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Molecular and antigenic characteristics of Newcastle disease virus isolates from domestic ducks in China

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ABSTRACT

Newcastle disease (ND) is one of the most devastating diseases to the poultry industry. The causative agents of ND are virulent strains of Newcastle disease virus (NDV), which are members of the genus *Avulavirus* within the family *Paramyxoviridae*. Waterfowl, such as ducks and geese, are generally considered potential reservoirs of NDV and may show few or no clinical signs when infected with viruses that are obviously virulent in chickens. However, ND outbreaks in domestic waterfowl have been frequently reported in many countries in the past decade. In this study, 18 NDV strains isolated from domestic ducks in southern and eastern China, between 2005 and 2013, were genetically and phylogenetically characterized. The complete genomes of these strains were sequenced, and they exhibited genome sizes of 15,186 nucleotides (nt), 15,192 nt, and 15,198 nt, which follow the "rule of six" that is required for the replication of NDV strains. Based on the cleavage site of the F protein and pathogenicity tests in chickens, 17 of our NDV isolates were categorized as lentogenic viruses, and one was characterized as a velogenic virus. Phylogenetic analysis based on the partial sequences of the F gene and the complete genome sequences showed that there are at least four genotypes of NDV circulating in domestic ducks; GD1, AH224, and AH209 belong to genotypes VIId, Ib, and II of class II NDVs, respectively, and the remaining 15 isolates belong to genotype 1b of class I NDVs. Cross-reactive hemagglutination inhibition tests demonstrated that the antigenic relatedness between NDV strains may be associated with their genotypes, rather than their hosts. These results suggest that though those NDV isolates were from duck, they still don't form a phylogenetic group because they came from the same species; however, they may play an important role in promoting the evolution of NDVs.

1. Introduction

Newcastle disease (ND) is a highly contagious viral disease that causes serious economic damage to the poultry industry (Alexander and Senne, 2008). The causative agents of ND are virulent strains of Newcastle disease virus (NDV), also known as avian paramyxovirus type 1 (APMV-1), which are members of the genus *Avulavirus* within the family *Paramyxoviridae* (Mayo, 2002). As a negative-sense, single-stranded, non-segmented, enveloped RNA virus, the NDV genome encodes six structural polypeptides: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin–neuraminidase (HN), and RNA-dependent RNA polymerase (L) (de Leeuw and Peeters, 1999). In addition, the P gene generates the V and W proteins via an RNA-editing mechanism (Steward et al., 1993). On the basis of genomic size and sequence analysis of the F gene, NDV strains can be separated into two clades, class I and class II, within a single serotype (Czeglédi et al., 2006). Class I viruses are comprise a single genotype and class II viruses further divided into 18 genotypes (Diel et al., 2012; Snoeck et al., 2013). Class I strains, with a genome size of 15,198 nucleotides (nt), are frequently isolated from wild birds and live-bird markets (LBMs), and most of them are avirulent (Czeglédi et al., 2006; Kim et al., 2007a,b). Class II strains include most virulent NDVs (Czeglédi et al., 2006), of which genotypes I to IV represent early sub-lineages with a genome size of 15,186 nt, whereas recent NDV isolates, genotypes V to IX, have a genome size of 15,192 nt (Czeglédi et al., 2006; de Leeuw and Peeters, 1999; Huang et al., 2004).

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NDVs have been categorized into three pathotypes: lentogenic, mesogenic, and velogenic. Velogenic strains could be further classified into viscerotropic velogenic and neurotropic velogenic strains. Both mesogenic and velogenic NDV are considered virulent by the World Organization for Animal Health (OIE, 2008). The pathogenicity of NDV isolates can be assessed by *in vivo* tests, including the mean death time (MDT) in 9-day-old specific pathogen-free (SPF) embryonated chicken eggs, the intracerebral pathogenicity index (ICPI) in 1-day-old chickens, or the intravenous pathogenicity index (IVPI) in 6-week-old chickens (OIE, 2008). The primary molecular determinant for NDV virulence is related to the fusion protein cleavage site and the ease by which cellular proteases cleave the fusion protein into F1 and F2 polypeptides (Collins et al., 1993; de Leeuw et al., 2003; Kianizadeh et al., 2001; Panda et al., 2004).

To date, ND has spread throughout the world (Spradbrow, 1988), and it is able to infect over 240 species from 27 of the 50 orders of birds (Kaleta and Baldauf, 1988). ND has been endemic in China since it was first described in 1946 (Liu et al., 2003). Genotype VII NDV viruses, the predominant circulating genotype in China, may have been responsible for disease outbreaks in chicken flocks during the past decade (Liu et al., 2003). Waterfowl are considered to be potential reservoirs of NDV, and they may show few or no clinical signs in response to NDV strains that are markedly virulent for chickens (Lee et al., 2009). However, ten representative isolates of NDVs obtained from outbreaks in waterfowl with severe clinical signs have been reported in China since 1997 (Jinding et al., 2005). In addition, it was reported that the mortality varied from 7% to 67% when four duck breeds were challenged with a virulent NDV isolated from a white Muscovy duck, implicating that breed of duck used for various pathogenicity indices could make a difference (Shi et al., 2011).

To characterize NDV strains in ducks, 18 NDV strains that were isolated from domestic ducks in southern and eastern China between 2005 and 2013 underwent genomic characterization and phylogenetic analysis in the present study. To accurately and precisely assess the virulence of duck-origin NDVs, both chickens and ducks were used as the experimental animals to conduct pathogenicity tests. In addition, the antigenic relatedness of NDVs from different genotypes and hosts were evaluated using the cross-reactive hemagglutination inhibition (HI) test.

2. Materials and methods

2.1. Eggs and animals

All of the SPF fertilized chicken and duck eggs, as well as SPF chickens and ducks, were obtained from the Harbin Veterinary Research Institute (HVRI), Chinese Academy of Agricultural Sciences, China. The SPF ducks were bred and provided by the Laboratory Animal Center, HVRI. The breed of duck is Anas platyrhyncha domestica. The SPF ducks were free of 10 pathogens including NDV, Avian influenza viruses, Duck enteritis virus, Duck hepatitis virus I, Avian aspergillus, Reticuloendotheliosis virus, Riemerella anatipestifer, Salmonella pullorum, Pasteurella multocida and Egg drop syndrome virus. The birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum*. All animal experimental procedures were reviewed and approved by the Ethical and Animal Welfare Committee of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China.

2.2. Virus isolation, identification, and purification

The eighteen NDV strains were isolated from domestic duck samples, most of which were obtained from Animal Influenza Laboratory of the Ministry of Agriculture and collected in different provinces in China as part of an avian influenza surveillance program between 2005 and 2013 (Fig. 1); 17 strains were isolated from cloacal swabs from clinically healthy ducks, and one strain was isolated from kidney samples from a diseased duck. All of the samples were diluted in phosphate-buffered saline (PBS) containing penicillin (1000 u/ml) and streptomycin (1000 u/ml), and were inoculated into the allantoic cavity of 9-day-old SPF eggs. The allantoic fluids were harvested either when the embryos died or after 3 days of incubation at 37 °C. Hemagglutination (HA) assays were used to identify NDV-positive embryos as described previously (OIE, 2008). Hemagglutinin (HA)-positive samples were tested for HI using four HA units of antigen and anti-La Sota chicken serum according to standard methods (OIE, 2008). The virulent viruses which can induce cytopathic effect plaque-purified in primary chicken embryo fibroblasts, and for the viruses of lower virulence, they were purified by end point dilution method using embryonated chicken eggs. Each of the purified viruses was used for preparing for viral stocks by inoculating the virus into embryonated SPF chicken eggs via the allantoic cavity and collecting the infectious allantoic fluid 72 h post-inoculation.

The virus isolation, proliferation, viral total RNA extractions and hemagglutination inhibition tests were conducted in purifier class II biosafety cabinet in the biosafety level-2 laboratory.

2.3. RNA extraction, RT-PCR, and genome sequencing

Viral RNA was extracted from infective allantoic fluid using the RNAiso Plus reagent (Takara, Dalian, China) according to the manufacturer's instructions. Primers, including those for amplifying the 5′ and 3′ ends of the genome, were designed based on consensus sequences of the most published complete genome sequences of class I and II viruses (Table 1). Complete genomes were amplified with a one-step RT-PCR kit (Takara) according to the manufacturer's instructions. The PCR products were purified using a gel extraction kit (Omega Bio-Tek, Norcross, GA, USA) and cloned into the pMD18-T Vector (Takara) following the manufacturer's instructions. Sequencing was conducted by the BGI Company (BGI, Beijing, China) or the AGCT (AGCT, Harbin, China).

2.4. GenBank accession numbers of NDV sequences

The complete genomic sequences of the 18 NDV isolates in this study were deposited in GenBank under accession numbers KM885150–KM885167 (Table 1).

2.5. Sequence comparisons and phylogenetic analysis

All sequences were compared with 44 other complete NDV genomic sequences and 71 partial NDV F gene sequences from GenBank (Table 2). The selected NDV reference strains were from different hosts, as we wanted to study whether duck-origin NDV formed an independent branch like pigeon paramyxovirus type 1 (Guo et al., 2013). The accession numbers of these NDV isolates are shown in Figs. 2 and 3. Nucleotide sequence editing, analysis, prediction of amino acid sequences, and alignments were conducted using the MEGALIGN program (version 3.1; DNASTAR, Madison, WI, USA). Phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstraps by comparing the nucleotide sequences of the complete genomes and the partial coding sequences of the F gene (374 nt) via MEGA5.0 software (Molecular Evolutionary Genetics Analysis, version 5.0).