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. The findings of that study demonstrated that wild birds were considered to be particularly high, because many waterfowl, including NDV, APMV-1, 4, 6, 8 and 9 [1] and are considered to be important carriers of APMV isolates in the field.

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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 APMV-1 or APMV-4 infections of overwintering migratory waterfowl. 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. The findings of that study demonstrated that wild birds were considered to be particularly high, because many waterfowl, including NDV, APMV-1, 4, 6, 8 and 9 [1] and are considered to be important carriers of APMV 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*), and other waterfowl species during the winters of 2006 to 2012 in the San-in region of western Japan. The samples were collected from overwintering waterfowl and submitted to the Avian Zoonoses Research Center, Tottori University, for viral isolation and identification.

**Results:** 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.
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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/ml and streptomycin at 10 mg/ml. 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 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 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 |
| 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 | 2/227 | 0/60 | 0/16 | 3/188 | 1.6 |
| Unidentified duck | 2/127 | 0/164 | 0/251 | 0/51 | 0/68 | 0/22 | 2/683 | 0.3 |
| Tundra swan | 0/17 | 1/135 | 0/101 | 0/2 | 0/8 | 1/263 | 0.4 |
| Mallard | 0/2 | 0/2 | 0 | 0 | 0 | 0 | 0 |
| White-fronted goose | 4/152 | 2/454 | 0/410 | 2/280 | 7/505 | 1/121 | 16/1967 | 0.8 |
| Gadwall | 2.6 | 0.7 | 1.4 | 0.8 | 0 | 0 |
| Isolation rate (%) | 2.6 | 0.4 | 0.7 | 1.4 | 0.8 | 0 | 16/1967 | 0.8 |

| Table 2. Hemagglutination inhibition titers of avian paramyxovirus (APMV) isolates against reference APMV antisera |
|---|---|---|---|---|---|---|---|
| Virus | Reference Antiserum |
| Homologous | APMV-1 | APMV-2 | APMV-3 | APMV-4 | APMV-6 | APMV-7 |
| Duck/Tottori/N12/2006 | 2,560<sup>a)</sup> | 640 | 128 | 5,120 | 640 | 640 |
| Duck/Tottori/2/2006 | 1,280<sup>b)</sup> | 2,560 | 640 | 40 | 40 |
| 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
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
(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 a ERQER-LV with the remaining isolate Duck/Tottori/N12/2006 possessing GKQGR-LI 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
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-
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 relationships 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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