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Surveillance of Avian Paramyxovirus in Migratory Waterfowls in the San-in Region of Western Japan from 2006 to 2012

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ABSTRACT. Relatively little is known about the distribution of avian paramyxoviruses (APMVs) among wild birds in Japan. Surveillance of APMV in migratory waterfowl was conducted in the San-in region of western Japan during winters of 2006 to 2012. A total of 16 avian paramyxoviruses consisting of 3 lentogenic Newcastle disease viruses (NDVs), 12 APMV-4 and 1 APMV-8 were isolated from 1,967 wild-bird fecal samples. The results show that NDV and APMV-4 are relatively widely distributed among wild waterfowl that migrate to Japan from northern regions. Phylogenetic analysis revealed that there was no genetic relationship between the isolates from wild birds and domestic poultry in Japan. However, surveillance of APMVs in wild waterfowl needs to be conducted due to the pathogenic potential of these isolates in domestic poultry.

KEY WORDS: avian paramyxovirus, migratory waterfowl, NDV, surveillance.

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Avian paramyxoviruses (APMVs), which belong to the genus *Avulavirus* in the family Paramyxoviridae, comprise nine antigenically distinct serotypes (APMV-1 to 9) [1]. Recently, new serotypes of APMVs, APMV-10, -11 and -12, were proposed, respectively [4, 17, 24]. Although APMV-1, which is synonymous with Newcastle disease virus (NDV), is highly pathogenic in poultry, the other APMV serotypes are also known to cause respiratory and reproductive diseases in chickens [26]. All APMV serotypes, except APMV-5, circulate widely in wild bird populations [10, 11, 21]. However, the information on the distribution of APMVs in wild birds is limited, especially in Japan.

Wild birds, particularly waterfowl, are known reservoirs of APMV-1, 4, 6, 8 and 9 [1] and are considered to be important carriers of APMVs. The potential for disease transmission is considered to be particularly high, because many waterfowl, such as geese, swans and ducks, overwinter in Japan after migrating from Alaska, the Russian Far East, eastern Siberia, eastern Mongolia and northeastern China [18].

While very little is known about the molecular and biological characteristics and pathogenicity of APMV serotypes 2–9, extensive research has been conducted on APMV-1 or NDV. NDVs have been divided into three major pathotypes on the basis of pathogenicity; lentogenic (low virulence), mesogenic (moderate virulence) and velogenic (high virulence) [1]. Furthermore, recent phylogenetic analyses have separated NDVs into two distinct sister clades, classes I and II, each of which contains several genotypes [1, 5]. The majority of viruses that have been reported to be velogenic in domestic poultry have been grouped in class II, while lentogenic strains are dominant in class I [9, 16].

We previously experimentally demonstrated that a non-pathogenic NDV isolate from wild waterfowl became highly pathogenic after several passages in chickens [20]. The findings of that study demonstrated that wild birds were potentially capable of transmitting and spreading precursors of velogenic viral strains to domestic poultry. Consequently, continuous surveillance of APMV, including NDV, in wild birds is important for providing information on the viruses in the field, as well as emerging velogenic viruses.

In this study, we conducted a survey for APMV in populations of overwintering migratory waterfowl from 2006 to 2012 in the San-in region of western Japan where 16 APMV strains were previously isolated. Consequently, we examined the pathogenic and phylogenetic relationships among the collected APMV isolates and compared them with other isolates in the field.

MATERIALS AND METHODS

Samples: A total of 1,967 fresh fecal samples were collected from tundra swan (*Cygnus columbianus*), mallard (*Anas platyrhynchos*), white-fronted goose (*Anser albifrons*

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Table 1. Isolation of avian paramyxovirus from fecal samples of migratory waterfowls in the San-in region of western Japan during the winters of 2006 to 2012

Species	Sample Year							Total	Isolation
	2006	2007	2008	2009	2010	2011	2012		Rate (%)
Spot-billed duck					5/87	0/5		5/92	5.4
Common teal			0/58			1/2		1/60	1.7
Eurasian wigeon	2/6	1/106				0/60	0/16	3/188	1.6
Unidentified duck				2/227	2/350	0/34	0/7	4/618	0.6
Tundra swan	0/17	1/135	0/101	0/2		0/8		1/263	0.4
Mallard	2/127	0/164	0/251	0/51	0/68		0/22	2/683	0.3
White-fronted goose		0/49				0/12		0/61	0
Gadwall	0/2							0/2	0
Total	4/152	2/454	0/410	2/280	7/505	1/121	0/45	16/1967	0.8
Isolation rate (%)	2.6	0.4	0	0.7	1.4	0.8	0		

Table 2. Hemagglutination inhibition titers of avian paramyxovirus (APMV) isolates against reference APMV antisera

Virus	Reference Antiserum					
	APMV-1	APMV-2	APMV-3	APMV-4	APMV-6	APMV-7
Homologous	2,560[a)]	640	128	5,120	640	640
Duck/Tottori/N12/2006	1,280[b)]	<	80	320	320	320
Duck/Tottori/2/2006	<[c)]	<	<	2,560	160	160
Duck/Tottori/126/2006	<	<	<	640	40	40
Duck/Tottori/T99/2006	<	<	<	1,280	<	80
Duck/Tottori/140/2007	<	<	<	1,280	<	40
Tundra swan/Shimane/91–94/2007	<	<	<	<	<	<
Duck/Tottori/453/2009	640	<	160	320	320	320
Duck/Tottori/481/2009	640	<	160	320	320	320
Duck/Tottori/114–115/2010	<	<	<	640	40	40
Duck/Tottori/99/2010	<	<	<	640	<	160
Duck/Tottori/237–238/2010	<	<	<	1,280	40	160
Duck/Tottori/250/2010	<	<	<	640	40	<
Duck/Tottori/251–252/2010	<	<	<	640	40	40
Duck/Tottori/264/2010	<	<	<	640	<	40
Duck/Tottori/267–268/2010	<	<	<	1,280	80	80
Duck/Tottori/22/2011	<	<	<	640	<	40

a) Expressed as a reciprocal of the highest dilution of the antiserum inhibiting hemagglutination units of the virus. b) Underlined numbers represent the highest titers of each virus in HI test using a panel of reference antisera prepared against 6 subtypes of reference strains of APMVs (APMV-1-4, APMV-6 and -7). c) <: less than 1:40

frontalis), common teal (Anas crecca), Eurasian wigeon (Anas penelope), spot-billed duck (*Anas poecilorhyncha*), gadwall (*Anas strepera*) and unidentified duck spp. (*Anas* spp.) during winter (from November to March) of 2006 to 2012. Samples were collected at eight different sites, Lake Koyama, Pond Nikko, Lake Togo, Tenjin River, Hino River, Ito Coast, Yonago Waterbirds Sanctuary and rice fields in the suburbs of Yasugi city, in the San-in region (Tottori and Shimane prefectures) of western Japan. The fecal samples were collected individually, placed in screw-cap tubes and stored at −80°C until analysis.

Virus isolation: Virus isolation was performed using a previously described method with a slight modification [19]. Each collected fecal sample was suspended at a concentration of approximately 20% in phosphate-buffered saline (pH 7.2) containing penicillin at 10,000 units/m*l* and streptomycin at 10 mg/m*l*. The suspension was centrifuged at 1,000 × *g* for 10 min. Aliquots of 200 *µl* of supernatant were then used to inoculate into the allantoic cavities of two 9- to 11-day-old embryonated chicken eggs, which were then incubated at 37°C for 3 days unless the embryo died. The inoculated eggs were then chilled to 4°C, and the allantoic fluid of each egg was tested for hemagglutination activity.

Serotyping: All hemagglutinating agents were identified in a hemagglutination inhibition (HI) test using reference antisera against APMV strains: APMV-1/goose/Alaska/415/91, APMV-2/Chicken/California/Yucaipa/56, APMV-3/turkey/Wisconsin/68, APMV-4/duck/ Mississippi/320/75, APMV-6/duck/Hong Kong/18/199/77 and APMV7/dove/Tennessee/4/75 [15]. Samples that tested positive for at least one of these antisera were identified as APMV. The methods used in the HI test followed established procedures [19].

Sequencing and BLAST search: Viral RNA was isolated from infected allantoic fluid by using QIAamp Viral RNA

Table 3. Avian paramyxovirus isolates from migratory waterfowl in western Japan

Virus	Collection Date	Sample Site	Host	Subtype
Duck/Tottori/N12/2006	2006.12.13	Pond Nikko, Tottori	Eurasian wigeon	NDV (class II)
Duck/Tottori/2/2006	2006.12.13	Pond Nikko, Tottori	Eurasian wigeon	APMV-4
Duck/Tottori/126/2006	2006.12.18	Lake Togo, Tottori	Mallard	APMV-4
Duck/Tottori/T99/2006	2006.12.18	Lake Togo, Tottori	Mallard	APMV-4
Duck/Tottori/140/2007	2007.11. 8	Lake Koyama, Tottori	Eurasian wigeon	APMV-4
Tundra swan/Shimane/91–94/2007	2007. 3.20	Yasugi-city, Shimane	Tundra swan	APMV-8
Duck/Tottori/453/2009	2009. 1.20	Pond Nikko, Tottori	Unidentified duck	NDV (class I)
Duck/Tottori/481/2009	2009. 1.20	Pond Nikko, Tottori	Unidentified duck	NDV (class I)
Duck/Tottori/114–115/2010	2010.11.18	Tenjin River, Tottori	Unidentified duck	APMV-4
Duck/Tottori/99/2010	2010.11.18	Tenjin River, Tottori	Unidentified duck	APMV-4
Duck/Tottori/237–238/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
Duck/Tottori/250/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
APMV/duck/Tottori/251–252/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
APMV/duck/Tottori/264/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
APMV/duck/Tottori/267–268/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
APMV/duck/Tottori/22/2011	2011.11. 5	Pond Nikko, Tottori	Common teal	APMV-4

Table 4. Pathogenicity of avian paramyxovirus (APMV) isolates

Virus	Serotype (Clade)	ICPI[a)]	MDT (hr)[b)]
Duck/Tottori/237–238/2010	APMV-4	0.16	>168
Duck/Tottori/2/2006	APMV-4	0.04	>168
Tundra swan/Shimane /91–94/2007	APMV-8	0.16	>168
Duck/Tottori/453/2009	NDV (class I)	0.00	>168
Duck/Tottori/481/2009	NDV (class I)	0.00	>168
Duck/Tottori/N12/2006	NDV (class II)	0.00	>168

a) ICPI: intracerebral pathogenicity index in 1-day-old chicks. b) MDT: mean death time (hr) for chicken embryos infected with one minimum lethal dose of virus.

Mini Kit (Qiagen, Valencia, CA, U.S.A.). The F genes coding full-length ORFs were amplified using PrimeScript™ Reverse Transcriptase (TaKaRa, Otsu, Japan) for RT and KOD Dash polymerase (Toyobo, Osaka, Japan) for PCR. After extraction from an agarose gel using a QIAquick Gel Extraction Kit (Qiagen), viral cDNA fragments were sequenced using a BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) on a 3130 × l Genetic Analyzer (Applied Biosystems). The F gene-specific primer sequences and conditions employed for RT, PCR and sequencing are available upon request. The DNA sequence data were edited and aligned using BioEdit software (ver. 7.0.8.0) [25] before being subjected to BLAST search analysis using the NCBI database.

Pathogenicity test: To assess the virulence of each APMV isolate, the mean death time (MDT in hours) of chick embryos at the minimum lethal dose and the intracerebral pathogenicity index (ICPI) in 1-day-old chicks were measured [3].

Phylogenetic analysis: Phylogenetic analyses of F gene segments were performed using ClustalX implemented in the MEGA 4 software package [23]. The phylogenetic tree was estimated using the Kimura 2-parameter nucleotide model, and the robustness of the clusters obtained by the neighbor joining algorithm was assessed using 1,000 bootstrap replicates.

RESULTS

In the winters of 2006 to 2012, a total of 1,967 fecal samples were collected from tundra swan (n=263), mallard (n=683), white-fronted goose (n=61), common teal (n=60), Eurasian wigeon (n=188), spot-billed duck (n=92), gadwall (n=2) and unidentified duck spp. (n=618) in the San-in region of western Japan (Table 1).

A total of 64 hemagglutinating agents were isolated and assayed in the HI tests using anti-APMV reference strain antisera. Of these, 15 samples that tested positive for at least one of these antisera were identified as APMV (Table 2). Some minor cross-reactions between two different serotypes of APMVs were found as reported previously [1]. Consequently, 3 isolates were identified as NDV, and 12 isolates were APMV-4, based on the highest titers in HI tests (Tables 2 and 3). Another isolate (Tundra swan/Shimane /91–94/2007), which did not react to any APMV antisera (serotypes 1–4, 6 and 7), was identified as APMV-8 by fusion (F) -gene sequencing and BLAST analysis (the highest homology was with APMV-8/goose/Delaware/1053/76; 98%).

Furthermore, a BLAST search for other APMV isolates corroborated the serological findings. Briefly, 12 APMV-4 isolates showed the highest homology with APMV-4/KR/YJ/2006 (more than 96%). Duck/Tottori/N12/2006 showed the highest homology with NDV/Pennsylvania/3167/2009

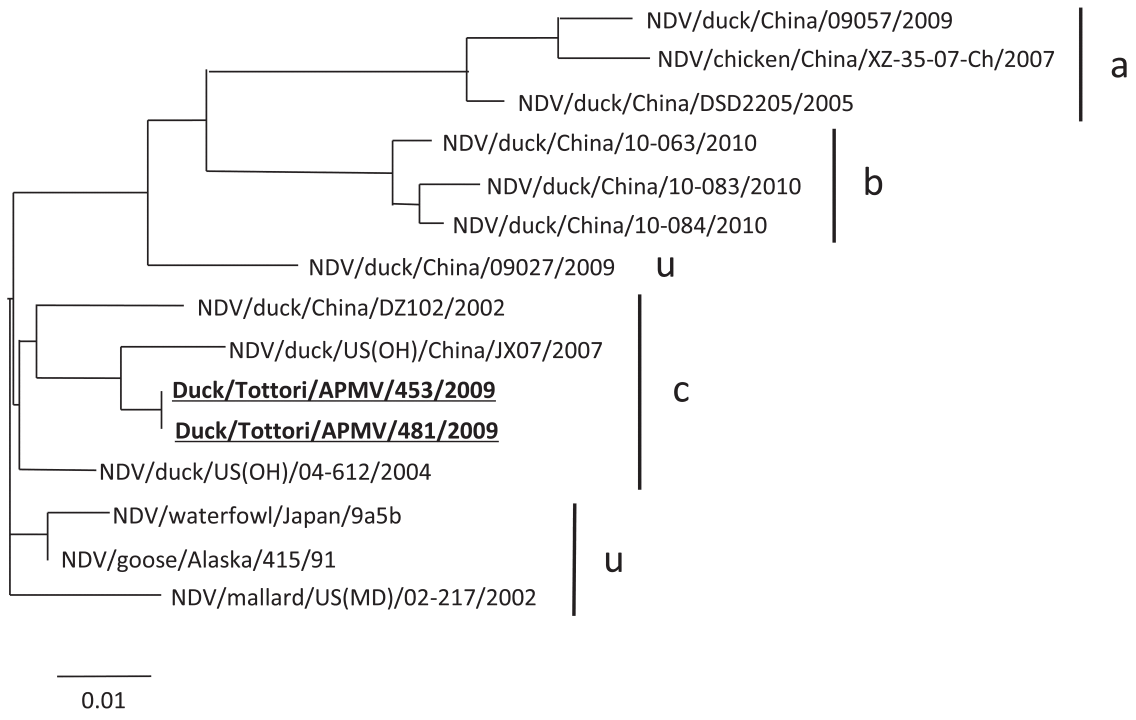

Fig. 1. Phylogenetic tree of F gene sequences from NDV isolates (class I). The phylogenetic tree was generated using the neighbor-joining algorithm with 1,000 bootstrap replicates in MEGA (4.0.2). Analysis was based on nucleotides 47–420 (372 bp) of the F gene. NDV isolates collected in this study were underlined. Letters a, b and c represent each sub-group, and "u" indicates "unidentified".

(99%), and Duck/Tottori/453/2009 and Duck/Tottori/481/2009 showed the highest homology with NDV/duck/China/08–046/2008 (98%). Consequently, a total of 16 APMV strains (tundra swan (n=1), mallard (n=2), common teal (n=1), European wigeon (n=3), spot-billed duck (n=5) and unidentified duck spp. (n=4)) were isolated. The overall rate of APMV isolation was 0.8% (Table 1).

Virulence of virus isolates was assessed by pathogenicity tests with chicken embryos and chicks (Table 4). The MDT of the 6 representative isolates was more than 168 hr, which is typical for avirulent viruses. The ICPI of these samples ranged from 0.00 to 0.16, which is also within the expected values for avirulent viruses. The amino acid sequence at the cleavage site of the F protein was deduced from the nucleotide sequence of the corresponding gene. Two of the 3 NDV isolates, Duck/Tottori/453/2009 and Duck/Tottori/481/2009, possess ^{112}ERQER-LV^{118} with the remaining isolate Duck/Tottori/N12/2006 possessing ^{112}GKQGR-LI^{118} at the fusion cleavage site; these characteristics were all typical of avirulent viruses [7].

A phylogenetic tree was constructed based on the partial sequences of the F genes together with those from Genbank (Figs. 1 and 2). The NDV isolates in the present study were divided into the two sister clades, Duck/Tottori/453/2009 and Duck/Tottori/481/2009, which belonged to the class I genotype c (Fig. 1), and the remaining isolate, Duck/Tottori/N12/2006, was included in the class II genotype I (Fig. 2).

To investigate the relationship between the NDV isolates from wild birds and field isolates from poultry farms in Japan, a phylogenetic tree was constructed (Fig. 3). The result showed that three NDV isolates in the present study were clearly distinguishable from any of the NDV isolates that had caused NDV outbreaks in Japan in recent years.

DISCUSSION

In the present study, a total of 16 APMVs were isolated from wild birds (Table 3). Of these isolates, APMV-4 strains were isolated at different sites in the San-in region in Japan relatively frequently during the study period. Conversely, APMV-8, which was isolated in 2007, was considerably rare, even at a global scale [6, 27]. Stanislawek *et al.* [22] isolated NDV and APMV-4 from wild ducks in New Zealand in 1997. In the United States, Goekjian *et al.* [11] reported that NDV, APMV-4 and APMV-6 were isolated from migratory waterfowl from 2004 to 2006. In this study, we found different serotypes of APMVs in wild bird species that had migrated into the San-in region, western Japan.

In the present surveillance study, the overall isolation rate of APMV was 0.8%, which is slightly lower than our previous survey in the same region in 1997–2000 (1.4%, 5 isolates/359 fecal samples) [19]. Another study in Japan reported that 11 NDV strains (prevalence rate: 0.46%) were isolated from 2,381 fecal samples of northern pintail from

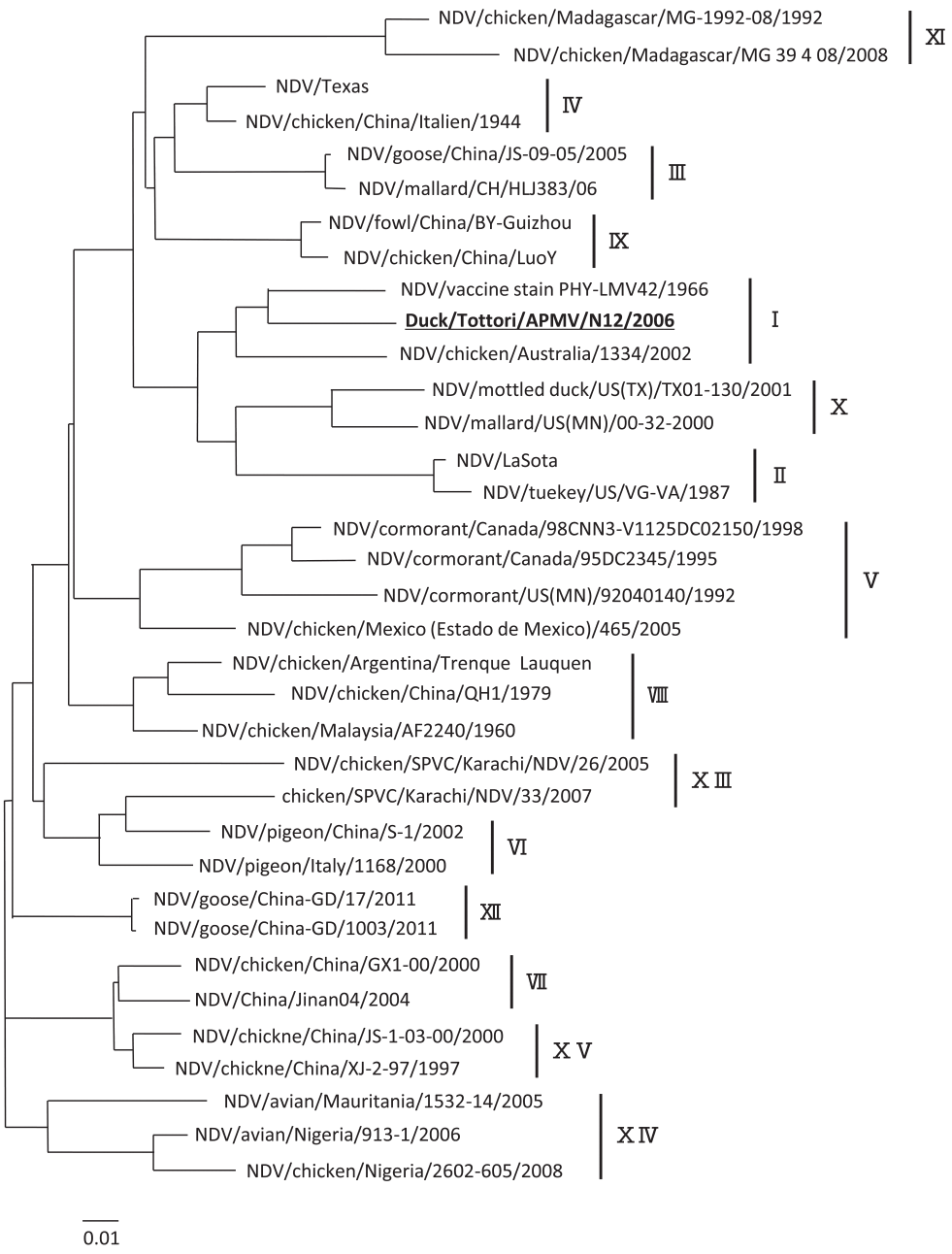

Fig. 2. Phylogenetic tree of F gene sequences from NDV isolates (class II). The phylogenetic tree was generated using the neighbor-joining algorithm with 1,000 bootstrap replicates in MEGA (4.0.2). Analysis was based on nucleotides 47–420 (372 bp) of the F gene. NDV isolates collected in this study were underlined. Roman numerals I − XV indicate each genotype.

2006 to 2008 in the Tohoku region in northeastern Japan [14]. The findings of these studies also support the notion that migratory waterfowl play an important role in the maintenance of APMVs in nature [1].

In cases where APMV-4 was isolated from waterfowl, the birds rarely exhibited clinical signs of viral infection [1, 6, 12, 21]. However, in chickens experimentally infected with APMV-4, all of the birds manifested symptoms of microscopic lesions in the trachea, lung, gut and pancreas [26]. Viral replication in chickens was also confirmed by isolation of the virus in embryonated eggs. It is therefore possible that the non-pathogenic APMV-4 that is maintained in populations of wild waterfowl has the potential to become pathogenic after transmission to and circulation within, domestic chicken populations.

Our previous report showed that a lentogenic NDV iso-

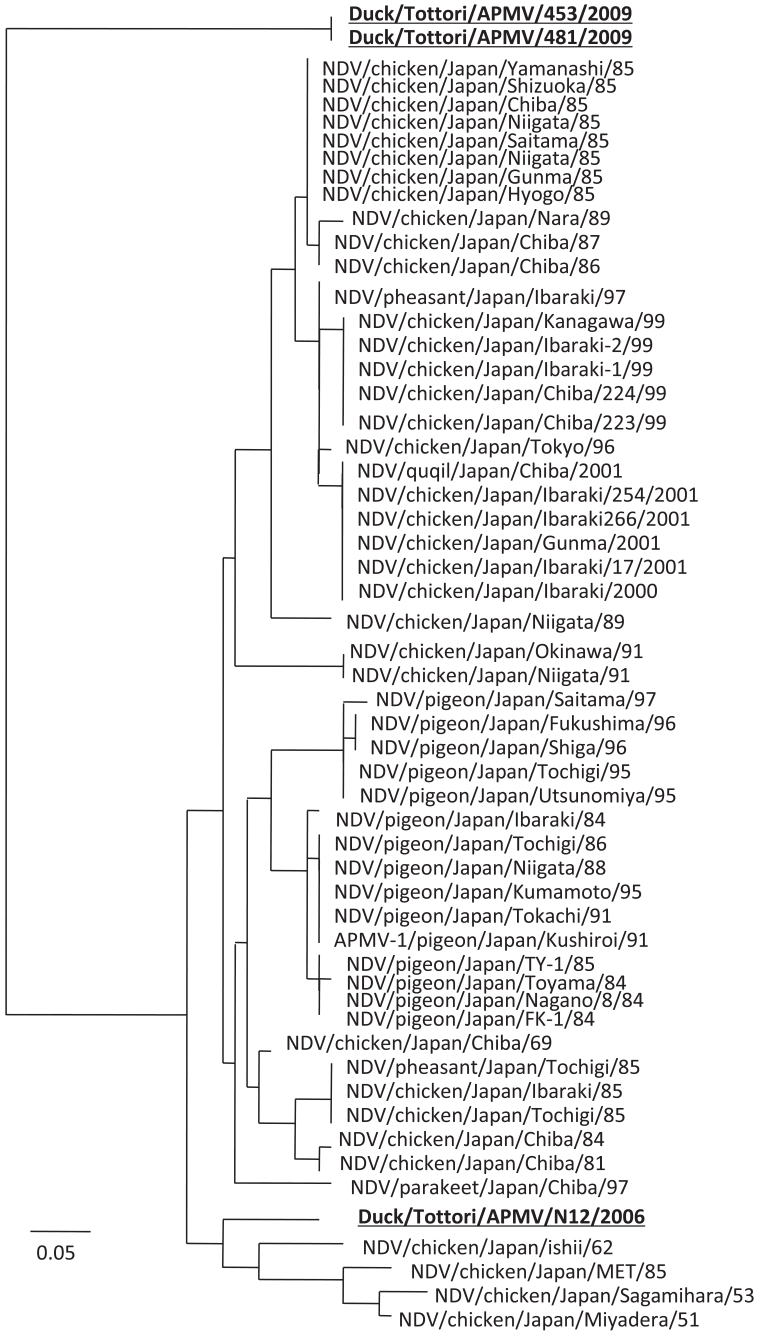

Fig. 3. Phylogenetic tree of F gene sequences from recent NDV isolates in Japan. The phylogenetic tree was generated using the neighbor-joining algorithm with 1,000 bootstrap replicates in MEGA (4.0.2). Analysis was based on nucleotides 47–420 (372 bp) of the F gene. NDV isolates collected in this study were underlined.

late from wild waterfowl becomes velogenic after repeated passage in chickens, causing 100% mortality in the infected birds [20]. The results suggested that circulation of lentogenic NDV isolates in poultry farm can result in viruses becoming velogenic. In the present study, an NDV class II strain, Duck/Tottori/N12/2006, was isolated in the field. Previous studies have shown that the majority of velogenic viruses in domestic poultry belong to class II [9]. Therefore, although the pathogenicity tests conducted in this study showed that the isolate was lentogenic, it could be a possible precursor virus in a future Newcastle disease outbreak in Japan.

Two outbreaks of Newcastle disease on poultry farms in

Ireland in 1990 [2] were caused by velogenic isolates that were very similar, both antigenically and genetically, to avirulent viruses isolated from feral waterfowl [8]. Moreover, genetic analysis of viruses isolated during outbreaks in 1998 to 2000 in Australia was also very similar to viruses isolated from birds in the wild [13]. Therefore, to investigate the genetic affiliation among the field isolates in Japan, phylogenetic analyses were conducted. The results revealed that there were no genetic relationship between the isolates obtained from wild birds and isolates from domestic poultry in Japan. Especially, class II NDV isolate, Duck/Tottori/N12/2006, was most closely related to the old isolate, NDV/chicken/Japan/ishii/62 (Fig. 3), indicating that the isolate is not a direct ancestor for the recent outbreaks in Japan. However, the pathogenic potential of the isolate to domestic poultry cannot be ignored. It is therefore necessary to continue surveillance of avian paramyxoviruses in wild waterfowl. Continued surveillance over multiple years will allow us to increase our understanding of the role of wild birds in the dissemination of APMVs in the field.

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