Genotyping of Newcastle Disease Viruses Isolated from 2001 to 2007 in Japan

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ABSTRACT. Seventeen isolates of Newcastle disease virus (NDV) were obtained from various prefectures in Japan during the years 2001–2007 and were genotypically analyzed by the reverse transcriptase-polymerase chain reaction (RT-PCR) method coupled with direct sequencing. These NDV isolates were classified into three genetic groups that had been reported previously, namely, genotypes I, VI and VII. The isolate from an aigamo duck was classified into genotype I with isolates mainly from waterfowl. All isolates from pigeons were classified into genotype VI, distinct from the remaining viruses in genotype VI. All isolates from chickens were classified into genotype VII, the predominant genotype responsible for most Newcastle disease outbreaks in the East Asian countries. Among the isolates from chickens, isolates after 2002 were genetically most closely related with isolates in Korea. The single isolate from a wild cormorant was also classified into genotype VII, although it was different from the recent NDV epidemic strain in Japan.

Key words: epidemiology, genotype, Newcastle disease virus.

Newcastle disease is one of the most serious diseases in the poultry industry. The causative agent of the disease is Newcastle disease virus (NDV), also designated avian paramyxovirus type 1 (APMV-1), which belongs to the genus Avulavirus within the family Paramyxoviridae [16]. NDV can infect a great variety of poultry or free-living birds [1], and such infections play a role in the spread of ND. For example, the ND outbreaks that occurred in Great Britain in 1984 are thought to have stemmed from feed contaminated by infected pigeons [1], and the outbreaks in cormorants in the United States, which occurred from 1989 to 1996, were traced to infected exotic psittacines [19, 20].

NDV is an enveloped, negative-stranded RNA virus containing a genome of approximately 15 kb [8]. NDV strains can be categorized as highly virulent (V: velogenic), intermediate (M: mesogenic) or nonvirulent (L: lentogenic) based on their pathogenicity in chickens [1]. The molecular basis for these differences of pathogenicity appears to be mainly determined by the amino acid sequence motif present at the cleavage site of the precursor fusion protein (F0) and the ability of cellular proteases to cleave the F0 protein of different pathotypes [4, 17]. The precursor fusion glycoprotein F0 has to be cleaved into F1 and F2 subunits for the progeny virus to be infectious and to be able to replicate in host cells. Based on phylogenetic analysis of the partial nucleotide sequence of F the gene, including the cleavage site, NDV strains have been classified into nine genotypes, I-IX [2, 5, 12, 13, 21].

In Japan, large outbreaks continued to occur until the ND live vaccine (Hitchner B1/47 strain) was applied in 1967. Fewer outbreaks have occurred in Japan since then, and those that have occurred have mainly erupted in small flocks that were not vaccinated against the disease, or had been vaccinated incorrectly. Previously, based on phylogenetic analysis of the above-mentioned F glycoprotein genes, we reported that NDV strains isolated in Japan up to mid-2001 could be classified into genotypes (I-III, VI, VII and VIII) [15]. In the present study, to define the epidemiology of or relationships among recent NDV isolates in Japan from 2001 to 2007, we used essentially the same procedure to characterize seventeen NDV isolates in Japan from various kinds of birds.

The NDV isolates were obtained from prefecture-based regional animal hygiene service centers in Japan (Table 1). Allantoic cavities of specific-pathogen-free embryonated eggs were used for virus propagation. The velogenicity of each isolate was judged on the basis of the time it took for the embryo to be killed and on the basis of the plaque and syncytia formation in the chicken embryo fibroblast (CEF) cultures, as described previously [15]. Viral RNA was extracted from infected allantoic fluids using a kit (ISOGEN-LS, Nippon Gene, Tokyo, Japan). Reverse transcription (RT), PCR amplification, sequencing and phylogenetic analysis were performed as described previously [15].

The expected sizes of DNA fragments (about 700 base pairs) were successfully amplified by RT-PCR from all of the employed NDV samples with NDV-F2 (5′-TGGAGC-CAAAACCACAACATGCGG-3′) and NDV-R2 (5′-GGAGGATGGTGCCAGCAT-3′) [15]. As the results of direct sequencing of the obtained PCR products, all of the isolates showed the deduced amino acid sequence 112RRQ(K/R)R116 at the C-terminus of the F2 protein, which was identical to a virulent motif, except for the aigamo duck isolate (JP/Hyogo-dk/02), which belonged to genotype I (Table 1). This motif was a prerequisite for high virulence, but none of the pigeon isolates were highly virulent, which
indicates that other properties might be influencing the full expression of virulence in isolates from pigeons.

Based on the phylogenetic analysis, we classified the seventeen NDV isolates used in this study into three genotypes, namely I, VI and VII (Table 1, Fig. 1). The isolate from the aigamo duck (JP/Hyogo-dk/02, which was isolated during surveillance) was classified into genotype I together with isolates mainly from waterfowl. This genotype I, has primarily been isolated from feral migratory waterfowl species, including mallards, baikal teals, and northern pintails in Far East Asia (Russia, Korea and Japan) [18] and North America (U.S.A.) [7].

All isolates from pigeons were classified into genotype VI, the genotype responsible for most of the Newcastle disease outbreaks in pigeons. The single isolate (JP/Nara-lk/05) from a laughing kookaburra (Dacelo novaeguineae), was classified into genotype VI, distinct from the remaining viruses in genotype VI, including pigeon isolates.

All isolates from chickens were classified into genotype VII, the predominant genotype responsible for most Newcastle disease outbreaks in the East Asian countries, including Japan [6, 10–12, 21–23]. Among the isolates from chickens, those collected in 2001 (JP/Nagano/01 and JP/ Miyagi/02) were clustered with isolates from 2000 to mid-2001, suggesting that they were derived from a common origin. However, isolates from 2002 to 2005, which were isolated in Okayama and Fukuoka prefectures located in western Japan, were also classified into genotype VII, clustered with NDV isolates from Korea and distinct from the isolates collected prior to 2001 in Japan. The single isolate from a wild cormorant (JP/Gifu-Ibaraki-co/05, which was isolated from cormorant without apparent clinical signs), which was examined as part of the surveillance of the avian influenza virus, was also classified into genotype VII, together with an isolate from a tern in Russia and apparently distinct from the other isolates collected in Japan.

Outbreaks of ND can be characterized by cocirculation of genetically distinct virus lineages. The phylogenetic analysis of NDV reported by several groups of the researchers revealed that genotype VII has been predominant in East Asian countries, including Korea, Taiwan and China, since the 1980s [6, 10-12, 21–23]. This genotype has also been the predominant pathogen in Japan, mainly in chickens, since 1985 [15]. The viruses isolated in Okayama and Fukuoka prefectures during the years 2002–2005 were genetically very close to Korean isolates. The isolate JP/Okayama–1/02 was identical to Korean SNU2084 strain (sequence identity of 100%), and isolate JP/Fukuoka-1/04 had a sequence identity of 99.5% compared with Korean SNU0169 strain, suggesting that isolates in Japan share an immediate ancestor with the Korean viruses. Interestingly, the highly pathogenic avian influenza viruses of the H5N1 subtype isolated in Japan were also genetically close to those isolated in Korea [14]. How were these viruses introduced into Japan? Several possibilities exist; feral birds, virus-contaminated material, and illegally imported infected birds are all possible sources. In Korea, genotype VII of NDV was isolated from wild ducks and owls [3, 9], which were genetically close to the prevalent genotype in chickens in Korea. Also in Japan, genotype VII of NDV (although genetically distinct from those isolated during the years 2002-2005) was isolated from a wild cormorant, suggesting that such wild birds were related to the dissemination of the virus. The surveillance of wild birds for NDV is important for understanding the epidemiology of a virus like avian influenza virus.

On the other hand, all isolates from pigeons were classified into genotype VI, and clustered with only viruses from pigeons, suggesting that they were transmitted mainly among pigeons. One isolate from a pet bird was also classi-

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**Table 1. NDV isolates from birds during 2001–2007 in Japan**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Abbreviation</th>
<th>Genotype</th>
<th>MDT*</th>
<th>Plaque formation test on CEF</th>
<th>Virulence</th>
<th>Fusion protein cleavage site</th>
</tr>
</thead>
<tbody>
<tr>
<td>APMV1/chicken/Japan/Nagano/2001</td>
<td>JP/Nagano/01</td>
<td>VII</td>
<td>59</td>
<td>+</td>
<td>V</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/pigeon/Japan/Kumamoto/2001</td>
<td>JP/Kumamoto-pg/01</td>
<td>VI</td>
<td>88</td>
<td>+</td>
<td>M</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/chicken/Japan/Miyagi/2002</td>
<td>JP/Miyagi/02</td>
<td>VII</td>
<td>54</td>
<td>+</td>
<td>V</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/pigeon/Japan/Hiroshima/2002</td>
<td>JP/Hiroshima-pg/02</td>
<td>VI</td>
<td>106</td>
<td>+</td>
<td>L-M</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/pigeon/Japan/Shizuoka/2002</td>
<td>JP/Shizuoka-pg/02</td>
<td>VI</td>
<td>102</td>
<td>+</td>
<td>L-M</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/chicken/Japan/Okayama-1/2002</td>
<td>JP/Okayama-1/02</td>
<td>VII</td>
<td>51</td>
<td>+</td>
<td>V</td>
<td>RRQR-F</td>
</tr>
<tr>
<td>APMV1/chicken/Japan/Okayama-2/2002</td>
<td>JP/Okayama-2/02</td>
<td>VII</td>
<td>51</td>
<td>+</td>
<td>V</td>
<td>RRQR-F</td>
</tr>
<tr>
<td>APMV1/pigeon/Japan/Mie/2002</td>
<td>JP/Mie-pg/02</td>
<td>VI</td>
<td>96</td>
<td>+</td>
<td>L-M</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/pigeon/Japan/Hyogo/2002</td>
<td>JP/Hyogo-dk/02</td>
<td>I</td>
<td>&gt;120</td>
<td>-</td>
<td>L</td>
<td>GKOGR-L</td>
</tr>
<tr>
<td>APMV1/pigeon/Japan/Saitama-1/2003</td>
<td>JP/Saitama-pg-1/03</td>
<td>VI</td>
<td>118</td>
<td>+</td>
<td>L-M</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/pigeon/Japan/Saitama-2/2003</td>
<td>JP/Saitama-pg-2/03</td>
<td>VI</td>
<td>114</td>
<td>+</td>
<td>L-M</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/laughing kookaburra/Japan/Nara/2005</td>
<td>JP/Nara-lk/05</td>
<td>VI</td>
<td>59</td>
<td>+</td>
<td>V</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/cormorant/Japan/Gifu-Ibaraki/2005</td>
<td>JP/Gifu-Ibaraki-co/05</td>
<td>VII</td>
<td>53</td>
<td>+</td>
<td>V</td>
<td>RRQR-F</td>
</tr>
<tr>
<td>APMV1/chicken/Japan/Fukuoka-1/2005</td>
<td>JP/Fukuoka-1/05</td>
<td>VII</td>
<td>59</td>
<td>+</td>
<td>V</td>
<td>RRQR-F</td>
</tr>
<tr>
<td>APMV1/pigeon/Japan/Tokyo/2006</td>
<td>JP/Tokyo-pg/06</td>
<td>VI</td>
<td>94</td>
<td>+</td>
<td>L-M</td>
<td>RRQKR-F</td>
</tr>
<tr>
<td>APMV1/pigeon/Japan/Niigata/2007</td>
<td>JP/Niigata-pg/07</td>
<td>VI</td>
<td>112</td>
<td>+</td>
<td>L-M</td>
<td>RRQKR-F</td>
</tr>
</tbody>
</table>

* MDT = mean death time in eggs in hours.
fied into genotype VI, distinct from the remaining viruses in genotype VI. This virus (JP/Nara-lk/05) was isolated from an outbreak among birds owned by a bird fancier who bred, the many kinds of birds, including the infected laughing kookaburra. The introduction route of this virus into this flock was unclear. Interestingly, this isolate was classified into genotype VI, which includes the isolate from a parakeet imported from Pakistan [15]. Hence, this isolate might have been introduced into Japan by the increasing trade of poultry, contaminated poultry products or pet birds harboring viruses from foreign countries. This suggests that the surveillance of the international bird trade for NDV is important for understanding the epidemiology of this virus.

ACKNOWLEDGMENT. We would like to thank the veterinary officials of each prefecture for their cooperation in collecting of the viral samples.

Fig. 1. Phylogenetic tree of NDV isolates based on nucleotide sequences from a portion (nt 47 to 420) of the F gene. The horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. The viruses employed in this study are italicized and underlined.

REFERENCES


