Characterization of Newcastle Disease Virus Isolated from Northern Pintail (Anas acuta) in Japan

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ABSTRACT. A field isolate of Newcastle disease virus (NDV) isolated from northern pintail (Anas acuta) in Tohoku district, northeast Japan, was characterized. Phylogenetic analysis of the fusion protein indicated that the isolate belonged to genotype I and was closely related to isolates from the Far East corresponded to the migration route for this bird species. The isolate had the typical avirulent cleavage site of the fusion protein 112GKQGR*L117. In addition, pathogenicity tests indicated the isolate to have avirulent characteristics. However, the isolate has been shown to cause fusion cytopathic effects and form plaques on chicken embryo fibroblasts (CEF) in the absence of trypsin. The present results suggest that the CEF-adapted NDV, which is avirulent, is circulating among waterfowl populations.

KEY WORDS: Newcastle disease virus (NDV), plaque formation, wild birds.

Newcastle disease (ND), which is caused by the ND virus (NDV), is one of the most serious diseases in the poultry industry [2]. Also called avian paramyxovirus type 1 (APMV1), NDV is an enveloped, negative-stranded RNA virus containing a genome of approximately 15 kb [10]. NDV can be classified into highly virulent (velogenic), intermediate (mesogenic), and nonvirulent (lentogenic) on the basis of pathogenicity in chickens [2]. NDV can infect a great variety of poultry and free-living birds [2], and such infections apparently play a role in the spread of ND. Since the first outbreak of ND in Japan recorded in 1930 [16], large outbreaks continued to occur until the development of the ND live vaccine (Hitchner B1/47 strain) in 1967. Fewer outbreaks have occurred in Japan since, and those that have occurred have erupted mainly in small flocks that were not vaccinated against the disease or had been vaccinated incorrectly.

Comparison of the nucleotide sequences among the different strains of NDV revealed two major groups [22], which could be further divided into three lineages [5, 20, 26] or eight genotypes [3, 7, 8, 28, 29]. Mase et al. [12] performed epidemiological analysis of the NDV isolates isolated from domestic fowl and pet birds in Japan between 1930 and 2001. However, there have been insufficient molecular analyses of the NDV isolates from waterfowl in Japan, and the relationship between the Japanese representative NDV isolates from chickens and the strains isolated recently from migrating waterfowl in Japan remains unknown.

The present study was performed to investigate NDV maintained in the duck population in their sites of overwintering in Tohoku district, northeast Japan, and to obtain basic information to allow efficient prevention and control measures to reduce and avoid the transmission of NDV from wild birds to poultry. In this study, we performed surveillance of NDV in migrating ducks, carried out phylogenetic analysis of fusion genes, and assessed the virulence of the isolates by pathogenicity tests.

Fresh fecal samples were put into phosphate buffered saline (PBS: 0.14 M NaCl, 2 mM KCl, 3 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2) containing antibiotics (penicillin 10,000 units/ml, streptomycin 10,000 µg/ml, gentamicin 5,000 µg/ml, amphotericin B 50 µg/ml) to give a 30% suspension. The suspension was centrifuged at 12,000 × g for 5 min. Aliquots of 200 µl of the supernatant were inoculated into the allantoic cavities of 10-day-old chicken embryos. The eggs were incubated for 3 days unless death of the embryo was detected. At the end of the incubation period or upon death of the embryo, the allantoic fluids were taken and tested for hemagglutination activity. Virus was isolated by 2 passages in 10-day-old eggs and identified by hemagglutination inhibition (HI) test using specific antisera to NDV strain Ishii.

Viral RNA was extracted directly from infected allantoic fluid using a commercial kit (ISOGEN-LS; Nippon Gene, Tokyo, Japan). Then, the reverse transcriptase reaction was carried out with MuLV reverse transcriptase (Perkin-Elmer Applied Biosystems Japan, Tokyo, Japan) with random hexamers, and the cDNA was amplified by PCR. We used two sets of PCR primers as described previously [12]. The PCR product was used as the template for sequencing on an Applied Biosystems 310 automated DNA sequencer using a Dye terminator Cycle sequencing FS Ready Reaction kit (Perkin-Elmer Applied Biosystems Japan). The purified PCR product was sequenced from both directions. The determined nucleotide sequences were analyzed with the GENETYX-Mac program (version 10.0; Software Development Corp., Tokyo, Japan).
Phylogenetic analysis was performed with Clustal X [25], and trees based on the nucleotide sequences from a portion (nt 47 to 420) of the fusion protein gene were constructed by the neighbor-joining method [19]. The nucleotide sequence data of the fusion protein gene reported in this study were obtained from GenBank. One thousand replicates were bootstrapped to construct a consensus phylogenetic tree.

Mean death time (hr) with the minimum lethal dose (MDT/MLD) in chicken embryos and intracerebral pathogenicity index (ICPI) in day-old chicks were measured to assess the virulence of the NDV isolate [1]. ICPI represents the mean score (0, normal; 1, sick; 2, dead) per chick per observation over the 8 days [1].

Chicken embryo fibroblasts (CEF) were prepared as described Takehara et al. [24]. CEF were infected in the absence or presence of trypsin (1 µg/ml). Plaque assay was performed using CEF and MDCK cells as described [24].

A total of 541 fecal samples were collected from northern pintail (Anas acuta) from January to March 2003 at Tsuo River in Tohoku district, northeast Japan. From a total of 541 samples, one hemagglutinating virus was isolated and identified as NDV by the HI test. The isolate was named strain APMV/northern pintail/Japan/Aomori/2003 (dk-Aomori/03).

The amino acid sequence at the cleavage site of the fusion protein gene was deduced from the nucleotide sequence of the corresponding gene of dk-Aomori/03. The nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB299230. The dk-Aomori/03 isolate possesses 112GKQGR*L117 at the cleavage site, which is an avirulent type.

Figure 1 shows a phylogenetic tree constructed on the basis of partial sequence (nt 47 to 420) of the fusion protein gene together with those from GenBank. The NDV isolate, dk-Aomori/03, was classified as genotype I by phylogenetic analysis, the same genotype as the Japanese lentogenic chicken isolate JP/Ishii/62. Comparisons of dk-Aomori/03 with four isolates in the Far East available in GenBank showed that dk-Aomori/03 was closely related to FarEast-dk/2713/01, FarEast-dk/2687/01, FarEast-an/3652/02, and FarEast-an/3658/02 with 99.0%, 98.4%, 98.4%, and 98.4% nucleotide identity, respectively.

MDT/MLD was determined using 10-day-old chicken embryos. The MDT/MLD of the isolate was more than 120 hr, which is typical for an avirulent virus. Pathogenicity test in day-old chickens by intracerebral inoculation was performed in the absence or presence of trypsin. None of the chicks showed adverse symptoms or died, and the ICPI of the isolate in chicks was 0.0, a value typical of non-pathogenic isolates.

The fusion protein of virulent NDV strains is cleaved by cellular proteases in cultured cells, while that of avirulent strains requires an extracellular protease, such as trypsin, for cleavage of the fusion protein to allow multiple replication to take place. Therefore, virulent strains form plaques on the cultured cells, while avirulent strains do not in the absence of trypsin. We examined the ability of dk-Aomori/03 to form plaques in the absence or presence of trypsin on CEF and MDCK cells. This strain formed plaques in the absence of trypsin on CEF (Fig. 2, Table 1), and these plaques were inhibited by antiserum specific to NDV strain Ishii (data not shown). However, dk-Aomori/03 did not form plaques in the absence of trypsin on MDCK cells. Cell-to-cell fusion was observed when dk-Aomori/03 infected CEF in the absence of trypsin (Fig. 3). Infectious NDV released from the infected cells was harvested and inhibited by antiserum specific to NDV strain Ishii (data not shown). To confirm virus adaptation to CEF, dk-Aomori/03 was serially passaged on CEF. After the third passage of dk-Aomori/03 on CEF, the virus showed 1:128 HA activity, indicating that this strain possesses adaptations to CEF (data not shown).

In this study, we investigated the presence of NDV in wild birds from geographic areas relevant to the poultry industry in Tohoku district, northeast Japan. Reports concerning the occurrence of NDV in this region were not available for the past ten years. As it has been suggested that non-virulent NDV maintained in different wild bird species may cause NDV outbreaks in poultry, we considered it important to search for the presence of NDV in these putative reservoirs. In the present study, one NDV strain was isolated from 541 fecal samples from northern pintail (Anas acuta).

Phylogenetic studies of NDV strains based on nucleotide sequences of the fusion protein genes classified the isolate into genotype I, the same genotype as many viruses often isolated from feral waterfowl populations throughout the world. Viruses classified into genotype I cause little or no disease in chickens and replicate primarily in the gut. One Japanese lentogenic chicken isolate, JP/Ishii/62 [9], was classified as NDV genotype I. However, most of the NDVs isolated in Japan before 1985 corresponded to genotypes from the previous worldwide panzootics (II, III, and VI) [12]. After 1985, NDV genotype VII began to replace genotype VI as the most prevalent genotype in domestic fowl in Japan [12]. Further, the more recently isolated (in 1995) lentogenic chicken isolate JP/MET/95 [13] was classified as NDV genotype II. Therefore, dk-Aomori/03 differed phylogenetically from isolates that caused outbreaks in domestic fowl in Japan. The dk-Aomori/03 isolate was closely related to isolates from the Far East, corresponding to the migration route of wild waterfowl.

The results of the pathogenicity tests indicated that dk-Aomori/03 was non-pathogenic for chicken embryos and chicks. In addition, the isolate possesses 112GKQGR*L117 at the cleavage site of the fusion protein gene, which is the avirulent type. In a previous study performed in northern Japan between 1976 and 1979 [27], all isolates of NDV from feral ducks were avirulent as determined from their MDT/MLD in chicken embryos and ICPI in day-old chicks. Interestingly, dk-Aomori/03 caused fusion and formed plaques in the absence of trypsin on CEF despite its status as an avirulent type. Furthermore, the addition of trypsin resulted in about 500-fold increase of plaque number (Table 1). Therefore, characterization of dk-Aomori/03 in
this study was unique compared with other avirulent viruses. To our knowledge, there have been no detailed reports of avirulent NDV forming plaques in the absence of trypsin on CEF.

It has been established that cleavage of the fusion protein of NDV is a major determinant for pathogenicity of NDV [6, 14, 15]. The fusion proteins of virulent NDV strains possess a pair of dibasic amino acids at the cleavage site and are cleaved in a wide variety of cells. Collins et al. [4] reported that 2 of 15 pigeon NDV isolates had the virulent type amino acid sequence (\textsuperscript{112}RRQKR\textsuperscript{*}F\textsuperscript{117}) at the cleavage site of the fusion protein gene. Pathogenicity index tests showed that these isolates were of the mesogenic and lentogenic types. We feel that the wide variety of pathogenicity of NDV strains for cultured cells is maintained in nature and that plaque formation may not always be correlated to the amino acid sequence at the cleavage site of the fusion protein gene. In this study, even after plaque cloning of the isolate dk-Aomori/03, the cloned virus caused cell-to-cell fusion and formed plaques on CEF in the absence of trypsin.
The mechanism of cleavage of the fusion protein is unknown. The formation of plaques in the absence of trypsin on CEF suggested that the fusion protein of dk-Aomori/03 may be cleavable by intracellular proteases. The results of this study indicated that dk-Aomori/03 was CEF-adapted NDV despite being the avirulent type.

The major breeding areas of ducks in Alaska are located close to Arctic regions. These areas are cold enough for viruses to survive for long periods in autumn when ducks leave for migration to the south. Okazaki et al. [18] reported that influenza viruses perpetuated in ducks nesting in Siberia. It is possible that the same is also true for NDV. Isolation of virulent NDV from wild aquatic birds has been reported [11, 23]. However, whether these virulent NDV emerged in the wild bird population or were transferred to wild birds from poultry remains unclear. In addition, it is unclear whether virulent NDV is perpetuated in wild waterfowl. As feral birds transmit NDV across regional and international boundaries, multiple genotypes of NDV may have been introduced into Japan by such birds. Previously [21], we demonstrated that NDV had infiltrated into ostrich farms in Japan. Thus, further surveillance of NDV in various

Fig. 2. Plaque morphology in the absence of trypsin on CEF.

Fig. 3. Cytopathic effect of the isolate dk-Aomori/03 strain in CEF culture.
Table 1. Virus titers of dk-Aomori/03 on CEF

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plaque formation (PFU/ml)</th>
<th>trypsin (−)</th>
<th>trypsin (+)*</th>
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<tbody>
<tr>
<td>dk-Aomori/03</td>
<td>3.5 × 10^6</td>
<td>1.7 × 10^9</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>0</td>
<td>6.6 × 10^6</td>
<td></td>
</tr>
</tbody>
</table>

*: trypsin 1 µg/ml.

migrating birds will be important to improve our understanding of the epidemiology of NDV, and further detailed investigations will reveal the mechanism of plaque formation in the absence of trypsin despite being an avirulent type strain.

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