NOTE *Avian Pathology*

Surveillance of avian paramyxovirus serotype-1 in migratory waterfowls in Japan between 2011 and 2013

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ABSTRACT. To further understand the epidemiology of avian paramyxovirus serotype-1 (APMV-1) in migratory waterfowls in Japan, we conducted the surveillance of this virus from feces derived from the migratory waterfowls collected in 41 Japanese prefectures between October 2011 and March 2013. Six APMV-1 viruses were isolated from total 661 samples. All isolates were identified as the avirulent (lentogenic) type on the basis of intracerebral pathogenicity tests. Genetic analysis showed that these viruses possessed the deduced amino acid sequence of ^{112}GKQGR-L^{117} or ^{112}ERQER-L^{117} at the cleavage site of the F0 protein, which was identical to the motif in the avirulent type. Phylogenetic analysis based on the partial fusion protein gene classified these APMV-1 isolates into 2 major genetic groups. Four isolates were classified as class II genotype I, and they were genetically closely related to strains isolated in Asian countries, including Japan. In contrast, two isolates were classified as class I, and they were genetically closely related to strains mainly isolated in the U.S.A.
KEY WORDS: avian paramyxovirus type 1, genotype, phylogeny

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Newcastle disease (ND) is prevalent worldwide, and it causes significant clinical and economic losses in the poultry industry. This disease is caused by specific strains of avian paramyxovirus serotype-1 (APMV-1), which belongs to the genus Avulavirus in the family Paramyxoviridae [10]. At present, there are 12 known serotypes of APMV [10], but all Newcastle disease virus (NDV) isolates are APMV-1, and therefore, NDV is synonymous with APMV-1. Furthermore, on the basis of their genome lengths and the nucleotide sequences, APMV-1 isolates have been classified into two major groups (class I and II) [2].

According to a recent definition by the World Organization for Animal Health (OIE), ND is defined as an infection of birds caused by APMV-1 that meets one of the following criteria for virulence: (a) the virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 0.7 or greater, or (b) multiple basic amino acids have been demonstrated in the virus at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein [10]. The term "multiple basic amino acids" refers to at least three arginine or lysine residues between positions 113 and 116. According to this definition, amino acid residues are numbered from the N -terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene; thus, 113–116 corresponds to residues located −4 to −1 from the cleavage site.

On the basis of the phylogenetic analysis of the above−mentioned F glycoprotein genes, the NDV strains isolated in Japan before 2007 can be classified according to 6 genotypes (class II genotypes I–III, VI, VII and VIII) [7, 8]. Among them, genotype VII is the predominant genotype responsible for most ND outbreaks in chickens, and this genotype is also predominant in East Asian countries, including Japan [6, 8, 11, 14]. Genetic analysis shows that recent isolates from Japan are very similar to Korean isolates, suggesting that isolates from Japan share an immediate ancestor with the Korean viruses. The highly pathogenic avian influenza viruses of the H5N1 subtype isolated in Japan were also genetically close to those isolated in Korea, which suggests that these viruses were introduced into Japan by migratory waterfowls [9, 16]. Migratory birds may be similarly involved in the dissemination of APMV-1 in this area.

Therefore, to further understand the epidemiology of APMV-1 in migratory waterfowls in Japan, we conducted the surveillance of this virus from feces derived from the migratory waterfowls collected from 41 Japanese prefectures between June 2011 and March 2013.

The fecal samples (one sample was made by pooling feces from 5 birds, principally) were periodically collected from feral birds, such as mallard, northern pintail and spot-billed duck, in each Japanese prefecture (Table 1). The collected fecal samples were placed in screw-cap tubes and stored at −80°C until virus isolation.

Each collected sample was suspended at a concentration of approximately 10% in Eagle's minimum essential medium containing antibiotics. The suspension was centrifuged, and the supernatant was inoculated into the allantoic cavities of 9- to 12-day-old specific pathogen- free chicken embryonated eggs. After inoculation, eggs were incubated at 37°C for 6 days, unless the embryo died. The inoculated

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Table 1. Numbers of fecal sample collected in 41 Japanese prefectures between October 2011 and March 2013

Prefecture	2011			2012						2013			Total
	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	
Hokkaido	6	6	6	6	6	6	12	6	3	5	3	5	70
Aomori					3		3			1		1	8
Iwate		3		3		3	3	3		3		3	21
Miyagi	1		3		3		3		3		3		16
Akita	1		1		2		1		3		3		11
Yamagata		***3***[*]				3	3	3		2		3	17
Fukushima		3											3
Ibaraki						3		6		3		3	15
Tochigi			3		3		3		3		3		15
Gunma		***1***											1
Saitama				3		3							6
Chiba	3		4		3		***3***		3		***3***		19
Kanagawa										3			3
Niigata	3		3		3		3		3		3		18
Ishikawa	2				2								4
Yamanashi		3				3	3	3		3		3	18
Nagano		3		3		3	3	3		3		3	21
Gifu							3		3		3		9
Shizuoka	3		3		3		3		3		3		18
Aichi							3	3		3		3	12
Mie	3		3		3		3		3		3		18
Shiga		3		3		***4***	3	3		3		3	22
Kyoto	3				3		3		3		3		15
Osaka		3					2	3		3		3	14
Nara				3			3	3		3			12
Wakayama	3		3		3		1		3		3		16
Tottori	3		3		3		3		3		3		18
Shimane								3		3		3	9
Okayama		3		3		3		3		3			15
Yamaguchi	3	3		3		3		3	3	3		3	24
Tokushima	3		3		3		3		3		3		18
Kagawa		3		3		3		3		3		***3***	18
Ehime	3		3		3		3		3		3		18
Kochi		3		3		3	3	3		3		3	21
Fukuoka	3		3		3		3		3		3		18
Saga		3		3		3						3	12
Nagasaki	3		3		3		4		6		6		25
Kumamoto		3		3		3	3	3		3		3	21
Miyazaki		3		3				3	3	3			15
Kagoshima	3		3		3				3		3		15
Okinawa		1		3				3		3		2	12
Total	49	50	47	45	55	46	86	60	60	59	54	50	661

*The samples from which APMV-1 isolated are shown in bold, italic and underlined.

eggs were then chilled to 4°C, and the allantoic fluids were harvested and tested to assess their hemagglutination activity. For negative samples, 2 allantoic fluids samples were pooled and repassaged in 2 new eggs to confirm negativity. APMV-1 was identified using the conventional hemagglutination inhibition assay with APMV-1 specific antiserum.

In total, 661 samples were collected during the surveillance period (Table 1), and 6 APMV-1 viruses were isolated from these samples (Table 2).

To evaluate the pathogenicity of isolates, the ICPI values were measured in 1-day-old chicks according to the procedure by OIE [10]. The ICPI values of these isolates were 0–0.2, and they were classified as the lentogenic type (avirulent virus). These values were similar to those of many isolates previously isolated from waterfowls [19].

In the genetic characterization of these isolates, viral RNA was extracted from infected allantoic fluids using a commercial kit (QIAamp Viral RNA Mini Kit, QIAGEN, Valencia, CA, U.S.A.), according to the manufacturer's instructions. After reverse transcription with SuperScript™ III (Life

Table 2. APMV-1 isolates in this study

Isolate	Abbreviation	Collection date	Host	Genotype	Fusion protein cleavage site
APMV-1/duck/Gunma/1/2011	JP/Gunma-dk/2011	Nov.13, 2011	Mallard and Spot-billed duck	Class II genotype I	^{112}GKQGR-L^{117}
APMV-1/duck/Yamagata/2/2011	JP/Yamagata-dk/2011	Nov.28, 2011	Northern Pintail	Class II genotype I	^{112}GKQGR-L^{117}
APMV-1/duck/Shiga/1/2012	JP/Shiga-dk/2012	Mar.12, 2012	Unidentified duck	Class II genotype I	^{112}GKQGR-L^{117}
APMV-1/duck/Chiba/1/2012	JP/Chiba-dk/2012	Oct.26, 2012	Northern Pintail and Spot-billed duck	Class I	^{112}ERQER-L^{117}
APMV-1/duck/Chiba/1/2013	JP/Chiba-dk/2013	Feb.13, 2013	Northern Pintail and Spot-billed duck	Class I	^{112}ERQER-L^{117}
APMV-1/duck/Kagawa/3/2013	JP/Kagawa-dk/2013	Mar.19, 2013	Eurasian Wigeon	Class II genotype I	^{112}GKQGR-L^{117}

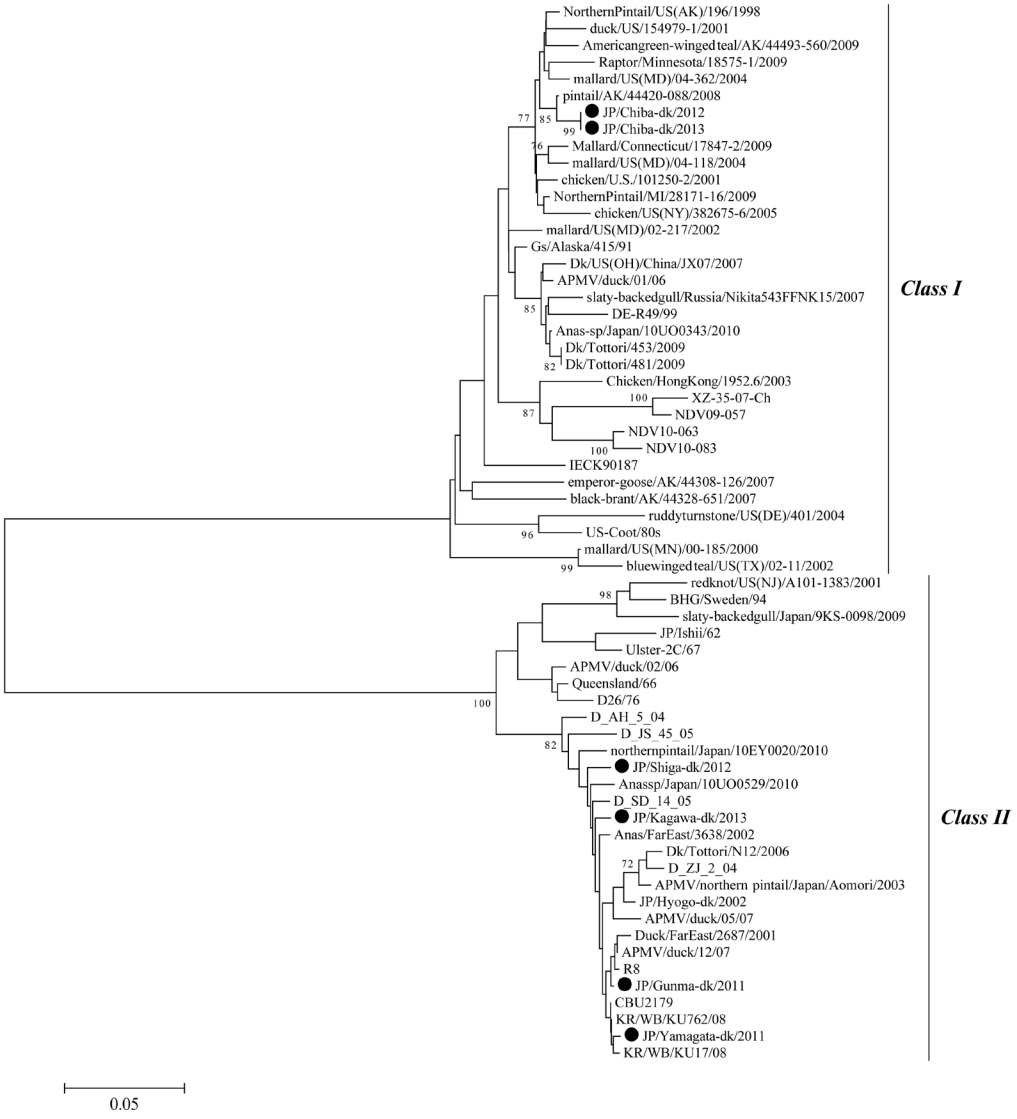

Fig. 1. Phylogenetic tree of the APMV-1 isolates based on nucleotide sequences from a portion (nt 47- 420) of the F gene. The horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. The isolates described in this study are shown by black circles. The tree was generated using the neighbor-joining algorithm, and alignments were bootstrapped 1,000 times. Bootstrap values >70 are shown.

Technologies, Gaithersburg, MD, U.S.A.) using random 9- mers, PCR was performed to amplify cDNA with Takara ExTaq (Takara, Tokyo, Japan), which consisted of 35 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec. The primer sequences and locations were as follows: MSF1 (4306 5′ -GACCGCTGACCACGAGGTTA- 3′ 4325) and #2 (5004 5′-AGTCGGAGGATGTTGGCAGC-3′ 4985). These sequences were numbered according to a previously published sequence (GenBank Accession. No. AF309418). The sequences amplified by these primers included the F protein cleavage site and the region used to construct a phylogenetic tree for genotyping the virus, as described previously [1].

DNA fragments with the expected sizes (approximately 700 bp) were successfully amplified by RT-PCR from all of the APMV-1 samples in this study. The nucleotide sequences obtained were analyzed using GENETYX-Mac (Software Development Corp., Tokyo, Japan). Phylogenetic analysis with available sequences from GenBank was conducted using the Clustal W program, and the tree was constructed by the neighbor-joining method [15] in MEGA6 [18].

The viruses possessed the deduced amino acid sequences of ^{112}GKQGR-L^{117}(class II) and ^{112}ERQER-L^{117}(class I), respectively, at the cleavage site of the F0 protein, which were identical to motif in the avirulent viruses, and this agreed with the results obtained in the ICPI tests.

Phylogenetic analysis based on the partial fusion protein gene classified these APMV-1 isolates into 2 major genetic groups (Fig. 1). Four isolates were classified as class II genotype I, and they were genetically closely related to strains isolated in Asian countries, including Japan. These class II genotype I viruses have mainly been isolated from feral migratory waterfowl species, including mallard, Baikal teal and northern pintails in the Far East of Asia [5, 13] and North America (U.S.A.) [4].

In contrast, 2 isolates were classified as class I, and they were genetically closely related to strains isolated mainly in the U.S.A. These class I viruses have also been isolated mainly from feral migratory waterfowl species, including mallards and northern pintails in the Far East of Asia [3, 5, 19] and North America [4]. According to analysis using the GenBank BLAST database, the sequences obtained from class I isolates in this study (index isolate: JP/Chiba-dk/2012) were closest to those of the northern pintail/AK/44420-088/2008 strain isolated in the U.S.A. (GenBank Accession No. KC503443), where the shared sequence similarity was 98.9%. Two independent groups have recently reported the isolation of class I APMV-1 strains (Dk/Tottori/453/2009 and Anas-sp/Japan/10UO0343/2010) [12, 19]. However, the JP/Chiba-dk/2012 isolate was genetically dissimilar to these isolates compared with the U.S.A. isolates, i.e., shared sequence similarities of only 94.9% and 95.5% with the Dk/Tottori/453/2009 and Anas-sp/Japan/10UO0343/2010 isolates, respectively. These results suggest the possible introduction of genetically diverse class I viruses into Japan, and these viruses have an intercontinental distribution similar to APMV-1 class II genotype I virus [12]. Shengqing *et al*. previously demonstrated that apathogenic APMV-1 isolate from wild waterfowls in Alaska, which belonged to class I, became highly pathogenic after several experimental passages in chickens [17]. Therefore, it is necessary to investigate the potential pathogenicity of isolates, even if they are found to be apathogenic types.

In this surveillance study, the APMV-1 viruses associated with the ND outbreaks in Japan were not isolated, and no pathogenic APMV-1 viruses were isolated. However, the recent isolation of highly pathogenic avian influenza H5N1 viruses in Japan that are genetically very similar to Korean isolates [9, 16] suggests their possible introduction via migratory waterfowls. For example, the virus strain JP/Yamagata-dk/2011, which is genetically very similar to the Korean isolate KR/WB/KU17/08 (99.5% sequence similarity; Fig. 1), was isolated in this surveillance study. Thus, continuous surveillance over many years will be required to further understand the epidemiology of this virus in migratory waterfowls in a similar manner to avian influenza viruses.

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