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Genotypic and pathotypic characterization of Newcastle disease virus isolated from racing pigeons in China

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ABSTRACT A Newcastle disease virus (NDV) isolated from an outbreak in racing pigeons in China was characterized in this study. Complete gene of the NDV isolate was sequenced and phylogenetic analysis. Pathogenicity experiment was carried out in pigeons, chickens, and ducks. Phylogenetic analysis revealed that the strain clustered with the Class II viruses, has highly phylogenetically similar to NDV strains isolated from pigeons in China, but was distant from the viruses prevalence in chickens and vaccine strains used in China. The deduced amino acid sequence of the cleavage site of the fusion (F) protein confirmed that the isolate contained the virulent motif $^{112}$RRQKR$^{117}$ at the cleavage site, but it caused no appearance disease in chickens and ducks. However, the isolate had virulence in pigeons, resulting in severe nervous signs and highly mortality. Pigeons were considered as a potential source of NDV infection and disease for commercial poultry flocks. Therefore, new vaccines to prevent the NDV infection in the pigeon flocks should be developed as soon as possible, and strict biosecurity measures should be taken to reduce the risk of pigeon Newcastle disease outbreaks.

Key words: Newcastle disease virus, racing pigeon, phylogenetic analysis, pathology

INTRODUCTION Newcastle disease is a highly contagious viral disease of poultry and other bird species caused by the Newcastle disease virus (NDV), a member of the genus Avulavirus in the family Paramyxoviridae (Mayo, 2002; Nagai et al., 1976). The genome of NDV is a nonsegmented, single-stranded, negative-sense RNA molecule of approximately 15,186 nucleotides (nt) that contains 6 genes encoding the 6 structural proteins (from the 3′ to 5′ terminus): nucleoprotein, phosphoprotein, matrix, fusion (F), hemagglutinin-neuraminidase (HN), and the large protein (Chambers et al., 1986). NDV strains can be classified as high virulence (velogenic), intermediate (mesogenic), or low virulence (lentogenic) based on various 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 1-day-old chicks, and the intravenous pathogenicity index (IVPI) in 6-week-old chickens. The primary molecular determinant for NDV pathogenicity is the amino acids of the F protein cleavage site (Nagai et al., 1976; Ogasawara et al., 1992).

NDV has a wide host range with most orders of birds that reported to have been infected by the virus. More commonly affected species include chickens, turkeys, pigeons, and ducks (King, 1996; Lana et al., 1988; Zhang et al., 2011). Variant strains of NDV are associated with infections of pigeons, known as pigeon paramyxovirus Type 1 (PPMV-1), which can be characterized by unique monoclonal-antibody-binding profiles (King, 1996; Lana et al., 1988). After its first emergence in the Middle East (late 1970s) (Lister et al., 1986), PPMV-1 spread worldwide (Alexander, 1985; Lister et al., 1986; Vindevogel and Duchatel, 1988), and several Newcastle disease outbreaks in chickens have been attributed to PPMV-1 (Alexander et al., 1984). Therefore PPMV-1 presents which makes it a real threat to the poultry industry.

The frequent isolation of PPMV-1 from pigeons across China necessitates an assessment of the risk potential of these viruses causing disease in poultry (Liu et al., 2006; Yu et al., 2001). In the present study, a PPMV-1 isolate from outbreaks in racing pigeons in China was pathotypically and genotypically characterized.
MATERIALS AND METHODS

Virus Isolation and Identification

In February 2012, a flock of racing pigeons displayed severe nervous signs in Shandong Province of China. Tracheal and cloacal swabs were collected from the diseased pigeons. Swabs samples were maintained in transport medium containing antibiotics and kept at 4°C during transportation to the laboratory. Samples were centrifuged for 10 min at 3,000 x g at 4°C and 0.2 mL supernatant was used to inoculate the allantoic cavities of 9- to 11-day-old specific pathogen-free (SPF) embryonated chicken eggs, and incubated at 35°C for 72 h. Allantoic fluids were then harvested and tested for haemagglutinin (HA) activity. Positive isolates were characterized by HA inhibition (HI) tests using NDV, H5, H9 subtype avian influenza virus, and egg drop syndrome virus antibodies. The pigeon virus showed HA activity, which was inhibited by NDV antibody, but revealed no cross-reaction to avian influenza virus and egg drop syndrome virus antibodies in HI test. The strain was designated Pigeon/China/SD039/2012. To purify the virus, 10-fold dilutions of a virus stock are prepared, and 0.1-mL aliquots are inoculated onto baby hamster kidney cell monolayers. After an incubation period, the monolayers are covered with a nutrient agar. After 48 h, each infectious particle produces a plaque. The virus was purified 3 times using a plaque technique before being propagated in the allantoic cavities of 10-day-old SPF embryonated chicken eggs. Virus stocks were stored at −80°C until use.

Animals

To investigate the biological characteristics and pathogenicity of the NDV isolate, SPF chickens, NDV antibody-negative pigeons, and NDV antibody-negative Beijing ducks were used. All animal research was approved by Beijing Administration Committee of Laboratory Animals under the leadership of the Beijing Association for Science and Technology; the approve identifier is SYXK (Beijing) 2007-0023.

Assessment of the Biological Characteristics of the Isolate

The pathogenic potential for the isolated viruses was evaluated using standard assay methods to determine the MDT in 10-day-old chick embryos, the ICPI in 1-day-old chickens, and IVPI in 6-weeks-old chickens. For MDT test, virus-infective allantoic fluid was diluted 10-fold in saline from 10^{-6} to 10^{-10}. For each dilution, 5 eggs were used to inoculate 0.2 mL diluted virus via the allantoic cavity. After 24 h, the eggs were examined for mortality at 2-h intervals for 120 h. The MDT was the mean time in hours for the lowest dilution to kill all eggs of that dilution. To test the 50% embryo infectious doses (EID50) of the isolate, virus-infective allantoic fluid was diluted 10-fold in saline from 10^{-1} to 10^{-8}. For each dilution, 3 eggs were used to inoculate 0.1 mL diluted virus via the allantoic cavity. After 72 h, virus infection was assessed by HA assay of egg allantoic fluid. EID50 was calculated using the method of Reed and Muench (1938).

Phylogenetic Analysis and Molecular Characterization

Viral RNA was extracted using RNeasy Mini Kit (Qiagen, Chatsworth, CA). reverse transcription and PCR was performed under standard conditions as previously described (Liu et al., 2006). Primer sequences were used to generate for the overlapping subgenomic cDNA fragments and for genome sequencing are available upon request. PCR products were purified using the QIA quick PCR Purification Kit (Qiagen, Valencia, CA), and then sequenced by the Beijing BGI-GBI Biotech Co., Ltd. Reference NDV genomic sequences were obtained from GenBank, and these included current vaccine strains, typical prevailing isolates in China, and the reference strains for each known NDV genotype. These NDV sequences and the complete coding sequences of the NDV isolate were aligned and analyzed using the ClustalW multiple alignment algorithm in the MegaAlign program of the DNASTAR software suite (version 3.1; DNASTar, Madison, WI). Phylogenetic analysis was performed using software MEGA 4.1. (DNASTar Inc.) by the distance-based neighbour-joining method (1,000 replicates for bootstrap).

Animal Experiments

Twenty 1-month-old NDV antibody-negative pigeons, twenty 6-weeks-old SPF chickens, and twenty 2-weeks-old NDV antibody-negative Peking ducks were randomly divided into 2 groups, respectively. The challenge procedure was birds were inoculated via the intranasal route with 10^6 EID50 of the virus in a 0.2 mL volume or 0.2 mL PBS as a mock-infected control. All birds were clinically monitored every day for signs of disease (e.g., disheveled feathers, lethargy, fever or paralysis) and mortality. Dead birds were immediately necropsied for the determination of gross lesions, and their tissue samples (trachea, lung, brain, spleen, small intestine, proventriculus, and kidney) were collected and fixed in 10% neutral buffered formalin for histological observation. In addition, antigens of NDV present in the tissue of virus inoculation birds were further examined using immunohistochemistry, which employed an HN-protein-specific mouse monoclonal antibody. Serum samples were collected from all birds before inoculation and at 14 dpi for NDV-specific antibody detection using an HI test with 1% chicken red blood cells.
**RESULTS**

**Biological Characteristic Assessment of the SD069 Isolate**

Initial biological characterization of the isolate strain SD069 included MDT, ICPI, and IVPI were determinations. The virus exhibited MDT of 96 h and the ICPI values were 0.6 for the moderately virulent isolate. The virus did not produce obvious signs or lesions in all the 10 chickens within 10-d obvious period, indicating the IVPI was 0.

**Sequence and Phylogenetic Analysis**

The nucleotide sequence data were deposited into the Genbank database and the accession numbers was KP861633. The total length of the strain was 15,192 nt. Compared with the NDV Lasota strain, the isolated strain bears a 6-nt insertion (TCCCCCA) in positions 1,647 to 1,648 nt of the nucleoprotein gene. Proteolytic cleavage site motifs (Residues 112 to 117) for the F0 protein in the isolate were analyzed. The strain has a virulent motif (\texttt{RRQKRF}) composed of multibasic amino acids at the precursor F (F0) cleavage site. This motif is commonly found in strains that are highly virulent in chickens (Collins et al., 1993; Rui et al., 2010). Phylogenetic analysis was conducted based on complete nucleotide sequences. Nucleotide sequence data of 32 NDV reference strains obtained from the GenBank database were used for comparison. As shown in Figure 1, the isolated strain clustered within Class II NDVs, and phylogenetically close to Genotype VI NDVs. The strain was highly...
similar to pi/CH/LLN/110713 (99.78%), a PPMV-1 strain isolated in China. However, the isolated strain was genetically distinct and phylogenetically distant from the vaccine strains used in China (B1, LaSota, and Mukteswar). Genomic blast analysis revealed that the isolated strain had sequence homologies of 80.9, 80.8, and 84.2%, respectively, with Strain B1, LaSota, and Mukteswar.

**Animal Experiments**

All the pigeons infected with SD069 exhibited slight depression and head tremor at 2 dpi. At 4 dpi, all SD069 infected pigeons were severely depressed and had ruffled feathers. Additionally, some had severe nervous signs such as incoordination accompanied by leg paralysis (Figure 2A). By 5 dpi, one infected pigeon was dead, and altogether a total of 5 pigeons died within the 14-d observation period. At necropsy, brain hyperemia and hemorrhage were found (Figure 2B), and severe hemorrhage could be seen in the lung (Figure 2C). The gastrointestinal tract was empty in all dead pigeons, and multifocal hemorrhages were observed in the mucosa of the muscular stomach (Figure 2D) and small intestine (Figure 2E). Spleens were enlarged and mottled. Hemorrhaging of liver, trachea, and kidney could also be seen occasionally. No obvious clinical signs were seen in mock-infected pigeons within the 2-wk period.

Histopathologically, hemorrhage, necrosis, and inflammation were observed in multiple organs. Necrosis of mucous epithelial cells were seen in the trachea (Figure 3A). Severe bronchopneumonia was observed in the lungs. These changes were characterized by interstitial edema and extensive of lymphocytes, neutrophils, and infiltration of plasma cells around the bronchiolitis (Figure 3B). Pathological changes in the glandular stomach were hemorrhage and atrophy of the proventriculus papillae, with mucus on the surface of papillae. Necrosis of the mucosal epithelia of the proventriculus was also observed. Lesions in the small intestine of pigeons infected with SD069 virus showed signs of enteritis, characterized by broken villi, dropout of epithelium, and numerous inflammatory cells infiltrates (Figure 3C). The cardiac muscle fibers were elongated and thinned with unclear transverse striation, and some myocardial cells showed evidence of slight granular degeneration (Figure 3D). Capillaries in renal interstitium were congested, and typical granular degeneration occurred in the epithelial cells of convoluted tubule, some epithelial cells swelled and fell off to lumen (Figure 3E). A large number of lymphocytes were observed disrupting and disappearing in the spleen, and fibrin deposits were present in necrotic areas of the spleen (Figure 3F). The most remarkable histological finding was observed in the brain. Congestion in the blood vessels, endothelial cell hyperplasia, and central chromatolysis in the cerebral neurons were observed in all the virus-infected pigeons. Mononuclear inflammatory infiltrations in the neuropil, glial cell infiltration, and perivascular cuffing were also observed in some pigeons (Figure 3G, H). From immunohistochemistry, viral antigen was detected in bronchial epithelial cells (Figure 3I), neurons, and gial cells (Figure 3J). In comparison, all tissues samples

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**Figure 2.** Gross photographs in visceral organs from pigeons after intranasal inoculation with NDV SD069. (A) Pigeons infected exhibited severe nervous signs; (B) Brain hyperemia and hemorrhage; (C) Severe hemorrhage in the lung; (D) Multifocal hemorrhages in the mucosa of muscular stomach; (E) Multifocal hemorrhages in the mucosa of small intestine.
Figure 3. Histopathology and immunohistochemical detection of NDV antigens on tissues from pigeons infected with NDV SD069. (A) Dropout and necrosis of mucous epithelial cells in the trachea (arrow); (B) Severe bronchopneumonia in the lung; (C) Dropout of broken villi, epithelium and numerous inflammatory cells infiltrates in the small intestine (arrow); (D) Cardiac muscle fibers showed granular degeneration (arrow); (E) Typical granular degeneration in the epithelial cells of convoluted tubule; (F) A large number of lymphocytes disrupting in the spleen, and fibrin deposits in necrotic areas; (G) and (H) Mononuclear cell infiltrates in the perivascular spaces (arrow) and neuronophagia (arrow); (I) Influenza virus antigen (arrow) in the epithelial cells of the bronchioles; (J) Influenza virus antigen (arrow) in neurons and glial cells.
strains. Furthermore, other protein precursors also play a role in characterizing the virulence and pathogenicity of NDV strains examined in the study contain a polybasic cleavage site motif, but it caused no appearance disease and had middle ICPI in chickens. This indicates that the PPMV-1 strain had a genome size of 15,192 nt, has highly similar with PPMV-1 strains isolated in China, but was phylogenetically distant from the virus prevalence in chickens and vaccine strains used in China. This confirmed that the origin of this PPMV-1 was pigeon. Based on the current definition of the World Organization for Animal Health an NDV strain that either has an ICPI 0.7 or carries a typical velogenic amino acid motif at the F protein cleavage site can be classified as velogenic. However, the F proteins of the PPMV-1 strains examined in the study contain a polybasic cleavage site motif, but it caused no appearance disease and has middle ICPI in chickens. This indicates that the virulence of NDV strains can be qualified rather than quantified by the analysis of cleavage site motifs, and that pathogenicity tests such as MDT and ICPI cannot be replaced by analysis of the cleavage site to precisely characterize the virulence and pathogenicity of NDV strains. Furthermore, other protein precursors also play a role in the virulence of NDVs (Dortmans et al., 2011).

In pigeon pathogenicity experiments, the most remarkable and consistent histopathologic changes were observed in the brain, and abundant viral antigens were observed in the brain of PPMV-1-infected birds. Therefore, the virus tropism for brain and the brain lesions account for the severe nervous signs and highly mortality observed in the racing pigeons flock. Although this PPMV-1 strain caused no appearance disease in chickens and ducks, the virus could effectively infected of the poultry as evidence of seroconversion, and previous investigators demonstrated that PPMV-1 could enhance virulence for chickens when serially passage through chickens (Dortmans et al., 2011; Kommers et al., 2003).

Another concern regarding PPMV-1 is its ability to spread from infected pigeons to nearby chickens. As racing pigeons have free-flying characteristics, they come into frequent contact with wild birds and domestic poultry. In one study, the contact infection rate of 2 different PPMV-1 strains in 4-week-old SPF chickens reached 100% (Kissi, 1988). If these PPMV-1 strains allowed circulating among chickens, they could evolve to have a greater disease-causing capability. The potential for such an occurrence has already been realized for NDV. The 1998 to 2000 NDV outbreaks reported in Australia, with clinical disease and mortality observed among commercial chickens, were caused by a virulent NDV strain that evolved from a low-virulence strain that was apparently circulating among chickens for a long time (Gould et al., 2001).

Vaccination has been widely used for prevention and control of the PPMV-1 disease in many countries including China, but the disease is still enzootic in pigeons in China (Liu et al., 2006; Yu et al., 2001). The most commonly used live vaccines LaSota and Clone-30 were close to Genotype II, while the prevailing PPMV-1 strains belong to Genotype VI. There are significant differences between the prevailing NDV strains and the current vaccine strains in their biology, serology, and genetics, which might be considered as the reasons for the Newcastle disease outbreaks in pigeons in recent years. On the other hand, the nature of racing pigeon sport requires that birds from different breeders and different regions are brought together to compete in distance races. The gathering of birds and transportation stress often causes the vaccination failure.

In summary, we isolated a PPMV-1 from a free-living racing-pigeon flock and we demonstrated that the PPMV-1 caused severe lesions among infected pigeons, mostly affecting the brain. Although the virus did not cause disease in chickens and ducks, the virus could effectively infect and replicate in these poultry. Pigeons were considered seriously as a potential source of NDV infection and disease for commercial poultry flocks. Therefore, new vaccines to prevent the PPMV-1 infection in the pigeon flocks should be developed as soon as possible, and strict biosecurity measures should be taken to reduce the risk of pigeon Newcastle disease outbreaks.

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