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Identification and pathotypical analysis of a novel VIk sub-genotype Newcastle disease virus obtained from pigeon in China

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ABSTRACT

Newcastle disease virus (NDV) can lead to a devastating disease to various avian species including pigeons. Genotype VI NDV is a major cause of Newcastle disease (ND) in *Columbiformes* (i.e. pigeons and doves). Here, we analyzed the genetic diversity of genotype VI based on the complete F gene sequences of 2 pigeon-origin isolates in northeastern China in 2015, along with 238 genotype VI strains available in GenBank. The phylogenetic tree and evolutionary distances revealed that the 2 new isolates were clustered into a new sub-genotype herein proposed as VIk. Although the 2 isolates contain the ^{113}RQKRF^{117} cleavage site, a feature generally associated with virulent NDV strains, the values of ICPI and MDT showed lentogenic characteristics. The challenge experiment demonstrated that the isolate Pigeon/China/Jilin/NG05/2015 was pathogenic to pigeons, causing lesions in multiple tissues and organs. The emergence and spread of the sub-genotype VIk viruses illustrated that the genotype VI NDV was undergoing evolutionary changes. It is necessary to pay close attention and carry out epidemiological surveillance in pigeons.

1. Introduction

Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus 1 (APMV-1) of the genus *Avulavirus* belonging to the family *Paramyxoviridae* (Afonso et al., 2016). ND is devastating to a wide variety of avian species including *Columbiformes* and is regarded as a constant threat to most birds reared domestically (Alexander, 2011). ND virus (NDV) has a negative-sense, single-stranded RNA genome, which contains 6 genes coding for nucleocapsid (NP) protein, phosphoprotein (P), matrix (M) protein, fusion (F) protein, haemagglutinin-neuraminidase (HN), and large polymerase (L) protein (Aldous et al., 2003).

Genetic analysis of the complete F gene nucleotide sequences has recently been proposed as the basis for a unified nomenclature and classification system for assigning NDV isolates (Diel et al., 2012). The classification criteria are as follows. Firstly, the genotype or sub-genotype should be supported by the phylogenetic topology. Secondly, the bootstrap values at the defining node of the genotype or sub-genotype should be >60%. Thirdly, the evolutionary distances between genotypes are not less than 0.1, while for sub-genotypes, the values should be between 0.03 and 0.1. Additionally, new genotypes or sub-genotypes can be designated only when the complete F gene sequences of at least 4 independent isolates without a direct epidemiologic link are available (Diel et al., 2012). The classification system allows researchers to objectively name new genetic groups. Based on these criteria, NDV is divided into 2 classes (I and II), of which class I is composed of only 1 genotype (genotype 1) and class II is divided into 18 genotypes (I–XVIII) (Courtney et al., 2013; Czeglédi et al., 2006; Diel et al., 2012; Snoeck et al., 2013b).

Viruses of genotype VI are considered as the major cause of ND in *Columbiformes*, which includes numerous species of wild and domestic pigeons and doves (Chong et al., 2014; Dimitrov

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et al., 2016). Pigeons were considered as a potential source of NDV infection for commercial poultry flocks (Alexander, 2011). It was reported that the use of feed that had been contaminated by infected pigeons had led to ND outbreaks in unvaccinated chickens (Alexander et al., 1984), which emphasizes the importance of continuous NDV surveillance in pigeons. In this study, two genotype VI NDV isolates from diseased pigeon flocks in Jilin province of China in 2015 were genetically analyzed based on the complete F and HN gene sequences. To further elucidated the characteristics of the isolates, clinicopathologic assessments in pigeons were also conducted.

2. Materials and methods

2.1. Ethics statement

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocols for animal studies were approved by the Committee on the Ethics of Animal Experiments of Jilin University (approval numbers 2015042715-4 for chicken eggs, 2015060612-3 for chickens, and 2015092215-2 for pigeons).

2.2. Virus isolation and identification

The NDV strains were obtained from diseased pigeon flocks in Jilin province of China in 2015 and isolated through the inoculation of 10-day-old embryonating specific pathogen-free (SPF) chicken eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) (Oie, 2008). The allantoic fluids were harvested and analyzed for the existence of NDV by haemagglutination (HA) and haemagglutination-inhibition (HI) assays using NDV-specific antiserum (Harbin Weike Biotechnology Development Company, Harbin, China). The isolates were then plaque-purified for three times on primary chicken embryo fibroblasts and amplified by passaging in 10-day-old SPF chicken embryos. Virus titers were expressed as 50% embryo infectious dose (EID_{50}/ml) by the end-point method of Reed and Muench. The virus-containing allantoic fluids were harvested and stored at −80 °C until use.

2.3. Pathogenicity tests

Intra-cerebral pathogenicity index (ICPI) tests in 1-day-old SPF chickens (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) and the mean death time (MDT) in 10-day-old embryonating SPF chicken eggs were performed according to the Office International des Epizooties manual of standards (Oie, 2008).

2.4. RNA isolation, RT-PCR and sequencing

Virus RNA was extracted from infective allantoic fluid with Trizol (Invitrogen, San Diego, USA) according to the manufacturer's instructions. In order to obtain the complete F gene sequences, a pair of primers was designed based on the NDV F nucleotide sequences from GenBank: 5′-CATTGCCAAGTACAATCCCTT-3′ (upstream primer), 5′-TAACGCAACTTTGCTGACTGC-3′ (downstream primer). Another pair of primers was designed to obtain the complete HN gene sequences: 5′-ACTAGGCTTCACAACATC-3′ (upstream primer), 5′-GATCACATCAGCATTGTC-3′ (downstream primer). The RT-PCR was performed using a one-step RT-PCR kit (Takara, Dalian, China) according to the manufacturer's instructions. The purified PCR product was ligated into pMD18-T vector and then transformed into *Escherichia coli* DH5α. The clone was sequenced by Sangon Biotech (Shanghai, China).

2.5. Phylogenetic analysis and genotype classification

A preliminary phylogenetic analysis was performed based on the complete F gene (1662 nt) sequences of 1358 isolates (2 sequences acquired in this study and 1356 sequences available in GenBank as of 12/21/2016) to screen the genotype VI virus strains (data not shown). A further phylogenetic analysis based on 240 F gene sequences (2 sequences acquired in this study and 238 sequences available in GenBank) of genotype VI NDV strains was conducted to show genetic diversity. Analysis of the best-fit substitution model was performed using MEGA5, and the goodness-of-fit of each model was measured by corrected Akaine Information Criterion (AICc) and Bayesian Information Criterion (BIC) (Tamura et al., 2011). The final tree was constructed using the General Time Reversible (GTR) model with a discrete gamma distribution (+G) allowing for invariant sites (+I) (Nei and Kumar, 2000). The F gene sequences used for the phylogenetic reconstruction were also used to infer the evolutionary distances within and between sub-genotypes. The evolutionary history was inferred by using the maximum likelihood methods (Tamura and Kumar, 2002), with statistical analysis based on 500 bootstraps.

The two complete HN gene sequences generated in this study, along with 155 complete HN gene (1716 nt) sequences available in GenBank (12/21/2016) were used for the phylogenetic analysis. Determination of the best-fit substitution model was performed using MEGA5, and the goodness-of fit for each model was measured by AICc and BIC (Tamura et al., 2011). The GTR+G+I model of nucleotide substitution was selected for the construct of the phylogenetic tree.

2.6. Clinicopathologic assessment in pigeons

A total of 20 clinically healthy pigeons, hatched in captivity, were used in the present study. All the pigeons were lack of NDV-specific HI antibodies and negative in RT-PCR for the presence of NDV in cloacal swab samples. The twenty 1-month-old pigeons were randomly divided into 2 groups. One group of 10 pigeons were inoculated with Pigeon/China/Jilin/NG05/2015 via the intranasal route with 10^6 EID_{50} of the virus in a 0.2 mL volume. The negative control group was inoculated with 0.2 mL phosphate-buffered saline (PBS). Pigeons were housed in negative-pressure isolators and were provided food and water *ad libitum*. All birds were clinically monitored every day for signs of disease and mortality. Two pigeons in each group were sacrificed on 5 day postinoculation (dpi) and tissue samples (trachea, lung, brain, liver, spleen, small intestine, pancreas, and kidney) were collected and fixed in 10% neutral

Table 1
Two pigeon NDV isolates characterized in this study.

Strains	F cleavage site	Genotype	ICPI[a]	MDT (h)[b]	GenBank accession no.	
					F gene	HN gene
Pigeon/China/Jilin/DH09/2015	RQKRF	VIk	0.61	98	KU527559	KU527562
Pigeon/China/Jilin/NG05/2015	RQKRF	VIk	0.68	93	KU527560	KU527563

[a] Values 0.7 or greater are identified as virulent.
[b] Mean death time (MDT) less than 60 h is indicative of a virulent NDV strain.