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A novel recombinant attenuated Newcastle disease virus expressing H9 subtype hemagglutinin protected chickens from challenge by genotype VII virulent Newcastle disease virus and H9N2 avian influenza virus

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ABSTRACT

Newcastle disease virus (NDV) and H9 subtype avian influenza virus (AIV) are two avian pathogens across the globe. Inasmuch as most poultry flocks worldwide are vaccinated with a live low-virulence or attenuated NDV vaccine, we embarked on the development of vaccine prototypes that would have dual specificities and would allow a single immunization against both avian influenza (AI) and Newcastle disease (ND). Therefore, in the present work, a cloned full-length copy of the genome of the lentogenic NDV strain rmNA-1 was selected as a backbone vector to construct three chimeric NDVs that expressed (i) the ORF encoding the HA, (ii) the ectodomain of HA fused with the transmembrane domain and cytoplasmic tail regions derived from the NDV F protein and (iii) the ectodomain of HA fused with a short GS linker and the GCN4 sequences, and designated as rmNA-H9, rmNA-H9F, and rmNA-H9 (ECTO), respectively. rmNA-H9, rmNA-H9F, and rmNA-H9 (ECTO) stably expressed the modified HA gene for 10 egg passages and the three recombinants were found innocuous to chickens. The insertion of the chimeric HA-F, rather than HA-ECTO or ORF of HA, resulted in a recombinant virus with enhanced incorporation of the HA protein into the viral surface. A single immunization of SPF chickens with the three recombinants induced NDV- and AIV H9-specific antibodies, and protected chickens against a challenge with a lethal dose of velogenic NDV or AIV H9N2. Remarkably, non-shedding of influenza virus and higher levels of H9 subtype HI titers were observed 7 days post challenge (dpc) in rmNA-H9F vaccinated chickens, than other recombinants. Furthermore, a prime-boost vaccination of chickens with rmNA-H9F induced higher levels of NDV- and H9- HI and secretory IgA, as well as reduced viral shedding and virus-induced gross lesions, compared with the commercial vaccine. Therefore, the recombinant rmNA-H9F is a promising bivalent vaccine candidate against NDV and H9 subtype AIV in chickens.

1. Introduction

The H9N2 avian influenza virus (AIV), characterized as a low-pathogenicity AIV (LPAIV), has been circulating worldwide in avian species. It causes significant economic losses due to reduced egg production or high mortality in co-infection with other respiratory pathogens (Webster et al., 1992). H9N2 AIV has been documented in many countries after its first report in the USA in 1966 (Homme and Easterday, 1970). In China, H9N2 AIV was first isolated from diseased chickens in 1994 (Chen et al., 1997). Since then, H9N2 viruses have rapidly spread across China and became the most prevalent subtype of AIV that circulating in poultry (Li et al., 2005, 2003). Although a vaccination program using inactivated adjuvanted vaccines has been ongoing in China since 1998, outbreaks caused by H9N2 viruses in chicken populations are still not efficiently controlled (Li et al., 2005; Wei et al., 2016b). The currently used inactivated oil-emulsion AIV vaccines have several disadvantages, such as the limited immune activating ability, inconvenient individual administration, and its

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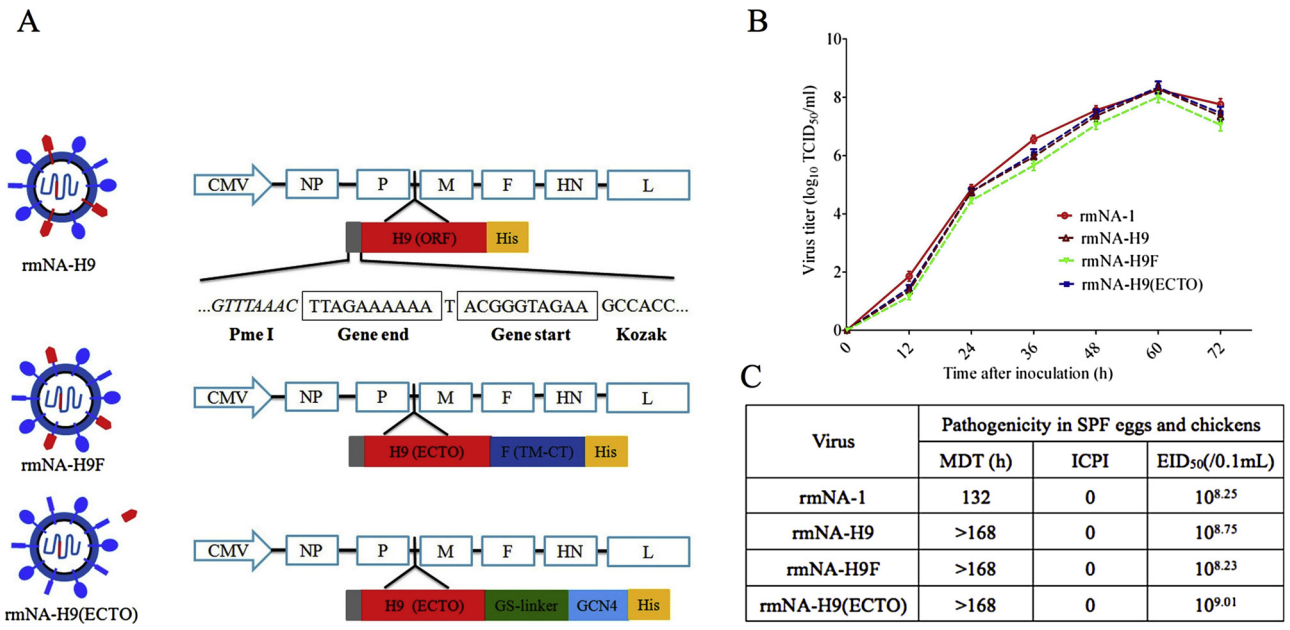

Virus	Pathogenicity in SPF eggs and chickens		
	MDT (h)	ICPI	EID_{50}(/0.1mL)
rmNA-1	132	0	$10^{8.25}$
rmNA-H9	>168	0	$10^{8.75}$
rmNA-H9F	>168	0	$10^{8.23}$
rmNA-H9(ECTO)	>168	0	$10^{9.01}$

Fig. 1. Construction and biological characterization of the three recombinant viruses. (A) Schematic illustration of cDNA constructs for rmNA-H9, rmNA-H9F, and rmNA-H9 (ECTO). (B) Comparison of viral growth kinetics of three rNDVs. (C) Pathogenicity assay in SPF eggs and chickens.

interference with routine surveillance by serological test, all of which limit their efficacy and application in the field. To overcome these problems, other approaches have been explored including DNA, subunit, and vectored vaccines.

Among these innovative approaches for the development of AIV vaccines, one of the most promising candidates is the vector-based vaccine. In particular, the Newcastle disease virus (NDV), that serves as a needed poultry vaccine itself, is used as an important vaccine vector for the development of bivalent vaccines against several avian pathogens (Kim and Samal, 2016). Newcastle disease (ND) is caused by virulent strains of NDV, having significant similarities to AIV in the poultry industry. Lentogenic NDV strains such as La Sota have been widely used as live vaccines against ND. The NDV strain La Sota belongs to genotype II and was isolated 70 years ago, whereas the most predominant genotype circulating in China and many other Asian countries is genotype VII. Antigenic differences between the prevalent circulating NDV strains and the vaccine strains lead to ND outbreaks in vaccinated poultry flocks (Miller et al., 2007; Qin et al., 2007). In contrast, ND vaccines developed from currently circulating genotype strains are with improved effectiveness (Hu et al., 2009). In a previous study, we developed a reverse genetics system for the generation of modified genotype VII NDV strains (Wang et al., 2015). NDV has several advantages as a vaccine vector candidate, and was proven in both human and other animals (Kim and Samal, 2016). Live ND vaccines are usually administered intranasally, that mimics the natural infection of NDV in chickens. It induces both systemic immune responses and local mucosal immune responses at the respiratory tract. Therefore, the NDV is ideally suitable as a vector for avian pathogens that invades through the respiratory tract, such as AIV, infectious bronchitis virus (IBV), and infectious laryngotracheitis virus (ILTV). In fact, NDV is one of the most commonly used vaccine vectors against AIV. Ge et al. reported a La Sota backbone-based recombinant NDV vaccine candidate against NDV and AIV (Ge et al., 2010)

The hemagglutinin (HA) protein is the major protective antigen on the AIV surface, making it a logical choice for influenza vaccine development (Ge et al., 2010; Goff et al., 2013; Wang et al., 2006). It is reported that the form of HA insertion determines the immunogenicity of HA-expressing DNA influenza vaccines (Wang et al., 2006). However, what is the optimal H9 HA antigen design for NDV vectored virus is still unknown. In this study, three recombinant Newcastle disease viruses (rNDVs) expressing different forms of the H9 HA were generated. Among which, the rmNA-H9 expresses a wild-type, full-length HA. The rmNA-H9F expresses a chimeric HA, which is the combination of the ectodomain of HA with the transmembrane domain (TM) and cytoplasmic tail (CT) of the NDV fusion glycoprotein. The rmNA-H9 (ECTO) expresses a truncated form of HA. The rNDVs were evaluated for the expression and incorporation of HA protein, the growth kinetics, the pathogenicity, and the protection efficacy against NDV and H9N2 AIV challenge in specific pathogen-free (SPF) chickens.

2. Materials and methods

2.1. Ethics statements

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocols for animal studies were approved by the Committee on the Ethics of Animal Experiments of Jilin University (approval numbers 2017061712-5).

2.2. Generation and characterization of three recombinant viruses

Three recombinant Newcastle disease viruses expressing the hemagglutinin (HA) of H9N2 avian influenza virus (A/chicken/Jilin/SJ150/2012) from an added gene were generated and designated as rmNA-H9, rmNA-H9F, and rmNA-H9 (ECTO). For the construction of rmNA-H9, the full length H9 HA was amplified with following primers: sense-5′-AGCTTTGTTTAAACTTAGAAAAAATACGGGTAGAACGCCGCC ACCATGGAAGTAGTATCACTA-3′ and antisense 5′-AGCTTTGTTTAAA CTTAGTG

GTGGTGGTGGTGGTGTATACAAATGTTGCA-3′. The regulation elements of NDV, the optimal Kozak sequence and His tag sequences were included. The second construct was designated as rmNA-H9F in which the ectodomain sequence of HA was fused with the sequence of the transmembrane and cytoplasmic tail of the NDV fusion protein gene (synthesized by Genewiz) and subsequently cloned into the NDV antigenomic cDNA. The last construct was designated as rmNA-H9 (ECTO), in which the ectodomain of H9 HA was fused with a short GS linker and the GCN4 sequence. This insertion sequence was synthesized (Genewiz)

and ligated into the NDV anti-genomic cDNA. The schematic diagram depicting the designs of the three rNDVs was shown in Fig. 1A. The rNDVs were rescued through the co-transfection of the full-length cDNA clone plasmid and helper plasmids into BHK-21 cells, as described previously (Wang et al., 2015). The expression of the H9 HA protein was examined by indirect immunofluorescence assay (IFA) in BHK-21 cells and western blotting in DF-1 cells as previously described (Cornelissen et al., 2012). The specific chicken polyclonal sera against NDV or H9 subtype AIV used in IFA and western blot analysis were produced by our lab. Fluorescein isothiocyanate-conjugated goat anti-chicken IgY (Thermo) or rabbit anti-chicken IgY-HRP (EarthOx) were used as secondary antibodies in the IFA and western blot analysis, respectively. The incorporation of the HA protein into the three rNDVs was determined by western blotting and immunoelectron microscopy (IEM) analysis as described previously (Basavarajappa et al., 2014). AIV subtype H9-specific mouse antiserum and goat anti-mouse IgG-gold antibody (Sigma) were used in IEM. The multistep growth kinetics of the three rNDVs was determined (Wang et al., 2015). The pathogenicity of the recombinant viruses was determined by mean death time (MDT) and the intracerebral pathogenicity index (ICPI) (Oie, 2015). rNDVs were passaged 10 times in 10-day-old SPF embryonated eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) to evaluate their genetic stabilities. MDT and ICPI were measured again after the 10 passages. Detection of the inserted gene was carried out by RT-PCR, the primers were available upon request.

2.3. Efficacy of a single vaccination in chickens

Ten 1-week-old SPF chickens were immunized through the oculonasal (o.n.) route with 10^6 EID_{50} of rmNA-1 in a 0.2 ml volume. Groups of 20 birds were immunized o.n. with 10^6 EID_{50} of recombinant virus rmNA-H9, rmNA-H9F or rmNA-H9 (ECTO). An additional group of 30 birds was administered with 0.2 ml phosphate-buffered saline (PBS). All the vaccinated chickens were bled weekly for hemagglutinin inhibition (HI) assays before challenge. At 21 days post vaccination (dpv), ten birds in group rmNA-1, rmNA-H9, rmNA-H9F, rmNA-H9 (ECTO), and PBS were challenged intranasally (i.n.) with 10^5 ELD_{50} of genotype VII virulent NDV strain Goose/China/Jilin/NA-1/1999 in a 0.2 ml volume. Another ten birds in group rmNA-H9, rmNA-H9F, rmNA-H9 (ECTO), and PBS were challenged i.n. with 10^6 EID_{50} in a 0.2 ml volume of H9N2 virus A/chicken/Jilin/SJ150/2012 (SJ150). Oropharyngeal and cloacal swabs were collected on 3, 5, and 7 days post challenge (dpc), which were used for virus detection using SPF eggs. All the birds were observed for clinical signs and mortality for 14 dpc.

2.4. Efficacy of prime-boost vaccination in chickens

18 one-week-old SPF chickens were immunized o.n. with 10^6 EID_{50} of rmNA-1. A group of 36 birds were immunized o.n. with 10^6EID_{50} rmNA-H9F. Another group of 33 birds were immunized with commercial inactivated oil-emulsion bivalent vaccine (inactivated NDV LaSota strain and A/Chicken/Shanghai/F/98 (H9N2), QYH biotech Co., Ltd., Beijing, China) through subcutaneous injection (s.i.) with the suggested dose. An additional 43 birds were inoculated o.n. with 0.2 ml PBS. A booster vaccination was performed at 21 dpv using the same procedure. All the vaccinated chickens were bled weekly for HI assays before challenge. 14 days post-booster (dpb), three chickens from each group were sacrificed for the collection of tracheal, lung and spleen tissue samples. Pathogen specific secretory immunoglobulin A (SIgA) against NDV and H9N2 AIV in the tracheal and intestine washings were analyzed by an indirect enzyme-linked immune sorbent assay (ELISA), as previously described (Rauw et al., 2009). In addition, flow cytometry was performed to analyze splenic $CD3^+CD4^+$ (Th) and $CD3^+CD8^+$ (Tc) lymphocyte subsets. At 14 dpb, 15 birds in each group were challenged i.n. with 10^5 ELD_{50} of NDV strain NA-1. Another 15 birds were challenged i.n. with 10^6 EID_{50} of H9N2 strain SJ150. Morbidity and mortality were recorded for 14 days post challenge. At each observation, the birds were scored: 0 when normal, 1 when sick, and 2 when dead (birds that were alive but unable to eat or drink were humanely killed and scored as dead at each of the remaining daily observations after death) (Liu et al., 2018). At 5 dpc, five chickens in every group were sacrificed, and tissues, including the liver, spleen, intestine, pancreas, stomach, trachea, lung, and heart were collected to evaluate the gross pathology. In addition, the lung tissues were used for histological observation. Oropharyngeal and cloacal swabs were collected on 3, 5, and 7 dpc for virus shedding detection as described above.

2.5. Statistical analysis

GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. Virus shedding, virus titers, antibody titers, and percentages of T lymphocytes were analyzed by ANOVA among groups, the Student *t*-test between two groups. A p value of < 0.05 was considered as a significant difference.

3. Results

3.1. Generation and biological characterization of the three recombinant viruses

Three rNDVs rmNA-H9, rmNA-H9F, and rmNA-H9(ECTO) were recovered from transfected BHK-21 cells and propagated in SPF embryonated chicken eggs. The presence of inserted HA gene in three rNDVs was confirmed by RT-PCR and sequencing (data not shown). The growth kinetics of three rNDVs were determined and in SPF embryonated chicken eggs at different time points after inoculation. A 50% tissue culture infective dose ($TCID_{50}$) of each virus was determined in DF-1 cells by IFA. The results showed that three rNDVs have similar growth patterns compared to rmNA-1 (Fig. 1B). At 60 h post-inoculation, rmNA-H9, rmNA-H9F, and rmNA-H9 (ECTO) reached the maximum titers of $10^{8.28}$ $TCID_{50}$/ml, 10^8 $TCID_{50}$/ml, and $10^{8.33}$ $TCID_{50}$/ml, respectively. Consistently, their EID_{50} values were $10^{8.75}$ EID_{50}/0.1 ml, $10^{8.23}$ EID_{50}/0.1 ml and $10^{9.01}$ EID_{50}/0.1mL. The MDT and ICPI values of three rNDVs demonstrated that they are with lentogenic characteristics (Fig. 1C) and there is no significant change after the 10 passages in SPF embryonated eggs. RT-PCR confirmed the presence of the AIV HA gene in each passage, and sequence analysis showed that the HA gene remains unchanged during the 10 passages. In addition, the insertion of HA protein decreased the virulence of rNDVs, indicated by prolonged MDTs (Fig. 1C)

3.2. Improved incorporation of AIV HA protein into rmNA-H9F virions

Expression of the AIV HA protein by rNDVs were examined by IFA assay (Fig. 2A) and western blot analysis (Fig. 2B). Incorporation of foreign glycoproteins into NDV particles has been previously documented for rNDVs expressing AIV H5 or H7 HA (Park et al., 2006; Veits et al., 2006). We postulated that the expression of the chimeric H9 HA protein containing the transmembrane and cytoplasmic tail regions of the NDV F protein would be associated with enhanced incorporation into virions. To address this question, NDV particles were purified by sucrose density gradient centrifugation. The amounts of H9 HA protein or NDV viral protein from rNDVs were measured by western blotting using anti-chicken H9N2 AIV polyclonal serum or anti-chicken NDV polyclonal serum. As expected, incorporation of chimeric H9 HA protein into rmNA-H9F virions was significantly increased compared with that of native H9 HA protein (Fig. 2B). In parallel with the western blot results, the IEM results indicated a higher level of incorporation of H9 HA into rmNA-H9F virions than to rmNA-H9 virions (Fig. 2C). In addition, there was no gold particle deposit on the envelope of purified rmNA-H9 (ECTO) virions (Fig. 2C), suggesting the truncated AIV HA protein was not incorporated into the viral particles. These data suggest

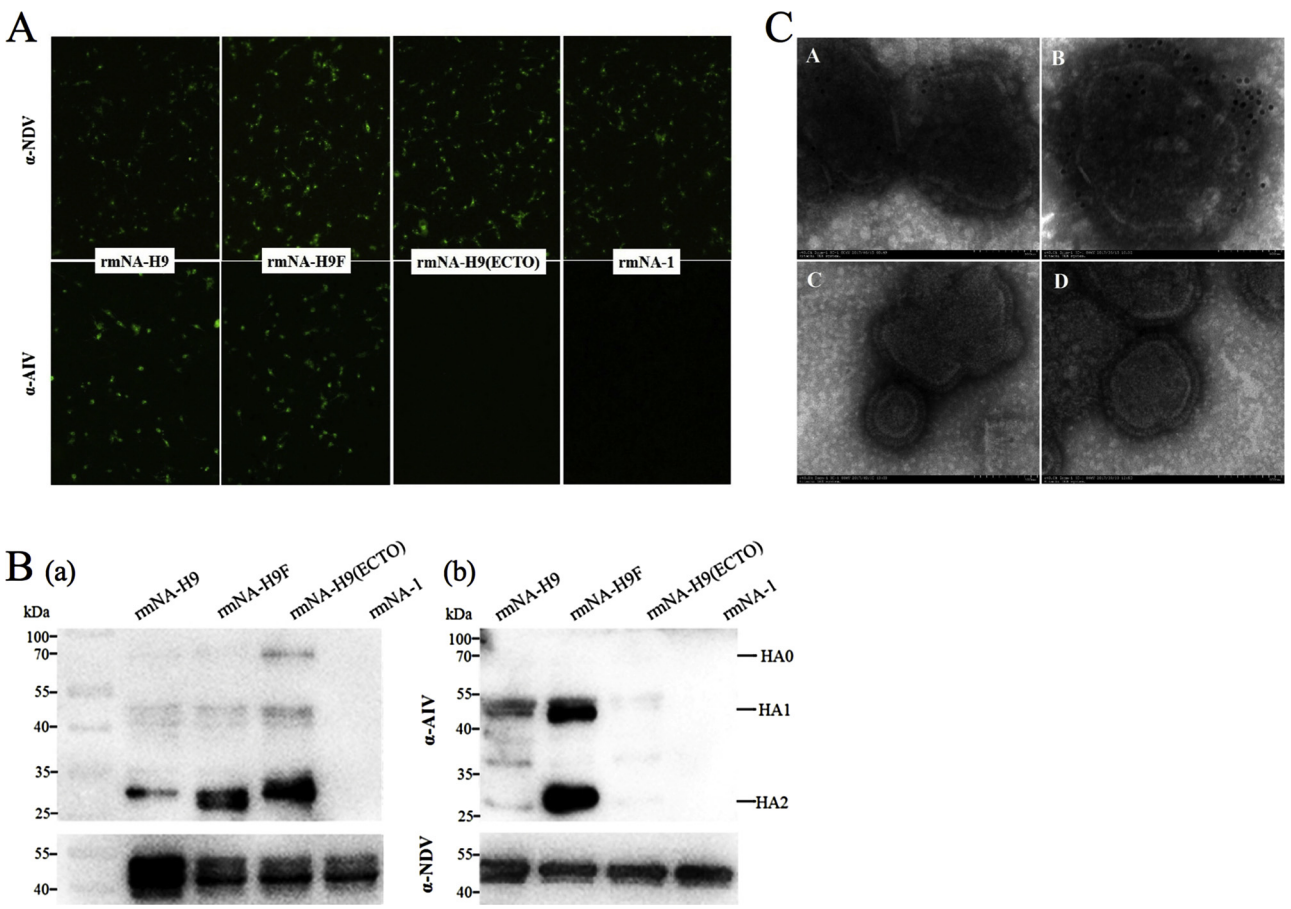

Fig. 2. Detection of expression of H9 HA. (A) H9 protein was expressed and detected in rmNA-H9, rmNA-H9F, rmNA-H9 (ECTO), and rmNA-1 infected BHK-21 cells using IFA. (B) Western blot analyses of rNDVs. (a) Combined lysate of cells and the cell culture supernatant were incubated with either AIV subtype H9-specific antiserum (α-AIV) or NDV-specific antiserum (α-NDV). (b) Western blot analyses of purified virions of rNDVs. Locations of marker proteins were indicated on the left, and the uncleaved (HA0) and processed forms (HA1 and HA2) of AIV hemagglutinin were indicated on the right. (C) Incorporation of the HA protein into rNDVs particles tested by Immunoelectron microscopy. (A–D) rmNA-H9, rmNA-H9F, rmNA-H9(ECTO), and rmNA-1.

that the chimeric H9 HA protein was more efficiently incorporated into the NDV envelope than the native HA protein.

3.3. rmNA-H9F protects chickens against NDV and AIV challenge through a single vaccination

The NDV HI antibodies induced by rmNA-H9, rmNA-H9 (ECTO), and rmNA-H9F increased gradually after vaccination, with similar dynamic changes to that of rmNA-1 (Fig. 3A), suggesting that the three rNDVs were as effective as the rmNA-1 strain. The mean H9-specific HI antibody in chickens immunized with rmNA-H9F was significantly higher than those of birds immunized with rmNA-H9 or rmNA-H9 (ECTO), reaching the protective level ($> 4\log_2$) at 21dpv (Fig. 3B).

All the chickens that had been immunized with three rNDVs were protected completely protected against virulent NDV strain NA-1 challenge, displaying neither clinical signs nor mortality (Fig. 3C). Oropharyngeal and cloacal viral shedding were completely inhibited at 7dpc (Table 1). In contrast, all the chickens in the unvaccinated control group died within 5 dpc. These results suggested that expression of the AIV HA protein does not interfere with the protective immune efficacy of rNDVs.

On the other hand, during H9N2 virus A/chicken/Jilin/SJ150/2012 infection, chickens in the rmNA-H9, rmNA-H9 (ECTO), and mock-vaccinated groups showed clinical signs, e.g., depression, anorexia, and ruffled feathers. In contrast, all chickens vaccinated with rmNA-H9F displayed no clinical signs post challenge. In addition, the rmNA-H9 and rmNA-H9 (ECTO) resulted with more and longer oropharyngeal and cloacal virus shedding than rmNA-H9F group (Table 2). No chickens of rmNA-H9F group shed virus through the oropharyngeal and cloacal routes at 7 dpc, whereas virus shedding was still detectable in rmNA-H9 and rmNA-H9 (ECTO) groups (Table 2).

3.4. rmNA-H9F induces both systemic and local immunity, and protects chickens against NDV and AIV challenge post a prime-boost vaccination

Based on the single vaccination results, rmNA-H9F was therefore selected for a further prime-boost vaccination experiment. NDV and AIV HI antibody titers around 7.7 $\log_2$ and 6.7 $\log_2$ were observed in rmNA-H9F vaccinated group at 14 dpb (Fig. 4A, B). The values of NDV and AIV HI antibody titers of commercial vaccine group were 9 $\log_2$ and 8.3 $\log_2$ at 14 dpb, respectively (Fig. 4A, B). Different from the result of serum antibody level, rmNA-H9F group showed significantly higher levels of NDV and H9 AIV specific SIgA than inactivated vaccine group at 14 dpb (Fig. 4C, D). In line with this, at 14 dpb, the percentages of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ splenic lymphocytes of the rmNA-H9F group were significantly higher than those of the control group (Fig. 4E). These results indicated that immunization with rmNA-H9F could induce profound humoral, mucosal, and cellular immune responses against NDV and H9 AIV in chickens.

The vaccinated chickens were then challenged with 10^5 ELD_{50} of virulent NDV virus two weeks post booster vaccination. As expected, rmNA-1 and rmNA-H9F provided full protection against virulent NDV challenge with no clinical signs or deaths (Fig. 4F, Fig. S1A). No gross lesions were observed in the rmNA-H9F and rmNA-1 vaccinated

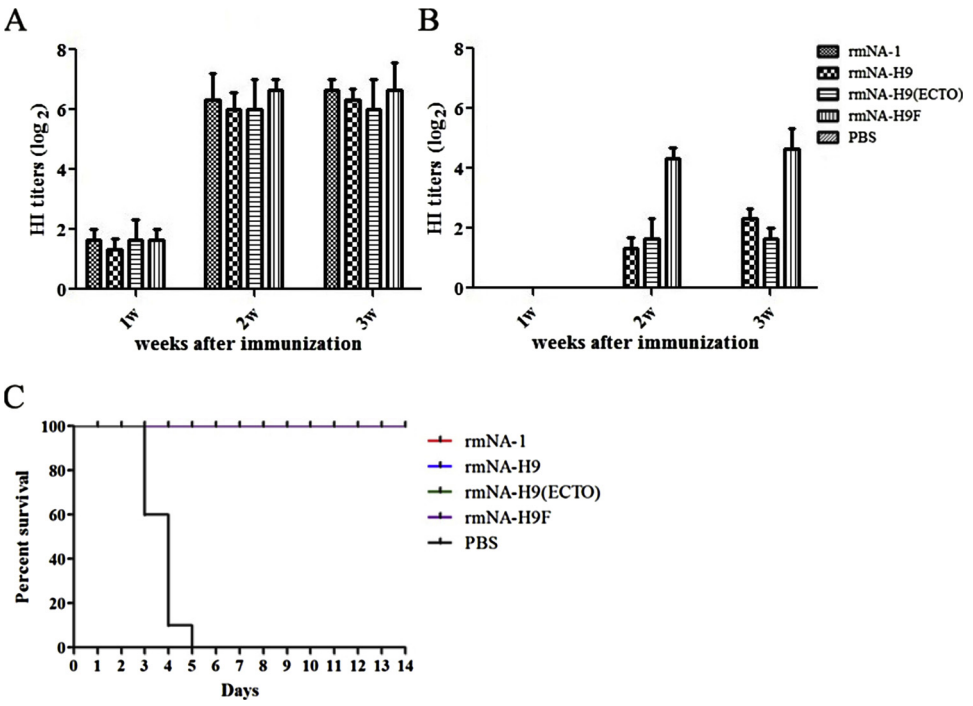

Fig. 3. Immunogenicity of rNDVs in SPF chickens post a single vaccination and the survival rate after challenge with virulent NDV. The NDV-specific HI titers (A) and H9-specific HI titers (B) induced by rmNA-1, rmNA-H9, rmNA-H9 (ECTO), and rmNA-H9F in chickens. (C) The survival rates of SPF chickens after challenge with virulent NDV.

Table 1
Virus detection in oropharyngeal and cloacal swabs of vaccinated and mock-vaccinated chickens challenge with virulent NDV at different time points.

Immunizing virus	Virus isolation from swabs shedding/total					
	3d		5d		7d	
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
rmNA-H9	3/10	1/10	1/10	2/10	0/10	0/10
rmNA-H9F[b]	4/10	2/10	2/10	3/10	0/10	0/10
rmNA-H9 (ECTO)[b]	4/10	2/10	2/10	3/10	0/10	0/10
rmNA-1	3/10	1/10	1/10	2/10	0/10	0/10
PBS	10/10	10/10	NA[a]	NA	NA	NA

[a] NA: not applicable due to death of chickens.
[b] Significance from rmNA-1 group, $P < 0.05$.

Table 2
Virus detection in oropharyngeal and cloacal swabs of vaccinated and mock-vaccinated chickens infected with H9N2 virus at different time points.

Immunizing virus	Virus isolation from swabs shedding/total					
	3d		5d		7d	
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
rmNA-H9[b,c]	5/10	2/10	3/10	4/10	1/10	2/10
rmNA-H9F[c]	3/10	1/10	2/10	3/10	0/10	0/10
rmNA-H9 (ECTO)[b,c]	6/10	3/10	4/10	4/10	2/10	1/10
PBSb	10/10	7/10	8/10	7/10	7/10	5/10
rmNA-1[b,c]	5/10	2/10	3/10	4/10	1/10	2/10

[b] Significance from rmNA-H9F group, $P < 0.05$.
[c] Significance from PBS group, $P < 0.05$.

chickens sacrificed at 5 dpc (data not shown). Moreover, no apparent histological changes were observed in the lung tissues of the two groups (Fig. 5). Virus shedding in oropharyngeal and cloacal swabs were completely inhibited in at 3 dpc (Table 3). As to the inactivated vaccine group, transient depression and loss of appetite were observed, but no deaths was detected (Fig. S1A). Hemorrhage in lung and, swelling and hemorrhage in bursas were also observed in inactivated vaccine group at 5 dpc (data not shown). Consistent with the gross lesions, hemorrhage in lung was seen in the histological examination (Fig. 5). In addition, 20% and 10% viral shedding rate in the oropharyngeal and cloacal swaps were detected in the inactivated vaccine group at 3 dpc (Table 3).

When challenged with H9N2 virus, neither clinical signs nor gross lesions were observed in the rmNA-H9F vaccinated chickens (data not shown). In the histological examination, no lesions were observed in the lung tissue in this group (Fig. 5). In addition, 90% of the vaccinated chickens were protected from virus shedding in the oropharynx and cloaca (Table 4). In contrast, although no clinical signs were observed in the inactivated vaccine group, hemorrhage in lung and bursas were observed (Fig. 5, Fig. S1B). Viral shedding rates in oropharyngeal and cloacal swabs were less in the inactivated vaccine group than mock-vaccinated birds at 3 dpc and 5 dpc (Table 4). No virus was detected in either oropharyngeal or cloacal swabs in birds of the inactivated vaccine group at 7 dpc (Table 4). As a comparison, hemorrhage in the lung, glandular stomach, and intestines were observed in the mock-vaccinated birds (Fig. 5).

4. Discussion

Newcastle disease virus and H9N2 avian influenza virus are two of the most important pathogens in poultry worldwide. Vaccination is an effective way to prevent the two pathogens. Live attenuated NDV vaccines could be applied in chickens efficiently via the intranasal route and induce both robust systemic antibodies, and mucosal and cell-mediated immunity (Kim and Samal, 2016). Attenuated NDV vaccines are considered as the most promising vectors. All the NDV strains belong to a single serotype, but genetic and antigenic differences are existing among isolates (Miller et al., 2010). When the vaccine strains and the challenge strains were genetically matched, the vaccination will result in an ideal immune response and protection efficacy (Dimitrov et al., 2017b). Therefore, a novel genotype-matched attenuated NDV vector rmNA-1 was used to develop a bivalent vaccine strain against genotype VII NDV and H9N2 AIV in this study. Three rNDVs expressing different forms of H9 HA as an added gene were developed through a reverse genetic system. A potential concern in the development of a recombinant virus expressing foreign antigens is whether the insertion

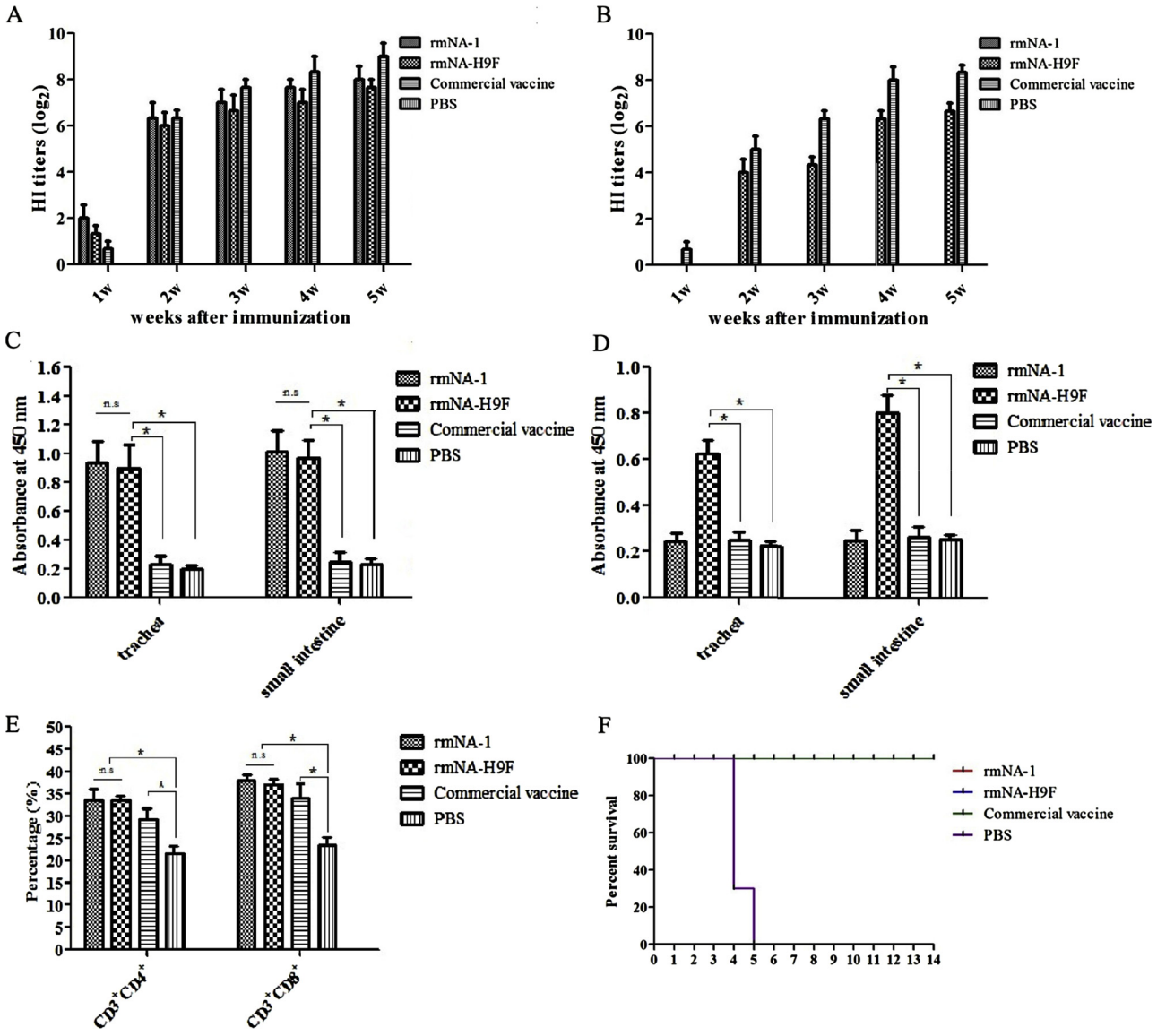

Fig. 4. Immunogenicity of rNDVs in SPF chickens post the prime-boost vaccination and the survival rate after challenge with virulent NDV. The NDV-specific HI titers (A) and H9-specific HI titers (B) induced by rmNA-1, rmNA-H9F, and commercial inactivated oil-emulsion vaccine in SPF chickens. NDV-specific SIgA (C) and H9N2-specific SIgA (D) in the tracheal and intestine washings. (E) The percentage of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ subsets in the splenic lymphocyte. (F) The survival rates of SPF chickens after challenge with virulent NDV.

of the HA gene would affect the replication, virulence and immunogenicity of the rNDVs. Our study showed that all the three rNDVs grew to high titers, comparable to the parental rmNA-1 strain, and the insertion of the HA gene slightly attenuated the virulence. Furthermore, all the three rNDVs induced effective NDV-specific HI antibodies and complete protection against virulent NDV challenge through a single dose vaccination. These results indicated that the introduction of foreign H9 HA gene into the rNDVs does not affect the safety, intrinsic replication, and its efficacy as a NDV vaccine.

The efficacy of NDV vectored vaccines are influenced by many factors. Among these factors, whether the foreign proteins are incorporated into viral particles and the incorporation level is undoubtedly important and critical for a successful chimeric vaccine development (Dimitrov et al., 2017b). It was previously demonstrated that replacement of the transmembrane and cytoplasmic tail regions of the AIV H5 or H7 HA protein with those of the NDV F protein generated an enhanced HA incorporation into NDV particles. It contributed to an improved AIV specific immune response compared to that of a NDV vector expressing the unmodified HA (Nayak et al., 2009; Park et al., 2006). Agree with previous study, in our study, the rmNA-H9F expressing a chimeric HA also induced effective H9-specific HI antibody production, protected chickens from clinical signs, and significantly reduced the viral shedding from both trachea and cloacae after a single dose immunization. However, immunization with rmNA-H9 or rmNA-H9 (ECTO) only elicited low AIV HI titers, which were below the protective level, and failed to protect chickens from clinical signs and virus shedding after challenge with H9N2 virus. The better immunogenicity against H9N2 AIV is mostly attributed to the enhanced incorporation of H9 HA into rmNA-H9F viral particles as HA presented on the viral particles will allow an efficient cross-linking of B cell receptors because they present HA molecules in a repetitive array (Bachmann and Jennings, 2010). The innate and adaptive immune systems have evolved to efficiently recognize such structures, which further result in a better immune response. Thus, compared to rmNA-H9 and rmNA-H9 (ECTO), the superior protective efficacy of rmNA-H9F against H9N2 AIV is the result of level H9 HA incorporation on the viral envelope.

In practical application, inactivated vaccines have to be repeatedly, intramuscularly and individually administered to poultry populations

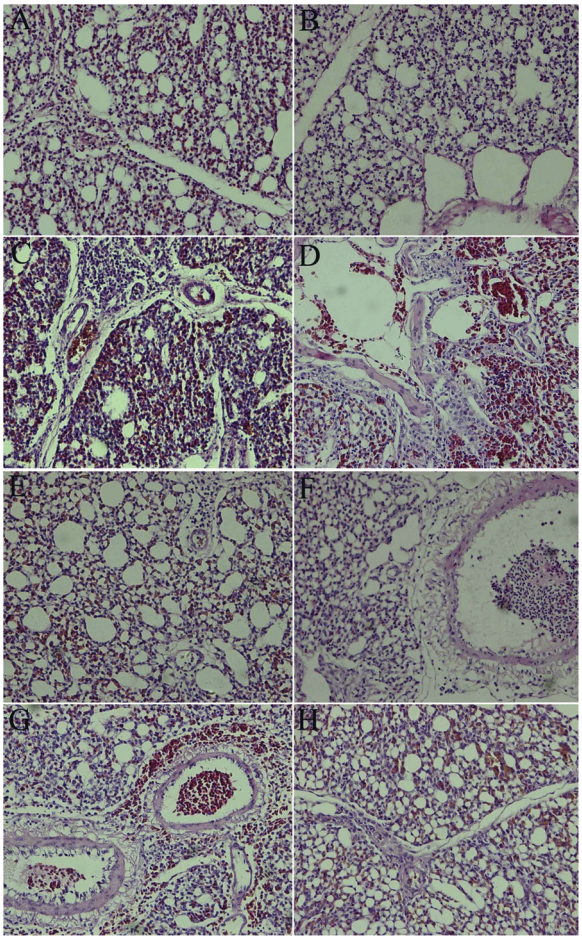

Fig. 5. Histological staining of lung tissue samples on 5 dpc (200× magnification). (A–D) Histopathological examination of lung from the chickens in rmNA-1, rmNA-H9F, commercial vaccine, and mock-vaccinated groups challenged with virulent NDV. (E–G) Histopathological examination of lung from chickens in rmNA-H9F, commercial vaccine, and mock-vaccinated groups challenged with H9N2 AIV. (H) The mock infected group.

Table 3
Virus detection in oropharyngeal and cloacal swabs of vaccinated and mock-vaccinated chickens challenged with virulent NDV at different time points.

Immunizing virus	Virus isolation from swabs shedding/total					
	3d		5d		7d	
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
rmNA-1[d]	0/10	0/10	0/10	0/10	0/10	0/10
rmNA-H9F[d]	0/10	0/10	0/10	0/10	0/10	0/10
Commercial vaccine	2/10	1/10	0/10	0/10	0/10	0/10
PBS	10/10	10/10	NA[a]	NA	NA	NA

[a] NA: not applicable due to death of chickens.
[d] Significance from commercial vaccine group, $P < 0.05$.

to prevent H9N2 AIV (Wei et al., 2016a). This process is time-consuming, laborious and expensive. In addition, intense stress caused by injection could hamper avian productivity including delayed growth and reduced egg production. Compared with inactivated vaccines, live attenuated influenza vaccines (LAIVs) can overcome above shortcomings. LAIVs can also induce sufficient mucosal and serum antibody production, and proficient cell-mediated immune responses. However, the safety concern of live H9N2 AIV vaccines totally limited their application in China. Thus, the development of novel H9N2 AIV vaccines is still a research hotspot. NDV has long been an attracting vector for

Table 4
Virus detection in oropharyngeal and cloacal swabs of vaccinated and mock-vaccinated chickens infected with H9N2 virus at different time points.

Immunizing virus	Virus isolation from swabs shedding/total					
	3d		5d		7d	
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
rmNA-H9F[e]	1/10	0/10	0/10	0/10	0/10	0/10
Commercial vaccine	3/10	2/10	2/10	0/10	0/10	0/10
PBS[e]	9/10	6/10	8/10	6/10	6/10	4/10

[e] Significance from commercial vaccine group, $P < 0.05$.

AIV antigens. Regarding the selection of NDV, the NDV strain NA-1 used to make the vaccine and to challenge the birds in this study was a classical genotype VII virulent NDV strain in China. It was isolated, well characterized and stored by our lab with a clear background information, which facilitate a fast construction of rNDVs. It allowed us to select the most efficient vaccine developing strategies for further research. For future investigation, the strategy can be readily applied to other predominant circulating strains. Additionally, the genetic background of several circulating genotype VII strains was investigated and it was found that the nucleotide and amino acid homology between NA-1 strain, go/CH/LHLJ/1/06 (GenBank No. KJ60716) and Goose/China/Jilin(Meihekou)/MHK-1/2010 (GenBank No. KM408752) were 99.9% and 99.8%, respectively (Chen et al., 2015). The high genetic similarity suggested that NA-1 strain may represent circulating strains to an extent.

In terms of immunogenicity efficacy, rmNA-H9F induced relatively high levels of H9-specific HI antibody titers after a booster vaccination. The HI titers in chickens immunized with inactivated vaccine were slightly higher than those immunized with rmNA-H9F. This could be the result of adjuvant in commercial vaccine, which will increase the immunogenicity of the vaccine (Ma et al., 2017). However, despite its high HI level induced by inactivated vaccine, chickens from this group displayed slight clinical signs after virulent NDV strain challenge. Several reasons may account for it. Firstly, the levels of NDV and H9N2 AIV specific SIgA in trachea and small intestine douche from rmNA-H9F vaccinated chickens were significantly higher than those of the inactivated vaccine group. SIgA is thought to be the major neutralizing antibodies against influenza virus during virus entry (Cox et al., 2004). Secondly, rmNA-H9F also significantly promoted the CD4+ and CD8 + T lymphocyte differentiation in the spleen, which is associated with more active cellular immunity. It is considered that CD4 + T cells contribute to stimulate B cell maturation and antibody responses, to enhance the functional activity of effector CD8 + T cells, and to release cytokines with immune regulatory activity. While CD8 + T cells mediate the CTL response which is responsible for the clearance of the virus during infection (Zhao et al., 2017). Thirdly, the genetic distance between NDV LaSota strain and current circulating strains was 18.3–26.6%, resulting with insufficient protection in the field (Dimitrov et al., 2017a). The incomplete protection of inactivated LaSota strain against genotype VII strain challenge observed in our study agreed with several other studies (Miller et al., 2007; Wajid et al., 2018; Yang et al., 2017). Another point is also worth noting, the HI titer in this study was measured with a commercial LaSota based standard antigen, which is heterologous to rmNA-1. According to Yang's study, HI level is around 2-fold lower when measured with a heterologous strain than a homologous strain (Yang et al., 2017). Thus, the high HI titer of LaSota group is also a result of genotype match with the standard antigen.

In summary, we constructed three rNDVs expressing different forms of H9 HA protein and evaluated their immunogenicity and protection efficacy against NDV and H9N2 challenge. Among them, the rmNA-H9F strain induced high levels of NDV- and H9-specific HI antibody. The rmNA-H9F strain also induced profound local mucosal immune

response and cellular immunity. The protective efficiency of rmNA-H9F against prevalent NDV and H9N2 virus was better than the that of the commercial inactivated oil-emulsion bivalent vaccine. This rNDV can be administered via the oculonasal route, mimicking the natural route of H9N2 infection and suitable for convenient applications in the field. Thus, rmNA-H9F could be considered as a live bivalent vaccine candidate against NDV and H9N2 AIV.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.11.006.

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