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Short communication

Newcastle disease virus (NDV) recombinant expressing the hemagglutinin of H7N9 avian influenza virus protects chickens against NDV and highly pathogenic avian influenza A (H7N9) virus challenges

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ABSTRACT

The emerged highly pathogenic avian influenza A (H7N9) (HPAI) viruses in China pose a dual challenge to public health and poultry industry. Thus H7N9 vaccines are in an urgent need. In this study, we constructed a Newcastle disease virus (NDV)-vectored vaccine (rLXHAF) expressing the hemagglutinin (HA) of H7N9 virus fused with the transmembrane/cytoplasmic tail domain of the NDV fusion protein. rLXHAF stably expressed the HA protein, exhibited similar growth kinetics and pathogenicity as the parental virus. rLXHAF induced positive NDV-specific hemagglutination inhibition (HI), virus neutralization (VN) and total IgY antibodies and completely protected chickens from NDV challenge. Unexpectedly, rLXHAF elicited undetectable HI and VN titers but high overall IgY antibody titers against H7N9 measured by ELISA. The vaccine provided 80% protection against HPAI H7N9 challenge. Virus shedding of NDV and H7N9 challenge strains was reduced. Our results suggest that rLXHAF is immunogenic and efficacious against HPAI H7N9 virus in chickens.

1. Introduction

Since 2013, mainland China has experienced five epidemic waves of avian influenza A (H7N9) virus, causing severe human infections with high mortality [1]. As of 15 June 2017, a total of 1533 laboratory-confirmed cases of human infection with H7N9 virus, including at least 592 deaths, have been reported (http://www.who.int/influenza/human_animal_interface/Influenza_Summary_IRA_HA_interface_06_15_2017.pdf?ua = 1). In the fifth wave, variant H7N9 viruses with an insertion of four amino acids in the hemagglutinin (HA) proteolytic cleavage site have been detected [2], indicating their pathotype switches from low pathogenic avian influenza (LPAI) to highly pathogenic avian influenza (HPAI). According to World Organization for Animal Health (OIE) reports, as of 5 June 2017, nine outbreaks of HPAI H7N9 virus in poultry in China have been reported, resulting in over 800,000 chickens being destroyed (http://www.oie.int/wahis_2/public%5C..%5Ctemp%5Creports/en_fup_0000024044_20170614_142756.pdf). Currently, HPAI H7N9 virus poses a dual challenge to public health and poultry industry. Under this circumstance, H7N9 vaccines are in an urgent need to contain H7N9 virus in poultry.

Newcastle disease (ND) is one of the most important infectious diseases for poultry and is endemic in many countries [3,4]. Strains of Newcastle disease virus (NDV) have been grouped into three main pathotypes on the basis of the clinical signs seen in infected chickens: velogenic, a form that presents with high mortality, severe haemorrhagic, respiratory or nervous signs; mesogenic, a form that presents with respiratory signs, but with low mortality; lentogenic, a form that presents with mild or subclinical respiratory infection. Lentogenic NDVs are usually used as live vaccines for poultry. In addition, lentogenic NDV is also a promising vector for the development of bivalent live poultry vaccines. Numerous NDV-vectored vaccines expressing protective antigens of different poultry viruses, including the HA genes of H5, H7 and H9 influenza viruses [5–7], viral protein 2 of infectious bursal disease virus [8], glycoproteins B and D of infectious laryngotracheitis virus [9] and the spike protein of infectious bronchitis virus [10], were shown to be highly immunogenic and efficacious in chickens.

In this study, a bivalent vaccine candidate based on a lentogenic NDV vector against both NDV and HPAI H7N9 was generated. The recombinant virus was evaluated *in vitro* and *in vivo* for HA expression, incorporation of HA into NDV virions, growth kinetics,

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pathogenicity, immunogenicity and protection against NDV and HPAI H7N9 virus.

2. Materials and methods

2.1. Ethics statements

All 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 ethics of Jiangsu Administrative Committee of Laboratory Animals. All experiments involving live virulent NDV and HPAI H7N9 virus were performed in animal biosecurity level-3 facilities.

2.2. Generation and characterization of the recombinant NDV containing H7 HA

First, the full-length cDNA clone of LX was constructed and infectious virus was rescued by reverse genetics as described previously [11]. In brief, $8X10^5$ BSR-T7/5 cells were seeded in a 6-well culture plate before transfection. Cells were firstly inoculated with modified vaccinia Ankara virus at a multiplicity of infection (MOI) of 1 and then transfected with a mixture containing the full-length cDNA, three supporting plasmids expressing the nucleoprotein, phosphoprotein (P) and large proteins and X-treme GENE HP DNA transfection reagent (Roche, Mannheim, Germany). Three days later, the supernatant and the cells were harvested. 0.3 ml of the mixture was inoculated into the allantoic cavity of 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECEs). To identify the rescued virus, the allantoic fluids were collected for hemagglutination (HA) assay.

Next, to facilitate insertion of the HA gene into the LX genome, the PacI site was introduced between the P and matrix (M) genes. The open reading frame (ORF) of the HA gene from a LPAI H7N9 strain (A/chicken/Zhejiang/JX148/2014, JX148) along with the NDV *trans*-acting signal sequences, including the gene end, intergenic and gene start sequences, was amplified. In addition, to promote the incorporation of the HA protein into NDV particles, the HA ectodomain was fused with a fragment encoding the transmembrane (TM)/cytoplasmic tail domain (CTD) of NDV fusion (F) protein by overlapping PCR. The final PCR products were cloned into the full-length NDV plasmid at the unique PacI site between the P and M genes. The recombinant viruses, rLXHA and rLXHAF, were rescued by reverse genetics as described above (Fig. 1A).

The expression of the HA protein was examined by immunofluorescence assay (IFA) and Western blotting in CEF. Further, the incorporation of the HA protein into two recombinant viruses was determined using Western blotting. Mouse mAb against the HA protein of a human-origin H7N9 virus (A/Anhui/1/2013) (Sino Biological, Beijing, China) and anti-NDV chicken serum were used in IFA and Western blotting. The multicycle growth kinetics of rLXHAF and rLX was determined in ECEs as described elsewhere [12]. Pathogenicity of rLXHAF was evaluated using the mean death time (MDT) test in ECEs as described previously [13].

2.3. Immunization and challenge studies in chickens

Twenty 3-week-old SPF chickens were immunized intranasally (i.n.) with 0.1 ml of the inoculum containing 5×10^6 50% embryo infectious dose (EID_{50}) of rLXHAF. Ten birds were immunized i.n. with 5×10^6 EID_{50} of rLX. Booster vaccination was conducted 2 weeks after the first vaccination using the same procedure. Ten chickens were inoculated with PBS as sham control. Blood samples were taken 2 weeks after each vaccination for determination of hemagglutination inhibition (HI) and virus neutralization (VN) antibodies. In addition, NDV-specific and H7 HA-reactive IgY antibodies were measured by ELISA.

NDV-specific IgY titers were determined using the commercial competitive ELISA kit (KERNEL, PA, USA). ELISA titers were calculated according to the manufacture's instruction. For H7-specific IgY measurement, ELISA plates were coated with 0.25 μg/ml concentration of the purified HA protein of A/Anhui/1/2013 (Sino Biological). 100 μl of serial dilutions of chicken sera was added to the plates and then 100 μl of HRP-conjugated goat anti-chicken IgY was added as a secondary antibody. For visualization, 100 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) liquid substrate was added to each well and the reaction was stopped by adding 50 μl of 3 N hydrochloric acid. Absorbance was measured at 450 nm. ELISA endpoint titers were defined as the highest serum dilutions at which the mean OD values of duplicate wells were >2-fold above the mean OD value plus 2 standard deviations for the sera from the sham control chickens [14]. Since unspecific reactions were observed for the sera from the empty vector-vaccinated animals, ELISA antibody titers induced by the recombinant vaccine were calculated by subtracting the value for the empty vector.

Two weeks after the second vaccination, chickens in each group were equally divided into two subgroups for i.n. challenge with virulent NDV (genotype VII, JS5/05) and HPAI H7N9 virus (A/chicken/Guangxi/GX100/2017, GX100) at the dose of 10^5 EID_{50}. The chickens were observed for 2 weeks post challenge (pc) for clinical signs and mortality. The survivors were bled and sacrificed at the end of challenge study. To monitor virus shedding, laryngotracheal and cloacal swabs were collected at day 3, 6 and 9 pc. Isolation of NDV and HPAI H7N9 challenge viruses from swab samples was performed by inoculating 9-day-old ECEs and the presence of virus was confirmed using HA test.

3. Results and discussion

3.1. Generation and characterization of the recombinant NDVs expressing H7N9 HA

The full-length cDNA clone of LX was constructed and the infectious virus (rLX) was successfully rescued using reverse genetics. The HA ORF and a chimeric HA cassette containing the HA ectodomain fused with the F TM/CTD were inserted into LX backbone between the P and M genes, respectively (Fig. 1A). Two recombinant viruses, rLXHA and rLXHAF, were successfully generated and the presence of the HA gene was confirmed by RT-PCR and sequencing (data not shown).

The expression of HA in two recombinant viruses in CEF was analyzed using Western blotting and IFA. Western blotting showed that at 24, 48 and 72 h post-inoculation (pi), the specific HA0 band with a molecular weight of ~66 kDa was detected in cells inoculated with two recombinant viruses (Fig. 1B). No HA protein was detected in cells inoculated with rLX and the cell control (Fig. 1B). In addition, when trypsin was added, only the precursor HA0 band was detected (data not shown). Further, to investigate whether the expression of the chimeric H7 HA containing the heterologous TM/CTD of the NDV F protein is associated with enhanced incorporation into NDV particles, the amounts of H7 HA protein and NDV viral protein from rLXHAF and rLXHA were measured by Western blotting. As expected, enhanced incorporation of the chimeric HA protein into NDV virions was seen for rLXHAF compared with that of rLXHA (Fig. 1C). The expression of HA was also confirmed in CEF inoculated with the recombinant viruses using IFA (Fig. 1D). After 10 successive passages in ECEs, the expression of HA in rLXHAF was detected (data not shown). These results demonstrated that the HA protein of H7N9 virus was expressed in the recombinant

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