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Phylogenetic characterization and virulence of two Newcastle disease viruses isolated from wild birds in China

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ABSTRACT

Wild birds are considered as a natural reservoir of Newcastle disease virus (NDV). However, there is no information about genotype IX NDV from wild birds, especially from *Columbiformes*. In this study, two genotype IX NDV viruses were isolated from wild birds. One was from Eurasian Blackbird, while the other was from Spotted-necked dove. After purification by plaque technique, complete genomes of both viruses were sequenced. Phylogenetic analysis of partial fusion (F) gene and complete genome indicated both strains belonged to genotype IX. Based on intracerebral pathogenicity index (ICPI), the virus from Eurasian Blackbird was velogenic virus, while the strain from Spotted-necked dove was lentogenic virus. However, both strains showed one of velogenic cleavage sites. In addition, the strain from Eurasian Blackbird showed greater replication ability and generated larger fusion foci in vitro than that of strain from Spotted-necked dove. Comparing all the corresponding protein sequences of both strains, there were only 9 different amino acid residues between them. Furthermore, after analysis of these differences, the information about lentogenic NDV with multi-basic cleavage site was presented.

1. Introduction

Since 1926, when the first Newcastle disease (ND) out broke, many countries have been reported this disease which causes enormous economic losses (Alexander, 2003). ND is caused by virulent strains of Newcastle disease virus (NDV) which is a member of genus Avulavirus in the family Paramyxoviridae (Mayo, 2002). The genome of NDV, is non-segmented, single-stranded, negative-sense RNA molecule with about 15 kb genome, encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein (L) (Alexander, 2003). During events of the transcription of the P gene RNA-editing generate V and W proteins (Peeters et al., 2004; Steward et al., 1993).

Based on some biological parameters, such as the mean death time (MDT) of chicken embryos infected with the minimum lethal dose of virus, the intracerebral pathogenicity index (ICPI) in one-day-old chickens and the intravenous pathogenicity index (IVPI) in six-week-old chickens, NDV strains are divided into high virulence (velogenic), intermediate (mesogenic) and low virulence (lentogenic) (Dortmans et al., 2011b). The precursor glycoprotein F0 is cleaved into F1 and F2 by wide host cell furin-like proteases, which is essential for viral virulence. F protein of lentogenic viruses with monobasic motifs (^{112}G-R/K-Q-G-R↓L^{117}) are cleaved by trypsin-like proteases in respiratory and intestinal tract, while virulent virus with multibasic motifs (^{112}R/G/K-R-Q/K-K/R-R↓F^{117}) are cleaved by furin-like proteases in whole body. Therefore, the virulence of stains can be predicted based on the cleavage site of F protein (Glickman et al., 1988; Peeters et al., 1999; Samal et al., 2011). However, some NDV strains, especially those from pigeon or dove, with the multibasic motif showed low virulence in chickens. The previous works indicated the virulence of some NDV strains of pigeon or dove origin could increase though passaging in chickens and the increase of virulence was due to some amino acid substitutes happened among proteins, especially appeared in RNP complex (Dortmans et al., 2011c; Fuller et al., 2007;). Moreover, Dortmans et al. (2010) proved the virulence of PPMV-1 enhanced when viral replication complex of PPMV-1 was replaced by that of virulent APMV-1. In addition, the virulence of NDV is also influenced by other factors, such as HN protein, V protein (Huang et al., 2003, 2004; Khattar et al., 2009; Panda et al., 2004; Yan et al., 2009). However, the virulence gap between these PPMV-1 strains and their progenies was based on artificial methods (Dortmans et al., 2011c; Fuller et al., 2007). There is little knowledge about factors that relates to virulence of natural NDV strains from pigeon or dove.

Recently, strains of NDV were divided into class I and class II which include I genotype and I- XVI genotypes based on F gene,

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respectively (Courtney et al., 2013; Diel et al., 2012a). In addition, According to phylogenetic analysis of complete genome or partial F gene sequence of NDV isolates, two major classes (class I and class II) were identified (Aldous et al., 2003; Miller et al., 2009; Miller et al., 2010). Class I viruses comprised at least nine genotypes, while Class II viruses consisted of genotypes I–XI (Maminiaina et al., 2010; Meng et al., 2012). Based on partial F gene sequence, genotypes VI and VII were further divided into nine (a–i) and five (a–e) subgenotypes, respectively (Chen et al., 2013; Munir et al., 2012). Except chickens, many species of birds, such as *Columbidae* (including pigeon and dove) tend to be infected by NDV (Guo et al., 2013; Kim et al., 2008; Meulemans et al., 2002). According to the phylogenetic tree, most of NDV strains from pigeon or dove were classified into genotype VI (Pchelkina et al., 2013), and a little information about other genotypes NDV from *Columbidae* was recorded (Diel et al., 2012c; Kim et al., 2008; Mase et al., 2009; Terregino et al., 2003; Tirumurugaan et al., 2011). Wild birds were considered the natural reservoirs of NDVs, mostly harbored lentogenic strains, and occasionally carry velogenic strains (Cai et al., 2011; Chen et al., 2013; Snoeck et al., 2013; Vidanovic et al., 2011; Xie et al., 2012). Studies on genetic diversity among strains of NDVs revealed that some of NDVs from wild birds were phylogenetically related with NDVs isolated from live-bird markets (Kaleta et al., 1985; Kim et al., 2007, 2012; Snoeck et al., 2013.

In this work, two NDV strains were isolated from wild birds (one was from Spotted-necked dove and another was from Eurasian Blackbird) in Shaanxi, China 2008. After complete genome sequencing, phylogenetic and biological characteristics analysis, we found both strains belonged to genotype IX, and one was velogenic, while another was lentogenic. Furthermore, we also analyzed differences of virulence between these strains in terms of their genomes and proteins.

2. Materials and methods

2.1. Viruses

In 2008, two NDV strains were isolated from wild birds (Eurasian Blackbird and Spotted-necked dove) in China. These strains were designed as Blackbird/China/08 (NDV-Blackbird) and SpottedDove/China/08 (NDV-Dove), respectively. To isolate viruses, the cloacae mucus and throat mucus collected from these birds and dissolved in PBS containing penicillin (1000 u/ml) and streptomycin (1000 u/ml). 200 μL solution was inoculated into allantoic cavity of nine-day-old specific pathogen-free (SPF) chicken embryos. Then, DF-1 cell was chosen to purified viruses via a plaque technique. Finally, using SPF nine-day-old chickens embryos propagated viruses. Virus stocks were stored at −80 °C.

2.2. Pathogenicity test

To evaluate the pathogenicity of two viruses, the intracerebral pathogenicity index (ICPI) was determined according to international OIE standards. 50 μl of allantoic fluid with HA titer >16 unites was diluted 10-fold in PBS without antibiotics and then inoculated one-day-old SPF chickens. At the same time, 50 μl PBS was inoculated one-day-old SPF chickens as control. The chickens were raised for 8 days, observed daily and scored 0, 1 or 2 to normal, sick or dead, respectively. Finally, calculation of ICPI was performed as described (OIE, 2004).

2.3. Animal inoculations

To assess whether these virus could cause disease in chickens, thirty three-week-old SPF chickens were divided into three groups. Each chicken in the first group was inoculated with NDV-Blackbird suspensions including 10^5 50% embryo infectious dose (EID_{50}). Half of suspension was injected though conjunctival sac and half though choanal cleft (Diel et al., 2012b). Each chicken in the second and the third group was inoculated with NDV-Dove suspensions including 10^5 EID_{50} and 100 μl PBS, respectively. All chickens were raised for 10 days and observed daily.

2.4. The growth kinetics of viruses

DF-1 cells were cultured in the 24 well plate. Each DF-1 cell was inoculated with NDV-Blackbird or NDV-Dove at an m.o.i. of 0.001. After adsorption for 1 h at 37 °C, 5%CO_2, shaking once each 20 min, the liquid was replaced by DMEM with 2% fetal calf serum. 100 μl supernatant was collected at every 8h in 72 h of post-inoculation. At the same time, 100 μl DMEM with 2% fetal calf serum was added. Then, the viral titers were determinated by plaque assay. Briefly, 10-fold serial dilutions of each supernatant were performed in DMEM without fetal calf serum. The confluent monolayer of DF-1 cells was inoculated with 10^{-1}–10^{-7} dilutions of supernatant. After adsorption for 1 h at 37 °C, 5% CO_2, shaking once each 20 min, the liquid was aspirated. Each monolayer of cell was overlaid with 500 μl DMEM including 2% fetal calf serum, 1% methylcellulose and cultured at 37 °C, 5% CO_2. After three days, DMEM was aspirated and cells was slightly washed with D-Hanks and fixed at room temperature for 15 min with 1 ml PBS with 5% formaldehyde. Finally, Plaques were stained with crystal violet.

2.5. Fusion phenotype of virus

To assess fusion phenotype of these two isolates, the monolayer of DF-1 cell was inoculated with NDV-Blackbird or NDV-Dove at an m.o.i. of 0.01, respectively. At 24 h post-inoculation, the microscope was used to photograph fusion foci.

2.6. Primer design, RT-PCR and sequencing

The complete genomes of NDV-Blackbird and NDV-Dove were amplified using 10 pairs of specific primers which were designed according to NDV nucleotide sequences with GenBank accession numbers HQ317394, FJ436302, FJ436303, FJ436304, FJ436305, FJ436306.

Trizol reagent (TAKARA, Japan) was chosen to extract totally viral RNA. Reverse transcription was conducted at 42 °C in 20 μL reaction volume including 100 ng total RNA, 25 μM Random Primers, 100 U MLV (TAKARA, Japan), 20 U RNase Inhibitor, 10 mM dNTPs. The PCRs were performed in a thermocycler in 25 μL reaction volume that contained 2 μL cDNA, Forward Primer (10 μM), Reverse Primer (10 μM), 2.5 mM dNTPs, 2.5 units TransS-tart Fast-Pfu DNA Polymerase (TransGenBiotech, China). Reactions were performed according to the following protocol: 95 °C for 2 min, followed by 40 cycles 95 °C for 20 s, 52, 53, 54, 55, or 56 °C for 30 s, 72 °Cfor 2 min, and 1 cycle 72 °C for 5 min.

The PCR products were purified using a Gel Extraction kit and cloned into PMD19-T vector (TAKARA, Japan). Then, being transformed into calcium-competent Escherichia coli DH5αcells (TransGenBiotech, China). The plasmids which contained each PCR fragments were purified and sequenced. To ensure the accusation of sequence, three clones of each fragment were sequenced at least on an ABI 3730 genetic analyzer (Beijing Genomics Institute, China). The sequences of complete genome of Blackbird/China/08 and SpottedDove/China/08 were submitted to GenBank under accession numbers KC934169–KC934170.