABSTRACT H9N2 subtype avian influenza virus (**AIV**) is a low pathogenic AIV, which is widely prevalent all over the world. The infection of H9N2 AIV often leads to secondary infection with other pathogens, causing serious economic losses to poultry industry. Up to now, several recombinant Newcastle disease viruses (**NDV**) expressing H9N2 AIV hemagglutinin (**HA**) protein had been developed. However, the efficacy of recombinant virus on tracheal and intestinal injury caused by H9N2 AIV was rarely reported. The aim of this study was to evaluate the efficacy of recombinant NDV expressing H9N2 AIV HA protein in respiratory and intestinal tract. In this study, based on Red/ET homologous recombination technology, H9N2 AIV HA gene was embedded into the genome of NDV LaSota vaccine strain to obtain the recombinant virus rNDV-H9. The recombinant virus rNDV-H9 showed similar replication kinetic characteristics with the parent LaSota strain and had good genetic stability. The immunization result showed that rNDV-H9 induced high HI antibody titer against H9N2 AIV. In the H9N2 AIV challenge experiment, rNDV-H9 could significantly reduce the virus shedding in trachea and cloaca. In addition, rNDV-H9 protected the barrier function of chicken intestinal mucosal epithelial cells and reduced the virus-induced inflammatory response to a certain extent, so as to inhibit the abnormal proliferation of *E. coli*. This study suggests that rNDV-H9 is a promising vaccine candidate against H9N2 AIV.

Key words: H9N2 avian influenza virus, Newcastle disease virus, HA protein, trachea, intestine

2022 Poultry Science 101:102078
https://doi.org/10.1016/j.psj.2022.102078

INTRODUCTION

H9N2 avian influenza virus (**AIV**), belongs to the influenza virus A genus of the *Orthomyxoviridae* family, is a kind of negative stranded RNA virus (Kariithi et al., 2020). H9N2 AIV was first reported in the United States in 1966 (Peacock et al., 2019). At present, H9N2 AIV was widely spread all over the world and had become the most prevalent AIV in China (Sun and Liu, 2015). H9N2 AIV is a low-pathogenic AIV, only causing mild respiratory symptoms after infection in chicken (Swayne and Pantin-Jackwood, 2006). However, H9N2 AIV is often co-infected with other avian pathogens, resulting in severe clinical symptoms and high mortality (Arafat et al., 2018; Kong et al., 2021). In addition, H9N2 AIV can also provide internal genes for other subtypes of viruses, resulting in zoonotic virus or highly pathogenic virus, which not only causes serious economic losses to poultry industry, but also poses a major threat to human health (Chen et al., 2014; Naguib et al., 2015; Zhu et al., 2018).

H9N2 AIV has multiple tissue tropisms and can replicate both in the respiratory and gastrointestinal tracts (Post et al., 2012). Numerous studies had shown that H9N2 AIV infection caused intestinal flora disorder and inflammation (Zhang et al., 2020a,b; Chrzastek et al., 2021). Alterations in the gut microbial composition of chickens led to significantly increased shedding of H9N2 AIV in trachea and cloaca, which was associated with impaired expression of type I interferon (**IFN**)

Received June 2, 2022.
Accepted July 20, 2022.

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(Yitbarek et al., 2018a). Therefore, the balance of gut microecology might play an important role in the body's resistance to influenza virus (Yitbarek et al., 2018b). Previously, it was reported that H9N2 AIV infection affected the microbial composition of the chicken ileum and destroyed the mucous layers and tight junctions, resulting in a significant upregulation of proinflammatory cytokines expression in intestinal epithelial cells and an inflammatory response (Li et al., 2018). In addition, H9N2 AIV infection could induce intestinal mucosal inflammation and promote the secretion of nitrates by the intestinal epithelium; moreover, intestinal symbiotic *E. coli* proliferated with nitrate as the electron receptor and translocated to other organs through the damaged intestinal barrier, resulting in secondary bacterial infection (Zhang et al., 2020a). These studies highlighted the role of chicken intestinal function and intestinal microbes in H9N2 AIV infection. Therefore, the evaluation of H9N2 AIV vaccines should not be limited to the detection of virus shedding.

So far, although biosecurity measures had been adopted, vaccination was still the main method to prevent and control H9N2 AIV (El et al., 2013; Krammer and Palese, 2015). Inactivated vaccines had been used in China to prevent H9N2 AIV infection in chickens since 1998, but there were still numerous reports of immunization failures (Wei et al., 2016; Ma et al., 2018; Liu et al., 2020a). Inactivated vaccines can effectively reduce viral shedding and prevent clinical symptoms in chickens (Khantour et al., 2021). However, inactivated vaccines have some disadvantages, such as limited protective effect, inconvenient immunization methods, and interference with routine serological monitoring, all of which limit the application (Xu et al., 2019a). Therefore, in order to overcome the shortcomings of existing vaccines, it is urgent to develop safe and effective new vaccines, including DNA, subunit and vector vaccines. Currently, several viral vector vaccines against H9N2 AIV had been developed, including Fowlpox virus (**FPV**), Turkey herpesviruses (**HVT**), and Newcastle disease virus (**NDV**) (Chen et al., 2011; Liu et al., 2019; Zhang et al., 2021). NDV belongs to the genus Avulavirus of family Paramyxoviridae (de Leeuw and Peeters, 1999). As a necessary vaccine for poultry, NDV is also used as a viral vector to develop bivalent vaccines against avian pathogens (Lee et al., 2021). A large number of studies had shown that recombinant vector vaccine expressing H9N2 AIV HA protein could provide effective protection against H9N2 AIV challenge. Besides, previous studies had generally evaluated the effect of the vector vaccine by detecting the virus shedding rate of H9N2 AIV in trachea and cloaca (Liu et al., 2019; Xu et al., 2019b; Zhang et al., 2021). Importantly, the efficacy of H9N2 AIV vaccine on intestinal microbiota and function requires more attention.

In this study, recombinant virus rNDV-H9 expressing H9N2 AIV HA protein was generated by Red/ET homologous recombination technology (Liu et al., 2016a). Additionally, the growth kinetics, genetic stability and immunogenicity of rNDV-H9 were assessed. The results showed that rNDV-H9 showed similar growth kinetics to the parental virus and had good genetic stability. rNDV-H9 could induce high levels of H9N2 AIV HI antibody titers and effectively reduce the shedding of H9N2 AIV in trachea and cloaca. Besides, this study evaluated the effect of rNDV-H9 on intestinal structure and function impairment caused by H9N2 AIV infection, so as to provide more theoretical basis for the evaluation of H9N2 AIV vaccine.

MATERIALS AND METHODS

Ethics Statement

Institutional and national guidelines for the use and care of laboratory animals were closely followed. The use of animals in this study was approved by the South China Agricultural University Committee for Animal Experiments (approval ID: SYXK2019-0136). The study was performed in positive pressure high-efficiency particulate air-filtered stainless-steel isolators with enclosed and ventilated environment, and feed and water were provided ad libitum.

Viruses and Cells

The H9N2 AIV A/chicken/China/GD2021 strain (GenBank No. MW548848.1), isolated from a chicken farm in Guangdong in January 2021, showed low pathogenicity to chickens. Both the NDV LaSota strain (GenBank No. JF950510.1) and the H9N2 AIV A/chicken/China/GD2021 strain in this study were propagated in 9-day-old specific pathogen free (**SPF**) chicken embryos. BSR-T7/5 cells, a cell line stably expressing T7 RNA polymerase, were generously provided by Prof. Youming Zhang from Helmholtz Institute of Biotechnology, Shandong University. Chicken embryo fibroblasts (**CEF**) were generated from 10-day-old SPF chicken embryos. The above cells were cultured in Dulbecco's modified Eagle's medium (**DMEM**) supplemented with 10% heat-inactivated fetal bovine serum (**FBS**), plus 1% Antibiotic-Antimycotic (10,000 I.U./mL of penicillin, 10,000 μg/mL of streptomycin) and incubated at 37°C with 5% CO_2.

Construction of Recombinant Plasmids

The HA or GFP genes containing the homologous sequences of the NDV genome were amplified by overlapping PCR. The heterologous genes were fused with gene-end (**GE**) and gene-start (**GS**) of NDV genome, and optimal Kozak sequence was added. Using Red/ET homologous recombination technology, the heterologous genes containing homologous sequences were recombined with NDV genome in *E.coli* DH10B expressing Redα/Redβ recombinant protein, and the correct recombinant plasmids were obtained, named pNDV-H9 or pNDV-GFP.

Rescue of Recombinant Viruses

According to the manufacturer's instructions, the recombinant NDV plasmid was co-transfected with pCI-NP, pCI-P, and pCI-L into BSR-T7/5 cells using lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA). After 48 h of transfection, the supernatant and cells were harvested. After three freeze-thaw cycles, the cell lysates were inoculated into 10-day-old SPF chicken embryos incubated at 37°C. 48 h postinoculation, the chicken embryo allantoic fluid was harvested.

GFP Identification

rNDV-GFP and LaSota were inoculated into CEF cells at MOI = 1, respectively. After culturing at 37°C with 5% CO_2 for 48 h, the expression of GFP was observed under inverted fluorescence microscope.

Stability Detection of rNDV-H9

In order to test the genetic stability, rNDV-H9 was continuously grown in chicken embryos for 15 generations. Refer to the manufacturer's instructions of Axyprep humoral Virus DNA/RNA Mini-Extraction Kit (Axygen, Union City, CA) to extract the viral nucleic acids of P1 (1st passage), P5, P10, and P15 generations. The full-length HA gene was amplified by HiScript II One Step RT-PCR Kit (Takara, Beijing, China) using the sense primer 5′-ATGGAGACAGTATCACTAA-TAAC-3′ and the antisense primer 5′-TTATATA-CAAATGTTGCATCTGC-3′. In addition, the total protein was harvested 48 h after infecting CEF cells with rNDV-H9 at P5, P10, and P15 generations to detected the expression of HA protein by Western blotting analysis.

Growth Kinetics of rNDV-H9

Ten-day-old SPF chicken embryos were inoculated with rNDV-H9 and LaSota strains at a dose of 100 50% egg infection dose (**EID_{50}**) via the allantoic route, respectively. The chicken embryo allantoic fluid was harvested and EID_{50} assay conducted at 12, 24, 36, 48, 60, and 72 h postinoculation, and the growth kinetic curve of the recombinant virus was drawn using GraphPad Prism 8 software.

Immunization and Challenge Test in Chickens

Forty healthy one-day-old SPF chickens were randomly divided into 4 groups of 10 chickens each. The chickens in the immunized groups were separately inoculated with 100 uL rNDV-H9 and rNDV via the intranasal route at a dose of $10^{6.0}$ EID_{50}, while the chickens in the control group were inoculated with the same amount of PBS. Ten days postimmunization (**dpi**), booster immunization was performed with the same immunization procedure. At 21 dpi, the chickens in the immunized groups and nonvaccinated/challenged (**NV/C**) group were challenged with A/chicken/China/GD2021 strain at a dose of $10^{6.0}$ EID50, while the chickens in nonvaccinated/nonchallenged (**NV/NC**) group were inoculated with the same amount of PBS. Tracheal and cloacal swabs of chickens in each group were collected 3, 5, 7, and 9 days postchallenge (**dpc**). In addition, 5 chickens in each group were randomly euthanized 5 dpc, and histological lesions were observed by necropsy. Then, ileal tissues were collected from the ileocecal junction of chickens in each group.

Detection of Virus Shedding

The levels of H9N2 AIV shedding in tracheal and cloacal swab samples were detected by quantitative RT-PCR (**RT-qPCR**) as previously described (Liu et al., 2020b). Briefly, a pair of identification primers was designed based on the M gene of the H9N2 AIV: the sense primer 5'-GACGCACAACATCGGTCTCATAGG-3' and the antisense primer 5'-GTGTGTCCCGACTGTTCTCA-TAGC-3'. RNA from the swab samples was extracted as a template for RT-qPCR detection.

Determination of Serum Antibody Titers

Blood samples were collected from chickens in each group at 7, 14, and 21 dpi. To determine antibody titres against H9N2 AIV, all serum samples were analyzed by hemagglutination inhibition (**HI**) assay.

Pathological Analysis of Trachea and Intestinal Tissue

Five days postchallenge with H9N2 AIV, 5 chickens in each group were randomly euthanized, and the trachea and ileum of the chickens were collected and fixed in 10% neutral formalin solution. The tissue samples were stained with hematoxylin and eosin (**H&E**) after embedding in paraffin, and histopathological changes were observed under an upright microscope.

Detection of Nitrate Metabolism

The ileal tissues at the ileocecal junction of the chickens were collected 5 dpc for the detection of nitrate metabolism. The intestinal tissue was lysed with cell and tissue lysate reagent, and the nitrate content was determined using the total nitric oxide assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

Detection of Genes Expression in Ileal Tissue

Total RNA of the same quality ileal tissue was extracted with TRIzol (Invitrogen, Carlsbad, CA)

Table 1. Primers for detection of genes expression in ileal tissue by RT-qPCR.

Gene	Forward (5'-3')	Reversed (5'-3')
TNF-α	TGTATGTGCAGCAACCCGTA	CCACACGACAGCCAAGTCAA
IFN-γ	ATCATACTGAGCCAGATTGTTTCG	TCTTTCACCTTCTTCACGCCAT
iNOS	AGTGGTATGCTCTGCCTGCT	CCAGTCCCATTCTTCTTCC
ZO-1	GCCTGAATCAAACCCAGCAA	TATGCGGCGGTAAGGATGAT
Occludin	GATGGACAGCATCAACGACC	CATGCGCTTGATGTGGAAGA
β-actin	CTGGCACCTAGCACAATGAA	CTGCTTGCTGATCCACATCT

according to the manufacturer's instructions. The total RNA was reverse transcribed into cDNA using the Genome Removal Reverse Transcription kit (Tiangen, Beijing, China), and gene expression level of ileum was detected using RT-qPCR as described previously (Table 1; Zhang et al., 2020b).

Statistical Analysis

Statistical analysis was performed using Prism 8 (Graph pad). The one-way analysis of variance (**ANOVA**) was adopted for multiple comparisons and $P < 0.05$ was considered statistically significant.

RESULTS

Construction and Identification of Recombinant NDVs

In order to obtain recombinant NDV plasmids of chimeric heterologous genes, the HA and GFP genes were inserted between the P and M intergenic regions of the NDV genome by Red/ET homologous recombination technology (Figure 1A). The recombinant NDV plasmids and the helper plasmids were co-transfected into BSR-T7/5 cells stably expressing T7 RNA polymerase by liposome method to obtain recombinant viruses rNDV-H9 and rNDV-GFP. To detect the expression of heterologous proteins, CEFs were infected with rNDV-H9 and rNDV-GFP, respectively. As shown in Figure 1B, the expression of GFP could be detected 48 h after rNDV-GFP infection. The expression of HA protein in rNDV-H9 was verified by western blotting. The result in Figure 1C indicated that the H9N2 AIV HA protein was successfully expressed with expected size (about 70 kDa).

Genetic Stability and Biological Characterization of rNDV-H9

To assess the genetic stability, the recombinant virus rNDV-H9 was continuously propagated in chicken embryos for 15 passages. First, the genetic stability of HA gene in rNDV-H9 was detected by RT-PCR. The result showed that rNDV-H9 of P1, P5, P10, and P15 passages all contained the complete HA gene (Figure 2A). In addition, the stability of HA protein expression in rNDV-H9 was detected by Western blotting, and the result showed that rNDV-H9 of P5, P10,

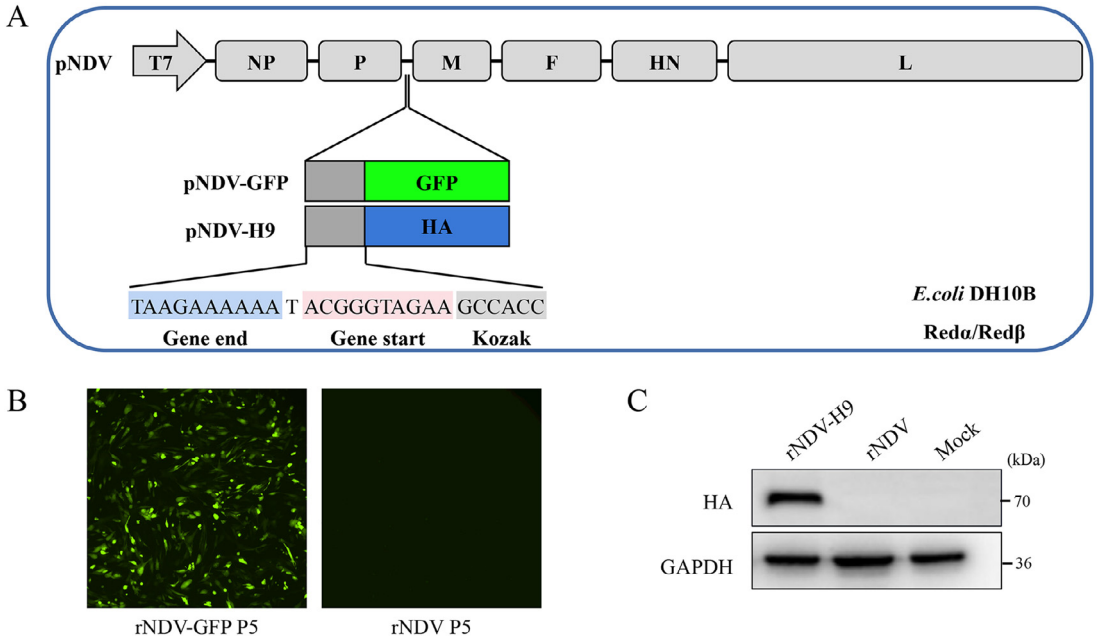

Figure 1. Construction and identification of recombinant NDV expressing heterologous genes. (A) Schematics for the construction of recombinant viruses rNDV-H9 and rNDV-GFP. (B) The expression of GFP was detected in rNDV-GFP infected CEF. (C) H9N2 AIV HA protein expressed by recombinant virus rNDV-H9 was detected by Western blotting.

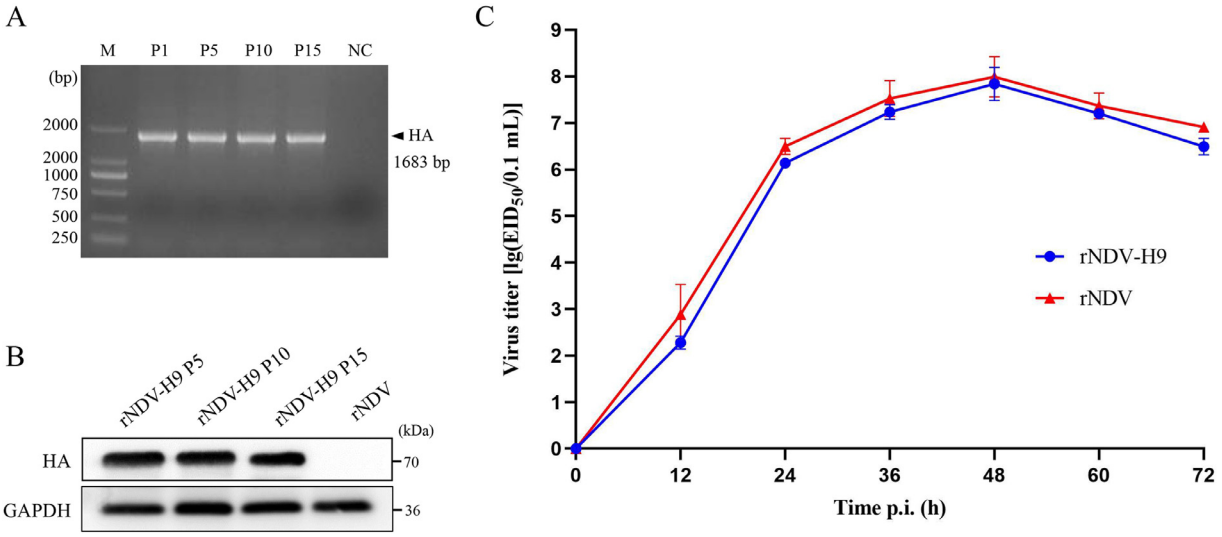

Figure 2. Genetic stability and biological characterization of rNDV-H9. (A) The genetic stability of the HA gene in P1, P5, P10, and P15 passages of rNDV-H9 was detected by RT-PCR. (B) The expression of HA protein in P5, P10, and P15 passages of rNDV-H9 was detected by Western blotting. (C) Growth kinetics of recombinant virus rNDV-H9.

and P15 passages could stably express HA protein (Figure 2B).

The growth kinetics of recombinant virus rNDV-H9 and parental virus rNDV were analyzed in chicken embryos. The result showed that rNDV-H9 had good growth kinetics in chicken embryos, and its replication pattern was similar to the parental virus rNDV (Figure 2C).

Immune Response Induced by rNDV-H9 in Chickens

The level of serum antibody is one of the important indexes to evaluate the immune response induced by rNDV-H9. The serum of chickens in each group was collected at 7, 14, and 21 dpi, and the antibody titers against H9N2 AIV were determined by HI method. Results as shown in Figure 3, there was no significant difference in HI antibody level among the groups at 7 dpi ($P > 0.05$). However, the HI antibody level in rNDV-H9 immunized group increased continuously, which was significantly higher than that in the control groups at 14 dpi and 21 dpi ($P < 0.01$).

Protective Effect Induced by rNDV-H9 in Chickens

Detection of virus shedding in trachea and cloaca was usually used as one of the criteria for evaluating H9N2 AIV vaccines. To evaluate the protective effect of rNDV-H9 against H9N2 AIV, tracheal and cloacal swabs were collected at 3, 5, 7, and 9 dpc for detection of virus shedding. The results showed that virus shedding in trachea remained high at 3 to 5 days post challenge with H9N2 AIV and then decreased gradually. From 3 to 9 dpc, the level of virus shedding in trachea of rNDV-

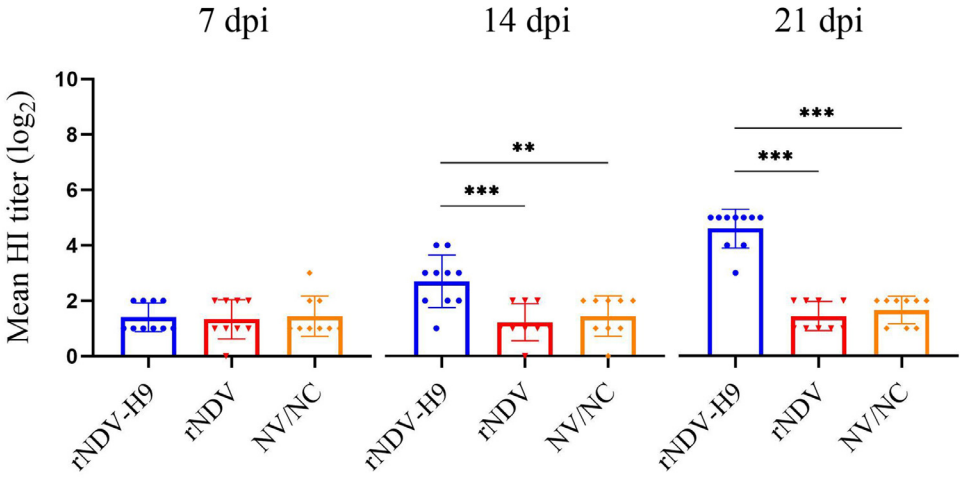

Figure 3. HI antibody titer against H9N2 AIV induced by rNDV-H9 in SPF chickens. Serum samples were collected from the chickens at 7, 14, and 21 dpi to detect the HI antibody titer against H9N2 AIV. Data is shown as mean values ± standard deviation. The differences between groups were analyzed using ANOVA (**$P < 0.01$, ***$P < 0.001$).

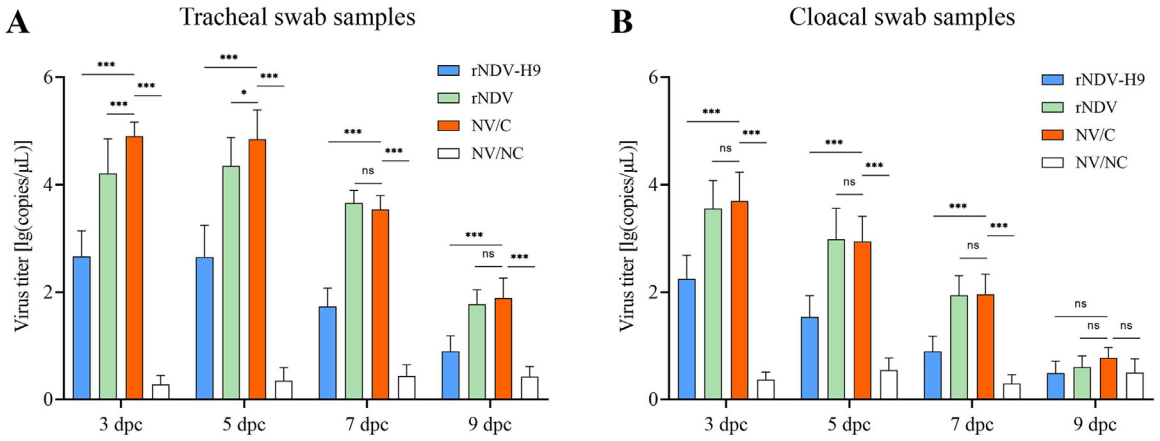

Figure 4. H9N2 AIV shedding in trachea and cloaca of the chickens was detected by RT-qPCR. At 21 dpi, the chickens in the immunized groups and NV/C group were challenged with H9N2 AIV at a dose of $10^{6.0}$ EID_{50}. Tracheal swab samples (A) and cloacal swab samples (B) were collected at 3, 5, 7, and 9 dpc to detect H9N2 AIV shedding by RT-qPCR. Data is shown as mean values ± standard deviation. The differences between groups were analyzed using ANOVA (*$P < 0.05$, ***$P < 0.001$, ns represents not significant).

H9 immunized group was significantly lower than that of NV/C group ($P < 0.01$; Figure 4A). In addition, the level of virus shedding in cloaca decreased gradually from 3 to 9 dpc, with no detectable virus shedding at 9 dpc. From 3 to 7 dpc, the level of virus shedding in cloaca of rNDV-H9 immunized group was also significantly lower than that of NV/C group ($P < 0.01$; Figure 4B). The above results indicated that rNDV-H9 could inhibit the shedding of H9N2 AIV in trachea and cloaca to a certain extent.

At 5 dpc, pathological studies were performed on trachea and intestinal tissue of the experimental chickens. Yellow cheese blocking of the trachea was observed in rNDV immunized group and NV/C group, while no obvious lesions were observed in rNDV-H9 immunized group and NV/NC group (Figure 5). In addition, histopathological studies showed that there were obvious pathological changes in the trachea and intestine of rNDV immunized group and NV/C group, such as mucosal epithelial damage, disappearance of cilia, infiltration of mucosal inflammatory cells, intestinal mucosal damage, and incomplete intestinal villi structure. No pathological changes in the trachea and intestine were observed in rNDV-H9 immunized group and NV/NC group (Figure 6).

rNDV-H9 Inhibited the Inflammatory Response Induced by H9N2 AIV

Tumor necrosis factor-α (**TNF-α**) and interferon-γ (**IFN-γ**) are important inflammatory cytokines. In addition, IFN-γ can induce the production of inducible nitric oxide synthase (**iNOS**) in intestinal epithelium, while iNOS is the only source of nitric oxide in the inflammatory response (Zhang et al., 2020a). Therefore, in order to evaluate the effect of recombinant virus rNDV-H9 on

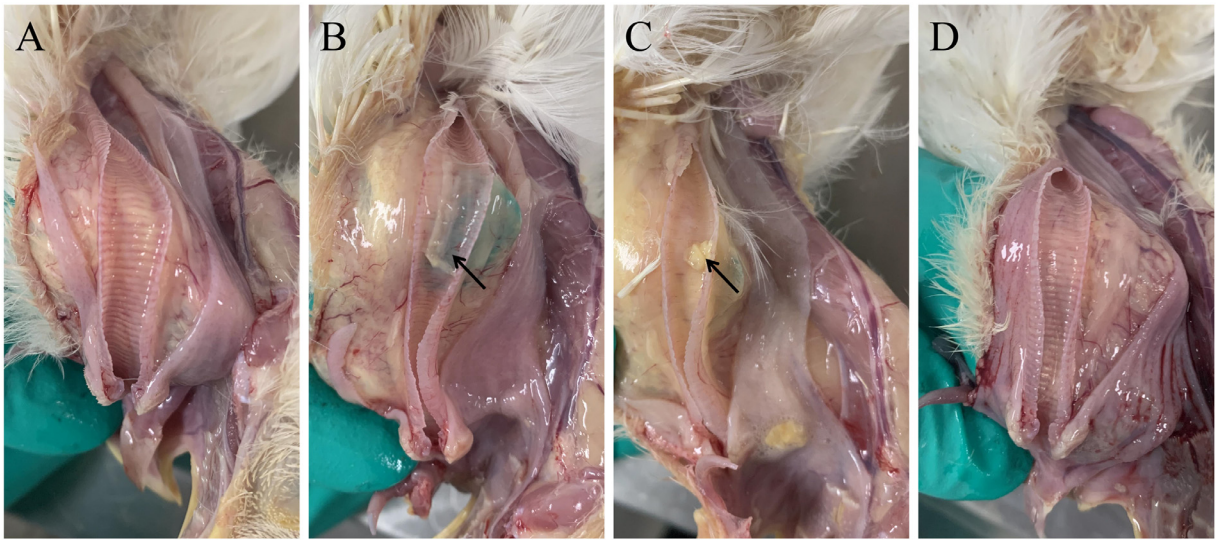

Figure 5. Representative postmortem examinations of chickens challenged with H9N2 AIV. (A) rNDV-H9 immunized group. (B) rNDV immunized group. (C) Nonvaccinated/challenged (NV/C) group. (B) nonvaccinated/nonchallenged (NV/NC) group. Black arrows mark the obstruction of the trachea by yellow cheese.

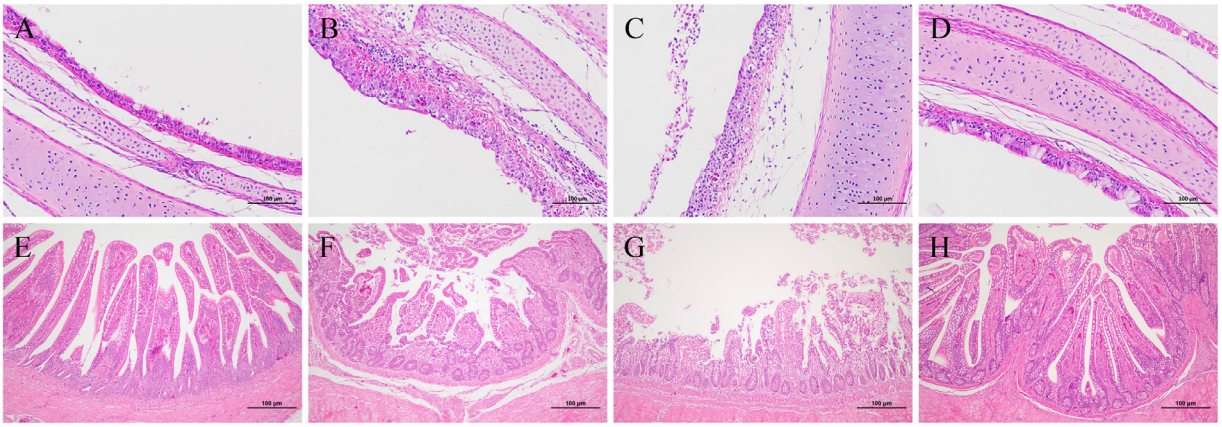

Figure 6. Histopathological analysis of trachea and ileum 5 days postchallenge with H9N2 AIV. (A−D) Tracheal pathological sections of rNDV-H9 immunized group, rNDV immunized group, NV/C group and NV/NC group. (E, F) Ileal pathological sections of rNDV-H9 immunized group, rNDV immunized group, NV/C group, and NV/NC group.

the inflammatory response, the expression of TNF-α, IFN-γ, and iNOS was detected by RT-qPCR. The results showed that compared with NV/NC group, the expressions of TNF-α, IFN-γ, and iNOS were all significantly upregulated in NV/C group ($P < 0.01$), indicating that the infection of H9N2 AIV would increase the expression of intestinal epithelial inflammatory cytokines. Moreover, the expression levels of TNF-α, IFN-γ, and iNOS in rNDV-H9 immunized group were significantly lower than those in NV/C group ($P < 0.01$), indicating that rNDV-H9 could inhibit the inflammatory response caused by H9N2 AIV infection (Figures 7A−7C).

rNDV-H9 Inhibited the Production of Nitrate in Ileal Epithelial Cells

Nitrate metabolism could be used as an index to detect the reproductive level of *E. coli* in chicken intestinal tract. Previous studies had demonstrated that the level of nitrate metabolism in the intestine was positively correlated with the reproductive level of *E. coli* (Zhang et al., 2020b). In this study, the ileal tissues at the ileocecal junction of chickens in each group were collected for the detection of nitrate content 5 dpc. Results as shown in Figure 7D, the nitrate content of ileal epithelium increased significantly after H9N2 AIV infection ($P < 0.01$), indicating the rapid proliferation of *E. coli* after H9N2 AIV infection. However, the nitrate content in rNDV-H9 immunized group was significantly lower than that in NV/C group, indicating that rNDV-H9 could inhibit the production of nitrate by ileal epithelial cells to a certain extent.

rNDV-H9 Inhibited the Injury of Tight Junctions in Chicken Ileal Epithelium After H9N2 AIV Infection

In order to evaluate the effect of recombinant virus rNDV-H9 on the barrier function of intestinal mucosal epithelial cells in chickens, the mRNA expressions of zonula occludens (**ZO**)-1 and occludin were determined by RT-qPCR. Results as shown in Figures 7E−7F, compared with NV/NC group, the mRNA expressions of ZO-1 and occludin in NV/C group decreased significantly ($P < 0.01$), indicating that the infection of H9N2 AIV would destroy the tight junction of intestinal epithelium. However, compared with NV/C group, the mRNA expressions of ZO-1 and occludin in rNDV-H9 immunized group were significantly upregulated ($P < 0.01$), indicating that rNDV-H9 had a certain protective effect on the structure and barrier function of intestinal mucosal epithelium (Figures 7E−7F).

DISCUSSION

So far, H9N2 AIV had spread widely in China, establishing stable lineages in commercial chicken flocks (Liu et al., 2016b). The mortality caused by H9N2 AIV was generally less than 20%, but it usually caused respiratory symptoms and secondary infection of other pathogens, resulting in serious economic losses to the poultry industry (Li et al., 2005; Sun and Liu, 2015). Although the inactivated vaccines against H9N2 AIV had been widely used in China, there were still a large number of reports of immunization failures, which posed a challenge to the effectiveness of H9N2 AIV inactivated vaccines (Wei et al., 2016; Xia et al., 2017). In addition, for the poultry industry, inactivated vaccines are inconvenient to administer and lack the induction of effective mucosal and cellular immune responses (Gu et al., 2017). Therefore, there is an urgent need to develop novel vaccines against circulating strains of H9N2 AIV.

NDV is considered to be a highly immunodominant and promising vaccine vector. Poultry vector vaccines based on NDV can effectively induce mucosal, humoral, and cellular immune responses (Dey et al., 2017; Saikia et al., 2019). In this study, SPF chickens were immunized with recombinant virus rNDV-H9, which induced high HI antibody titers against H9N2 AIV (Figure 3). In addition, in contrast to HVT and FPV vectors, NDV

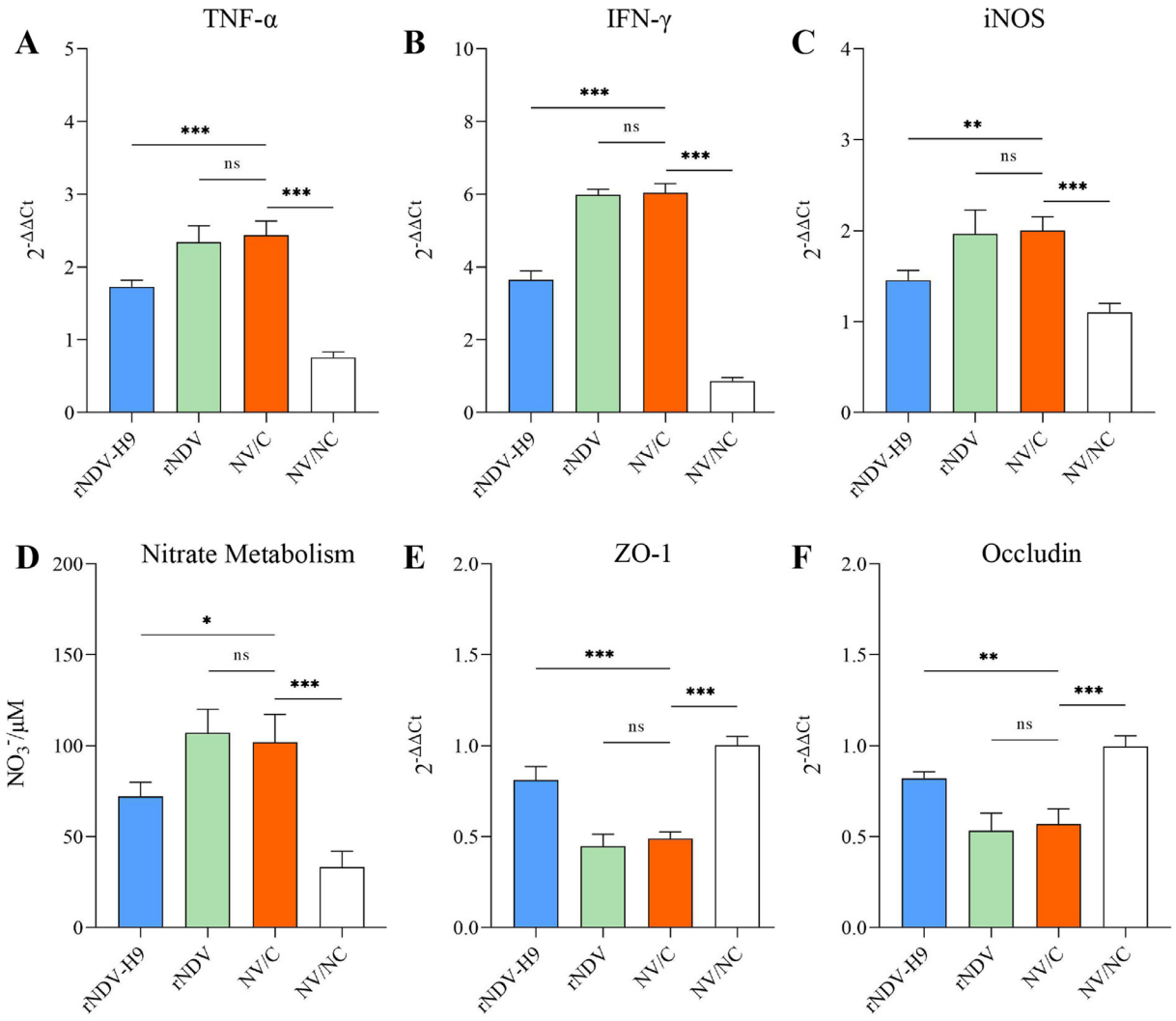

Figure 7. Detection of the genes expression in ileal epithelium of SPF chickens 5 days post-challenge. (A−C) Detection of mRNA expression of pro-inflammatory genes TNF-α, IFN-γ and iNOS in ileal epithelium of different treatment groups. (D) Detection of nitrate metabolism. (E, F) Detection of expression levels of tight junction proteins ZO-1 and occludin in ileal epithelium. Data is shown as mean values ± standard deviation. The differences between groups were analyzed using ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns represents not significant).

encodes only seven proteins and is thus less competition for immune responses between viral vector proteins and foreign proteins (Kim and Samal, 2016; Kamel and El-Sayed, 2019). Because the sequences between adjacent genes will weaken transcription, the mRNA transcription level of NDV genome decreases gradually from 3' end to 5' end (Dey et al., 2017). Theoretically, the closer the inserted foreign gene is to the 3' end of NDV genome, the higher the expression level is. However, many studies focusing on NDV vector vaccines had shown that the site between the P and M genes was considered to be the best choice for insertion of foreign genes (Xu et al., 2019a,b; Lee et al., 2021; Qiao et al., 2021; Zhang et al., 2021). In this study, the NDV LaSota strain was used as the backbone, and the HA gene was inserted between the P and M genes (Figure 1A). Genetic stability analysis of the rescued recombinant virus rNDV-H9 showed that rNDV-H9 could stably express HA protein after 15 generations of continuous passage on chicken embryos (Figure 2B). The results of growth kinetics showed that the replication characteristics of rNDV-H9 were similar to that of the parental virus LaSota strain, indicating that the insertion of HA gene had little influence on the proliferation characteristics of NDV (Figure 2C).

Virus shedding in trachea and cloaca is an important index for evaluating the effectiveness of vaccines against H9N2 AIV. Previous studies had shown that recombinant NDV expressing HA protein of H9N2 AIV could effectively reduce the shedding of H9N2 AIV in trachea and cloaca (Xu et al., 2019a; Zhang et al., 2021). Consistent with previous studies, in this study, the recombinant virus rNDV-H9 expressing HA protein of H9N2 AIV protected chickens from pathological damage to trachea and intestine after challenging with H9N2 AIV and significantly reduced virus shedding in trachea and cloaca. Notably, there were significantly differences of virus loads in tracheal swabs between rNDV alone and NV/C-AIV challenge groups at 3 and 5 dpc (Figure 4A), which probably caused by the mucosal immune response induced by NDV (Kapczynski et al., 2013). However, evaluating the efficacy of vaccines against H9N2 AIV by detecting virus shedding alone is not comprehensive.

Previous studies had shown that infection with H9N2 AIV induced an inflammatory response in intestinal mucosa, prompting the secretion of nitrates from intestinal epithelial cells; moreover, the intestinal symbiotic *E. coli* proliferated with nitrate as the electron receptor, crossing the damaged intestinal mucosal barrier and translocating to other organs for secondary bacterial infection (Li et al., 2018; Zhang et al., 2020a). Therefore, in order to comprehensively evaluate the protective effect of recombinant virus rNDV-H9, the detection of intestinal inflammatory response, tight junction and nitrate metabolism need to be included. The infection of H9N2 AIV could induce the abnormal expression of proinflammatory factors in chickens, resulting in the decline of immunity (Guan et al., 2015; Zhang et al., 2020b). Our results showed that the transcript levels of TNF-α and IFN-γ in rNDV-H9 immunized group were significantly lower than those in NV/C group, demonstrating that rNDV-H9 could effectively inhibit the inflammation induced by H9N2 AIV infection (Figures 7A−7C). Moreover, nitrate metabolism is an important indicator of *E. coli* reproduction in the intestine (Zhang et al., 2020a). rNDV-H9 could effectively inhibit the production of nitrate in ileal epithelial cells, indicating that rNDV-H9 could inhibit the abnormal proliferation of *E. coli* to a certain extent (Figure 7D). In addition, the tight junction proteins of intestinal epithelial cells are critical for the intestinal mucosal barrier, and disruption of tight junction proteins leads to increased intercellular permeability (Chasiotis et al., 2012). ZO-1 protein and occludin are usually used as key proteins to evaluate intercellular tight junction structures (Suzuki, 2013; Zhang et al., 2020b). In this study, H9N2 AIV infection resulted in significant downregulation of the expression levels of ZO-1 protein and occludin (Figures 7E and 7F). Importantly, rNDV-H9 could inhibit the downregulation of ZO-1 and occludin expression levels, indicating that rNDV-H9 could effectively protect the intestinal mucosal barrier.

In conclusion, this study constructed a recombinant virus rNDV-H9 expressing H9N2 AIV HA protein, and comprehensively evaluated its protective effect against H9N2 AIV challenge. The immunization assay showed that rNDV-H9 could induce high HI titers against H9N2 AIV and effectively reduce the shedding of H9N2 AIV in trachea and cloaca. Furthermore, rNDV-H9 could inhibit the inflammatory response and abnormal nitrate metabolism caused by H9N2 AIV infection, effectively protecting the barrier function of intestinal mucosa. Thus, the recombinant rNDV-H9 strain might be a potential vaccine candidate against H9N2 AIV.

ACKNOWLEDGMENTS

This work was supported by the Key Research and Development Program of Guangdong Province (2020B020222001), the Natural Science Foundation of Guangdong Province (grant no. 2019A1515012006), the Construction of Modern Agricultural Science and Technology Innovation Alliance in Guangdong Province (2020KJ128), the Special Project of National Modern Agricultural Industrial Technology System (CARS-41), the National Modern Agricultural Industry Science and Technology Innovation Center in Guangzhou (2018kczx01), the Provincial Science and Technology Special Fund Project for Zhongshan City (major special project + Task list management mode) (2021sdr003), the Chief expert Project of Agricultural Industry Technology system in Guangdong Province (2020LJ128, 2021KJ128) and the International Science and Technology Cooperation Project of Guangdong Province (grant no. 2016A050502042).

DISCLOSURES

The authors declare that there are no conflicts of interest.

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