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A novel genotype VII Newcastle disease virus vaccine candidate generated by mutation in the L and F genes confers improved protection in chickens

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ABSTRACT

Administration of vaccines combined with the good management and strict biosecurity is an effective way for Newcastle disease (ND) control. However, vaccine failure is continuously reported in some countries mainly because the antigenic difference between the used vaccine and field strains even they are of one serotype. Therefore, development of antigen-matched ND vaccines is needed to improve the vaccine efficacy in birds. In this study, we introduced four site mutations, K1756A, D1881A, K1917A and E1954Q, respectively, into the large protein gene of the virulent genotype VII Newcastle disease virus (NDV) G7 strain using reverse genetics technology. Four rescued NDVs were sharply attenuated for the pathogenicity in chickens. One of these mutants, E1954Q, was further manipulated by replacing the F cleavage site sequence of typical velogenic strains with that of the LaSota vaccine, resulting in a new mutant, G7M. Biological characterization showed that G7M was safe and genetically stable after serial passages in embryos and chickens. Vaccination of chickens with G7M induced a progressive elevation of the homologous antibodies and markedly higher $CD8^+$ T cell percentage, T cell proliferation and IFN-γ than LaSota. G7M conferred full protection against genotype VII NDV challenge, and more importantly, it effectively reduced the challenge virus replication and shedding in chickens. Together, our data suggest that G7M is a promising genotype VII vaccine candidate, and the novel attenuation approach designed in this study could be used to develop new antigen-matched NDV vaccines.

1. Introduction

Newcastle disease (ND) is a highly contagious disease in chickens, which can lead to huge economic losses to the poultry industry. At least four worldwide ND outbreaks have been documented since it was first identified in 1926 (Dimitrov et al., 2017, 2016). The causative agent of ND is a virulent strain of Newcastle disease virus (NDV), a member of the Paramyxoviridae family, which is enveloped, non-segmented, negative-stranded RNA virus. The NDV genome is about 15 Kb and encodes at least six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein (L).

All NDV strains belong to a single serotype. However, like all other RNA viruses, NDV is constantly evolving. Currently NDV strains have been grouped genetically into two distinct classes (class I and class II). Class I lentogenic (low virulence) NDVs are primarily isolated from wild aquatic birds and domestic poultry, while class II velogenic (highly virulent) viruses are predominant in domestic fowl and some wild birds. According to the characteristic of nucleotide sequence of the F gene, nine genotypes (1–9) in class I, and eighteen different genotypes (I–XVIII) in class II have been identified (Dimitrov et al., 2016; Shabbir et al., 2012). Among them, genotype VII NDVs have been involved in fatal infection to poultry since the early 1990s in Asia, Europe and Africa. Since 2000s, the genotype VII NDVs have gradually become the predominant strains in chicken flocks and have been evolved into different sub-genotypes (VIIa-i) (Dimitrov et al., 2016; Munir et al., 2012a, b; Qin et al., 2008; Wu et al., 2011).

Vaccination of flocks is considered an effective way to control ND around the world. An intensive vaccination program has been implemented in China for a long time. Immunization with live or inactivated commercial NDV vaccines (genotype I or II) has effectively reduced ND outbreaks. However, sporadic infections of velogenic NDVs

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in vaccinated flocks or backyard farms were reported frequently (Hu et al., 2009). Previous studies showed that the antigenic differences from classes and genotypes between vaccines and field strains were correlated to the protection efficacy in flocks (Miller et al., 2013, 2009; Miller et al., 2007). The vaccine failure in China was partly due to the antigenic variation of the currently circulated genotype VII NDV strains from the commonly used genotype I or II vaccine strains. In 2009, Hu et al. (Hu et al., 2009) generated an attenuated genotype VII ND vaccine candidate by replacing the F cleavage site sequence of the genotype VII ZJ1 strain with that of the LaSota vaccine using reverse genetics. This experimental vaccine conferred a better protection than LaSota against genotype VII NDV challenge. However, the genetic stability of this vaccine candidate and the vaccine-induced cellular immune response were not fully investigated.

In the present study, we developed an antigen-matched genotype VII NDV vaccine candidate (G7M) by introduced mutations into the L and F genes of the virulent genotype VII G7 strain (Liu et al., 2015) using reverse genetics technology. The vaccine safety and genetic stability and the induced immune responses and protection against genotype VII NDV challenge were evaluated in chickens. Our results suggest G7M is a promising genotype VII NDV vaccine candidate, and the novel attenuation approach could be used to develop other antigen-matched NDV vaccines.

2. Materials and methods

2.1. Cells, viruses and animals

BHK-21F cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, CA, USA). The viral stock of NDV G7 strain was prepared as described previously (Liu et al., 2015). The vaccinia virus expressing T7 RNA polymerase (VVT7) was kindly provided by Dr. Zhigao Bu as a gift (Harbin Veterinary Research Institute, CAAS). Specific pathogen free (SPF) chicken eggs and chickens were purchased from Merial Vital Laboratory Animal Technology Co., Ltd (Beijing, China). The protocol for animal studies was approved by the Committee on the Ethics of Animal Experiments of the institution (SYXK (G)-2015-0003).

2.2. Plasmid construction, site-directed mutagenesis and virus recovery

The full-length cDNA of G7 was cloned into a pBR322 vector flanking with a T7 promoter sequence and the hepatitis delta virus (HDV) ribozyme and the T7 terminator sequences (Ge et al., 2007, 2015; Zhao et al., 2014), and the resulting plasmid was designated pFL-G7. The NP, P, and L genes were cloned into the EcoRV site in pBluescript KS(+) (pBSK) plasmid, respectively, to support virus rescue. Site-directed mutagenesis was employed to introduce the L protein gene mutations, K1756A, D1881A, K1917A and E1954Q, respectively into pFL-G7 with specific primers (primer sequences available upon request), resulting in four full-length cDNA clones. Furthermore, the F cleavage site sequence (^{112}RRQKRF^{117}) in the pFL-G7(E1954Q) plasmid was replaced with that of LaSota (^{112}GRQGRL^{117}) as described in the previous study by Hu et al. (Hu et al., 2009). The resulting recombinant plasmid was designed as pFL-G7-E1954Q + F. The sequences of these recombinant plasmids were verified by sequencing (BGI, Beijing, China).

Virus rescue was performed as described in the previous studies (Estevez et al., 2007; Ji et al., 2017; Zhao et al., 2014). The rescued viruses were passaged by a three-round limited dilution purification in SPF chicken embryos for the viral stock preparation. Viral RNAs extracted from six rescued viruses, rG7, rG7-K1756A, rG7-D1881A, rG7-K1917A, rG7-E1954Q and rG7-E1954Q + F (G7M), were analyzed by RT-PCR and sequencing to confirm the presence of the designed mutations.

2.3. Biological characteristics of recovered viruses

The virulence of the recovered viruses was determined by the mean death time (MDT), the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) as described previously (Liu et al., 2015). Genetic stability of the G7M virus was assessed by serially passaging in 9-day-old SPF hen embryos for 15 times and SPF chickens for 5 times. The L and F genes in G7M at each passage were examined by RT-PCR and sequence analysis. Five 10-day-old SPF chicks were intramuscularly injected with 2 mL of G7M (5.62×10^9 EID_{50}/mL) for the safety test. The swabs from pharynx and cloaca were collected on days 2, 4, 6, 8, 10, 12 and 14 post-infection and the clinical signs were monitored until day 15 post-infection.

2.4. Immunization and challenge experiment

Forty-week-old SPF white Leghorn chickens were discretionarily allocated into three immune groups. Birds in control group (n = 10) were shame-vaccinated with 100 μL of phosphate buffer saline (PBS). Birds in G7M group (n = 15) and LaSota group (n = 15) were vaccinated with 100 μL of live G7M and LaSota (1.0×10^7 EID_{50}/mL), respectively, per bird via a combined intranasal and eye-drop route. Serum samples were collected via wing-web bleeding at 1, 2 and 3 weeks post-vaccination (pv). At three weeks pv, chickens were challenged intramuscularly with 10^6 EID_{50} of G7 viral stock in a 0.5-mL volume per bird. The tissues (spleen, bursa, liver, pancreas and brain) and swabs from throat and cloaca were collected from each chicken on days 2, 4 and 6 post-challenge (dpc) for virus isolation. The clinical signs of the rest chickens were observed daily until 10 dpc.

2.5. Antibody and cell-mediated immunity

Serological test: The haemagglutination inhibition (HI) test was performed by using 1% chicken red blood cells (RBCs) with 4 haemagglutination (HA) units of the NDV-specific antigen (G7M or LaSota) per well according to the standard protocol (Miller et al., 2009).

PBMC isolation and analysis by flow cytometry: At 1, 2 and 3 weeks pv, Peripheral blood mononuclear cells (PBMC) from ten chicken blood samples in each group were purified by Ficoll density gradient centrifugation following the manufacture's instruction (Biolite Biotech Co., Ltd, Tianjin, China). The purified PBMC were used for the $CD4^+$ and $CD8\alpha^+$ analysis by flow cytometry. One hundred microliters of PBMC (1.0×10^6 cells) were incubated with PE-labeled mouse anti-chicken $CD3^+$; FITC-labeled mouse anti-chicken $CD4^+$; Cy5-labeled mouse anti-chicken $CD8\alpha^+$ monoclonal antibodies (Southern Biotechnology, USA) for 30 min in dark. Then, the cells were washed twice with PBS containing 2% FBS and an aliquot of 1×10^4 cells per sample was analyzed quantitatively in a BD Accuri C6 flow cytometer using Cflow plus software (BD BioSciences, UK). Unstained cells were used as negative control to assess the background signals.

Lymphocyte proliferation assay: To analyze antigen-specific cellular proliferation at the indicated times, chicken PBMC were isolated at 1, 2 and 3 weeks pv. 1.0×10^6 PBMC were plated in 96-well plates and incubated at 37 °C, 5% CO_2 for 12 h. β-propiolactone(BPL)-inactivated G7 (10 μg/well) was added in triplicate wells for 48 h followed by adding CCK-8 (Biolite Biotech Co., Ltd, Tianjin, China) (10 μL/well) to each well. After incubated for another 2 h, reading was taken at 450 nm optical density (OD) on a microplate ELISA reader (Bio-Rad, USA). Results were expressed as the mean stimulation index (SI) as previously described (Gao et al., 2013).

IFN-γ in Sera by ELISA: The chicken sera from each group at the indicated times were separated by centrifugation and stored −20 °C for use. IFN-γ in sera was determined by using the commercial ELISA kit following the manufacturer's instructions (Abbexa, Cambridge, UK). The chicken IFN-γ concentration of the sample was interpolated from the standard curve.