Chimeric Newcastle disease virus-vectored vaccine protects chickens against H9N2 avian influenza virus in the presence of pre-existing NDV immunity

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Abstract

A chimeric Newcastle disease virus (NDV) vector (NDV/AI4-TFHN) was constructed with the replacement of the ectodomains of the fusion and hemagglutinin-neuraminidase proteins by those from avian paramyxovirus type 2. The chimeric virus induced high antibody response in chickens pre-immunized with NDV. A recombinant vaccine candidate, NDV/AI4-TFHN-H9, expressing the hemagglutinin of H9N2 avian influenza virus, was generated, on the basis of the chimeric NDV vector mentioned above. The NDV/AI4-TFHN-H9 vaccine elicited H9-specific hemagglutination inhibition antibodies in chickens pre-immunized with NDV vaccine, and reduced the numbers of chickens shedding virus after H9N2 challenge. NDV/AI4-TFHN-H9 could serve as an alternative vaccine for the prevention of H9N2 infection in commercial poultry flocks.

H9N2 subtype avian influenza virus (AIV) is endemic in many countries in the Eurasian continent, leading to significant economic losses due to a drop in egg production and

high morbidity and mortality associated with co-infection with other viruses and/or bacteria [1, 2]. Recently, mammals and human infections of H9N2 AIVs have been reported [3–5]. H9N2 AIVs have been recognized to undergo reassortment with multiple subtypes and to serve as donors of the six internal genes for generating zoonotic viruses, such as H5N1, H7N9 and H10N8 viruses [6–8]. Therefore, the prevention and control of H9N2 virus infection in poultry is one of the key points to both animal and public health, and it is necessary to develop new and more efficacious H9N2 vaccines.

Newcastle disease virus (NDV) is a promising vector for live vaccine development due to some advantages, including a high virus yield in embryonated chicken eggs, an induction of robust humoral and cellular immunity, and the potential for mass administration via drinking water or aerosol spray. Avirulent NDV strains have been used as vectors to express hemagglutinin (HA) of H9N2 subtype AIVs, and these vaccines are generally immunogenic and efficacious in chickens [9, 10]. However, the maternal Newcastle disease (ND) antibodies present in commercial chickens severely interfere with the replication of the vectored vaccine viruses and thus impair the efficacy of this kind of vaccine [11, 12].

Although NDV (renamed as avian avulavirus-1 (AAvV-1) recently) and avian paramyxovirus serotype 2 to 10 (APMV-2 to 10) are members of the genus *Avulavirus* in the *Paramyxoviridae* family [13, 14], significant antigenic

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differences exist between them [15, 16]. Fusion proteins (F) and hemagglutinin-neuraminidase (HN) are the surface glycoproteins possessing major antigenic epitopes of NDV [17–19]. Therefore, chimeric NDVs carrying functional surface glycoproteins of APMV-2 and APMV-8 show low cross-reactivity with antiserum against NDV, and have been reported as alternative vaccine vectors against H5 subtype HPAIV in the animals with maternally derived ND antibodies [20, 21]. In order to develop a novel live H9 vaccine for massive and convenient application in commercial chickens, the envelope proteins of NDV were replaced by the corresponding protein of APMV-2 in this study and the application of NDV-vectored H9 AI vaccine was facilitated in chickens with pre-existing NDV immunity.

Toward this end, a chimeric NDV vector based on an attenuated genotype VII NDV (NDV/AI4) with high

reproductivity in embryonated chicken eggs [22] was firstly developed in which the ectodomains of the F and HN proteins were replaced by those from the antigenically distinct avian paramyxovirus serotype-2 (APMV-2) [16, 21, 23] strain T4 that was gifted from Dr. Jixun Zhao (China Agricultural University). The coding regions for the ectodomains of the APMV-2/T4 glycoproteins and the transmembrane/cytoplasmic (TM/CT) domains of the NDV glycoproteins were fused in frame by overlapping PCR as previously described [24–26]. The resultant chimeric F and HN genes were used to replace the corresponding sequences in the cDNA clone of NDV/AI4. The chimeric virus was generated by reverse genetics as described previously [22] and was designated as NDV/AI4-TFHN (Fig. 1A). Briefly, BSR-T7/5 cells were inoculated with the T7 RNA polymerase-expressing recombinant vaccinia Ankara virus before

Fig. 1 Construction and biological properties of the rescued NDV viruses. (A) Schematic design of the chimeric NDV constructs used in this study. The ectodomains of the F and HN genes from NDV/AI4 were replaced by those of the APMV-2 strain T4. The HA gene from A/chicken/Jiangsu/WJ57/2012 (H9N2) was cloned into the P and M junction of NDV/AI4-TFHN-H9 under the control of a set of NDV gene start (GS) and gene end (GE) transcription signals. (B) The expression of the HA protein in the virus-infected cells was

examined by immunofluorescence assay (IFA). CEFs were infected with vaccine virus and WJ57 strain at an MOI of 1.0, and chicken anti-H9N2 serum was applied. (C) The expression of HA protein in the virus-infected cells was detected by Western blotting. CEFs were inoculated with NDV/AI4-TFHN-H9 of the same amount as that used in the IFA test. Lysates of the infected cells at 36 h post inoculation were detected with chicken anti-H9N2 serum

transfection. The cells were then transfected with a plasmid mixture containing the chimeric full-length cDNA pNDV/AI4-TFHN, three supporting plasmids expressing the nucleoprotein (NP), phosphoprotein (P) and large proteins (L) using X-treme GENE HP DNA transfection reagent (Roche, Mannheim, Germany). The supernatant and the cells were harvested 72 h post-transfection and inoculated into the allantoic cavity of 9-day-old SPF embryonated chicken eggs. To identify the rescued virus, the allantoic fluids were harvested for hemagglutination (HA) and hemagglutination-inhibition (HI) assays 4 days later.

To investigate the biological properties of chimeric virus, virus titers in embryos (50 % egg infectious dose, EID₅₀) and two virulence indices, mean death time (MDT) in embryos and intracerebral pathogenicity index (ICPI) in 1-day-old chickens, were determined [13]. The results indicated that NDV/AI4-TFHN replicated efficiently in SPF embryonated chicken eggs, and the virus titer reached 10^{8.5} EID₅₀/0.1 ml. The MDT value of this recombinant virus was more than 120 h, and the ICPI value was 0.00 (Table 1). Since the homologous TM/CT domains are important for virus replication and the F and HN proteins play a major role in viral pathogenesis [26], the chimeric vector was apparently unimpaired in terms of growth capacity and was safe to be used as a viral vector candidate.

The antigenic cross-reactivity of the chimeric virus NDV/AI4-TFHN with the parental virus NDV/AI4 was evaluated by cross HI test. The antisera used in the HI test were prepared from the SPF chickens vaccinated with inactivated oil-emulsified NDV/AI4 and APMV-2/T4. The results showed that the antiserum of APMV-2/T4 had high HI titers against NDV/AI4-TFHN, which is not surprising since it carries homologous F and HN proteins. In contrast, the antiserum of NDV/AI4 exhibited low HI titers against NDV/AI4-TFHN that harbours heterologous F and HN proteins. These results showed that there was an apparent antigenic variation between the chimeric NDV vector and the parental virus (Table 2).

The influence of the ND antibody on the immunogenicity of the chimeric NDV vector was examined in the SPF chickens that were immunized with 40 µl of inactivated

Table 2 Cross Hemagglutination inhibition (HI) titers of antisera against different antigens

Antigen	Antiserum	
	NDV/AI4	APMV-2/T4
APMV-2/T4	2 ^a	768
NDV/AI4	256	8
NDV/AI4-TFHN	2	768

^aHomologous titers are indicated in bold and mean titers are presented

oil-emulsified LaSota at 1 day of age. At 3 weeks post-immunization (pi), when the average HI titer against NDV reached 7 log₂, five chickens per group were immunized with NDV/AI4 or NDV/AI4-TFHN at a dose of 10⁶EID₅₀/0.1 mL via the oculonasal route. Another five birds were mock-inoculated with PBS as a control group. Furthermore, fifteen 3-week-old SPF chickens were divided into three groups and were immunized with NDV/AI4, NDV/AI4-TFHN or PBS, respectively, following the same procedures described above. None of the inoculated animals showed clinical signs during the observation period of three weeks. The serum samples were obtained 3 weeks pi, and the HI titers were measured against APMV/T4 and NDV/AI4, respectively. The results of seroconversion showed that the level of HI antibody induced by NDV/AI4 (HI titer was 7 log₂) and the chimeric virus NDV/AI4-TFHN (HI titer was 7.2 log₂) was comparable in SPF chickens. However, in the chickens with pre-existing ND antibodies, no significant changes in the NDV-specific antibodies in the birds vaccinated with NDV/AI4 were observed when compared with the control birds (ND HI titer was 3.2 log₂). In contrast, the HI antibody titers against APMV-2 reached up to 7.2 log₂ in birds vaccinated with NDV/AI4-TFHN (Fig. 2A). Therefore, the immune efficacy of NDV/AI4 as a vector was strongly affected by ND antibodies, while the NDV/AI4-TFHN as a chimeric vector could effectively induce the corresponding antibody in chickens with high level of ND antibodies.

Based on the chimeric NDV vector platform, a live vector vaccine candidate for H9N2 subtype AIVs was generated. The HA gene of H9N2 AIV WJ57 strain (A/Chicken/Jiangsu/WJ57/2012), which belonged to the dominant S genotype in China [27], was inserted between the phosphoprotein (P) and matrix (M) genes of the chimeric antigenomic cDNA (Fig. 1A). The recombinant virus was rescued as described above and was designated as NDV/AI4-TFHN-H9. To evaluate the genetic stability of the rescued virus, we serially passaged it 10 times in 9-day-old SPF embryonated chicken eggs. The presence of the chimeric F and HN genes from APMV-2 strain T4 and the HA gene from the H9N2 strain WJ57 was confirmed by RT-PCR and sequencing (data not shown) of these related

Table 1 Biological properties of parental and chimeric NDV vectored vaccine viruses

Virus	Pathogenicity		Virus titer	
	MDT (h)	ICPI	EID ₅₀ /0.1 ml	HA (log ₂)
APMV-2/T4	> 120	0.00	10 ^{8.3}	8
NDV/AI4	> 120	0.13	10 ^{9.1}	10
NDV/AI4-TFHN	> 120	0.00	10 ^{8.5}	10
NDV/AI4-TFHN-H9	> 120	0.00	10 ^{8.3}	9

Fig. 2 Hemagglutination-inhibition (HI) titers induced by the vaccines. (A) 3-week-old SPF chickens (upper left) or 3-week-old SPF chickens with NDV-specific antibodies (HI titer was 7 $\log_2$) (upper right) were inoculated with the NDV vaccine strain, the chimeric NDV strain or PBS. HI antibody titers against APMV-2/T4 and NDV/AI4 were detected at 3 weeks pi. (B) NDV/AI4-TFHN-H9 vaccination in SPF chickens with NDV-specific antibodies, HI antibody titers

against NDV and H9 AIV detected at weeks 1, 2 and 3 post-immunization (pi) were shown. Statistical analysis was calculated by GraphPad Prism 5.0 (GraphPad Software, California San Diego, USA) using ANOVA followed by Turkey's test with a significance level of $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***), respectively. ns = no significant difference

genes in the final passage. NDV/AI4-TFHN-H9 replicated efficiently in SPF embryonated chicken eggs, and the virus titer reached $10^{8.3}$ EID₅₀/0.1 ml (Table 1). The MDT value of the recombinant virus was more than 120 h, and the ICPI value was 0.00 (Table 1). These results revealed that the rescued recombinant vaccine candidate replicated efficiently in chicken embryos and were safe for chickens.

The expression of the HA protein was detected by indirect immunofluorescence (IFA) and Western blotting in the CEF cells infected with the recombinant virus (Fig. 1 B and C). Our IFA results demonstrated clearly the expression of HA protein in NDV/AI4-TFHN-H9 infected CEF cells with both positive and negative controls (Fig. B). The HA protein expressed in the NDV/AI4-TFHN-H9 infected cells was in the form of the HA0 precursor (~70 kDa) and the HA1 subunit (~50 kDa) as determined

by Western blotting (Fig. 1 C). In addition, the HA was stably expressed for the virus at different passages (data not shown).

To evaluate the immunogenicity and efficacy of the H9 HA-expressing recombinant vaccine candidate in the presence of the NDV-specific antibody, twenty 3-week-old SPF chickens were first immunized with the inactivated LaSota at 1 day of age and were then divided into two groups when the average HI titer against NDV reached 7 $\log_2$. Subsequently, the chickens were inoculated with 10^6 EID₅₀/0.1 mL of NDV/AI4-TFHN-H9 or PBS via the oculonasal route. The serum samples were collected at week 1, 2 and 3 pi. At 3-week pi, all the chickens were challenged with 10^6 EID₅₀/0.1 mL of A/Chicken/Anhui/AH320/2016 (H9N2). To monitor the shedding of the challenged virus, oral and cloacal swabs were collected at 3, 5, 7 days post-challenge (dpc). Three birds

Table 3 Frequency of virus isolation in swabs from NDV/AI4-TFHN-H9 vaccinated and control groups

Group	No. swabs positive in virus isolation/total no. of days post challenge					
	3 dpc ^a		5 dpc		7 dpc	
	T ^b	C ^c	T	C	T	C
NDV/AI4-TFHN-H9	3/10 ^d	5/10	2/10	5/10	0/7	0/7
Control	10/10	10/10	10/10	10/10	3/7	5/7

^aDays post challenge; ^bTracheal swabs; ^cCloacal swabs

from each group were necropsied at 5 dpc. The trachea, lung, spleen, heart and liver were collected and fixed by formalin. The fixed tissues were embedded in paraffin, sectioned at 3 µm and then routinely deparaffinized and stained with haematoxylin and eosin for histopathological analysis. Isolation of the H9N2 challenge virus from the swab samples was performed by inoculating 9-day-old SPF embryonated chicken eggs, and the presence of the virus was confirmed by HA and HI tests. The seroconversion rate of the recombinant virus NDV/AI4-TFHN-H9 was 90%, and the average HI titer against H9N2 reached 5.8 log₂ at 3 weeks pi (Fig. 2B). After challenge with H9N2 virus, some birds in control group showed depression and ruffled feathers, while an increase in oral mucus was observed in 6 out of 10 control birds at 5 dpc. In contrast, NDV/AI4-TFHN-H9-vaccinated chickens displayed no clinical signs post challenge. Virus isolation was performed from the oropharyngeal and cloacal swabs at 3, 5, and 7 dpc in the experimental chickens. At 3 and 5 dpc, some NDV/AI4-TFHN-H9-vaccinated chickens shed the virus via the oropharyngeal and cloacal routes (Table 3). No virus was detected in either the oropharyngeal or cloacal swabs in NDV/AI4-TFHN-H9-vaccinated birds at 7 dpc, whereas 5 out of 7 control birds still shed the virus. Histopathological changes were evaluated at 5 dpc. Mild tracheal mucosal epithelial shedding was observed in 2 out of 3 non-vaccinated chickens while no tracheal lesions were detected in chickens immunized with NDV/AI4-TFHN-H9. In the lung, mild to moderate haemorrhage and interstitial congestion occurred in 2 out of 3 birds in the non-vaccinated group, however, mild congestion was observed in 1 out of 3 vaccinated chickens. Mild lymphocytosis and congestion

were observed in the thymus in non-vaccinated birds, but no lesions were seen in the immunized birds (Fig. 3). These results indicated that the recombinant NDV/AI4-TFHN-H9 could effectively protect chickens against H9N2 AIV infection in the presence of pre-existing NDV immunity.

Vaccination is considered as one of the principal strategies for controlling H9N2 AI. Previous report showed that recombinant NDV-vectored vaccine candidates conferred protection against H9N2 AIVs infection in chickens [9, 10]. However, owing to the interference of the maternal ND antibodies acquired by intensive ND vaccination in commercial poultry flocks, vaccination failure is often seen when live ND vaccines or NDV-vectored vaccines are administered in young chickens. In this study, a chimeric NDV vector carrying the F and HN proteins from APMV-2 was generated. The results of our vaccination study in chickens pre-immunized with ND vaccine clearly demonstrated that the chimeric virus elicited high level of antibody specific for APMV-2 HN, suggesting that this chimeric vector can evade the interference from pre-existing NDV immunity. Based on this chimeric NDV vector, a novel recombinant vectored vaccine against the H9 AIV infection was developed. This vaccine can provide effective protection in chickens with high ND HI titer from H9N2 challenge in terms of reducing the numbers of chickens shedding virus and alleviating histological damages of the trachea, lung and thymus. Thus, the chimeric NDV vector-based live H9 AIV vaccine candidate NDV/AI4-TFHN-H9 is immunogenic and efficacious against H9N2 AIV infection in the presence of pre-existing NDV immunity and should be a valuable tool in addition to the conventional killed vaccines.

Fig. 3 Photomicrographs illustrating haematoxylin and eosin (HE) staining on sections of trachea, lung and thymus at day 5 post challenge. Magnification in each panel was shown

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Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

Ethical approval Animal experiments were approved by the Jiangsu Administrative Committee for Laboratory Animals (Permission number: SYXK-SU-2007-0005), and complied with the guidelines of

Jiangsu laboratory animal welfare and ethical of Jiangsu Administrative Committee of Laboratory Animals.

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