

Characterization of novel avian paramyxovirus strain APMV/Shimane67 isolated from migratory wild geese in Japan

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ABSTRACT. An avian paramyxovirus (APMV) isolated from goose feces (APMV/Shimane67) was biologically, serologically and genetically characterized. APMV/Shimane67 showed typical paramyxovirus morphology on electron microscopy. On hemagglutination inhibition test, antiserum against APMV/Shimane67 revealed low reactivity with other APMV serotypes and *vice versa*. The fusion (F) protein gene of APMV/Shimane67 contained 1,638 nucleotides in a single open reading frame encoding a protein of 545 amino acids. The cleavage site of F protein contained a pair of single basic amino acid (VRENRL). The nucleotide and deduced amino acid sequences of the F gene of APMV/Shimane67 had relatively low identities (42.9–62.7% and 28.9–67.3%, respectively) with those of other APMVs. Phylogenetic analysis showed that APMV/Shimane67 was related to NDV, APMV-9 and APMV-12, but was distinct from those APMV serotypes. These results suggest that APMV/Shimane67 is a new APMV serotype, APMV-13.

KEY WORDS: APMV, avian paramyxovirus, F gene, phylogenetic analysis, serotype

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Avian paramyxovirus (APMV) belongs to the genus *Avulavirus* in the *Paramyxovirinae* subfamily of the family *Paramyxoviridae*. Members of family *Paramyxoviridae* are characterized by pleomorphic enveloped particles that contain a single-stranded, negative sense RNA genome [10]. APMV is classified into nine distinct serotypes based on serological tests, such as hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests [1]. APMV-1, also known as Newcastle disease virus (NDV), causes severe disease in chickens and huge economic losses for poultry industries. Thus, APMV-1 is the most extensively characterized APMV serotype. In addition to APMV-1, APMV-2, 3, 6 and 7 are reportedly associated with disease in poultry. APMV-2 causes respiratory disease in chickens and turkeys, while APMV-3, 6 and 7 cause respiratory disease or egg production disorder in turkeys. On the other hand, APMV-4, 5, 8 and 9 have not been reported to infect poultry. APMV-4, 8 and 9 were mainly isolated from waterfowl, such as ducks and geese, while APMV-5 was isolated from budgerigar and is associated with diarrhea and high mortality [1]. Three APMVs isolated from the rockhopper penguin in the Falkland Islands, the common snipe in France and the Eurasian wigeon in Italy have recently been proposed to be new serotypes of APMV, APMV-10, 11 and 12, respectively [4, 12, 19]. The intracerebral pathogenicity index test using one-day-old chicks suggested that APMV-10 and 12 showed

little or no virulence in chickens, resembling the lentogenic NDV.

Since 1979, we have surveyed for influenza A viruses and APMVs in the San-in district of western Japan. During this surveillance, numerous influenza A viruses and APMVs have been isolated from the feces of migratory waterfowl flying from Siberia or northern China [5, 16, 20]. APMV/Shimane/67/2000 (APMV/Shimane67) examined in this study was isolated from the feces of geese collected in 2000 in Shimane prefecture. Here, we report the biological, serological and genetic analyses of APMV/Shimane67. The results suggested that APMV/Shimane67 belongs to a new serotype APMV-13.

MATERIALS AND METHODS

Viruses and cells: APMV/Shimane67 was isolated from goose fecal samples collected in Shimane prefecture, the wintering location for wild waterfowl, such as ducks, geese and swans. Virus isolation was performed as described previously [16].

Electron microscopy: Purification of APMV/Shimane67 was conducted by differential centrifugation and two successive sedimentations through a 10 to 50% sucrose gradient as described previously [6]. The purified virus particles stained with 2% phosphotungstic acid (pH 6.8) were observed under the JEM-100CX electron microscope (JEOL, Tokyo, Japan) at 80 kV.

Hemagglutination (HA) and hemagglutination inhibition (HI) test: HA and HI tests were performed using established procedures as described previously [5]. For HI test, chicken hyperimmune sera against APMV/Shimane67, NDV/goose/Alaska/415/91 (NDV/AK/415), APMV-2/chicken/California/Yucaipa/56 (APMV-2/Yucaipa), APMV-3/turkey/Wis-

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consin/68 (APMV-3/WI), APMV-4/duck/Mississippi/320/75 (APMV-4/MS), APMV-6/duck/Hong Kong/D199/77 (APMV-6/HK/D199) and APMV-7/dove/Tennessee/4/75 (APMV-7/TN) were prepared as described previously [20].

Propagation of APMV/Shimane67 in cultured cells: Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK), African green monkey kidney (Vero) and baby hamster kidney (BHK21) cells were grown in Eagle's minimal essential medium (E-MEM) containing 10% fetal bovine serum (FBS). Human embryonic kidney (293) and chicken embryo fibroblast (DF-1) cells were grown in Dulbecco's modified Eagle's medium (D-MEM) containing 10% FBS. Cell monolayers prepared in 6-well plates were infected with 10^4 50% egg infectious dose (EID_{50}) of APMV/Shimane67. After adsorption for 1 hr at 37°C, cells were washed three times with phosphate buffered saline (PBS, pH7.2) and were incubated at 37°C in E-MEM or D-MEM containing 0.3% bovine serum albumin and 0.5 µg of TPCK-treated trypsin (Sigma-Aldrich, St. Louis, U.S.A.). The virus titer of each cell culture supernatant was determined as follows. Ten-fold diluted supernatant (100 µl) was inoculated into the allantoic cavities of five 10-day-old embryonated chicken eggs and were incubated at 37°C for 3 days unless death of the embryo was detected. After the inoculated eggs were chilled at 4°C, their allantoic fluids were tested for hemagglutination activity. The virus titer was calculated using the method of Reed and Muench [13].

Pathogenicity test: The mean death time (MDT) at the minimum lethal dose for chicken embryos and the intracerebral pathogenicity index (ICPI) in 1-day-old chickens were measured in order to assess the virulence of this virus, as described previously [2].

Nucleotide sequencing and phylogenetic analysis: Viral RNAs were extracted from purified APMV/Shimane67 using the QIAamp Viral RNA Mini Kit (QIAGEN, Tokyo, Japan), and were transcribed to cDNA using F10 primer and RAV-2 reverse transcriptase (TaKaRa Bio, Otsu, Japan) in accordance with the manufacturer's instructions. F10 primer was designed to bind with the gene-stop signal region of the matrix gene and gene-start signal of the F gene of NDV/AK/415 [21]. PCR was carried out using Pwo DNA polymerase (Roche, Mannheim, Germany) and a cycling sequence of 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, which was then followed by a final extension at 72°C for 5 min. PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN) and were sequenced using the Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) and a Gene Rapid DNA sequencer (Amersham Pharmacia Biotech). Nucleotide sequences of PCR and sequencing primers are shown in Table 1. The nucleotide and deduced amino acid sequences of APMV/Shimane67 F gene were compared to those of published APMVs by Clustal X [11]. Phylogenetic trees were generated using the Neighbor-Joining (NJ) method with 1,000 bootstraps.

Table 1. Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5' to 3')
For PCR	
F10	ATTAGAAAAACACGGGTAGAA
FR1215	CAACGACAGATGACCTGTTG
F270	GAAACATTATCCCGCATTCTAAC
HNR39	GAATGAGCAATTTGATAACCCAG
For Sequencing	
F10	ATTAGAAAAACACGGGTAGAA
FR258	GCAATGATTGTTTGGCACAATC
F270	GAAACATTATCCCGCATTCTAAC
F588	TAACCCCTGAATCCAGGCTAGG
F905	GTTCAACCTTACTAGAAAACATTAGC
F1090	CATGTCTCAATGGAAATCTAAGTGA
FR1215	CAACGACAGATGACCTGTTG
FR1545	GTGATTGCAATTATTAAGCATACCCTG
F1689	ACTCCCCATCCAGCCACAT
FR1746	ACCTACATGTTGGAGTGCCA
HNR39	GAATGAGCAATTTGATAACCCAG

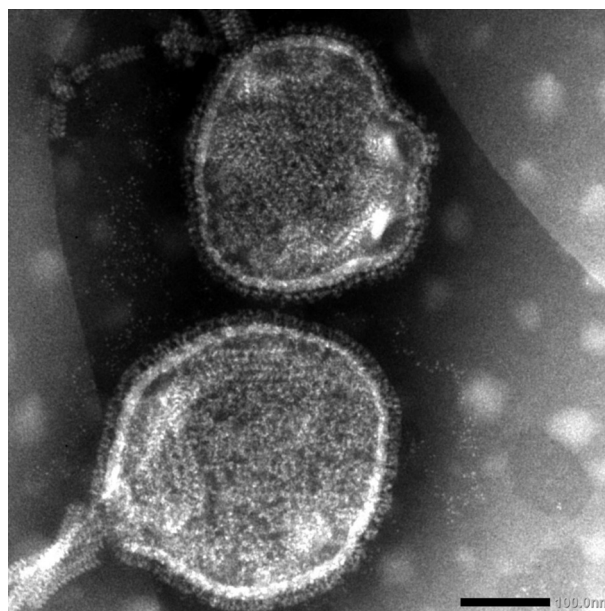


Fig. 1. Negative contrast electron micrograph of APMV/Shimane67 virus showing a partially disrupted particle with nucleocapsid emerging. $\times 200,000$.

RESULTS

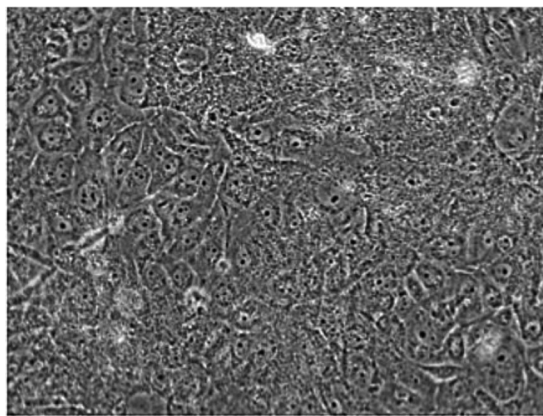
Electron microscopy: Electron microscopy of APMV/Shimane67 showed typical characteristics of a paramyxovirus. Virions were spherical or pleomorphic, enveloped particles (250–300 nm in diameter), and virion surfaces were covered with spiked projections of about 8–10 nm in length (Fig. 1).

HI test: In order to investigate the antigenic relationships between APMV/Shimane67 and other serotypes of APMV, HI tests were carried out (Table 2). As APMV-5 has no HA activity, and APMV-8 to 12 were not available in our labora-

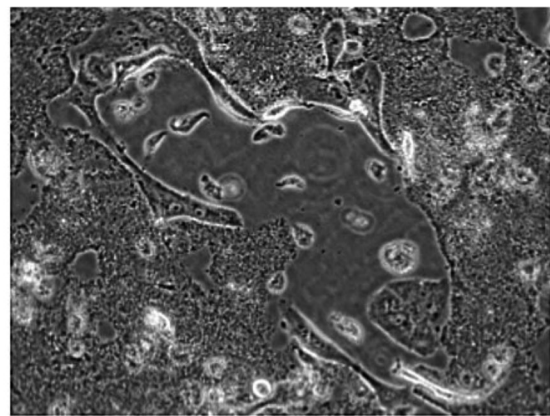
Table 2. Antigenic analysis of APMV/Shimane67 by HI tests with antisera against representative APMV strains

Antigen	Antiserum to						
	APMV/Shimane67	NDV/AK/415	APMV-2/Yucaipa	APMV-3/WI	APMV-4/MI	APMV-6/HK	APMV-7/TN
APMV/Shimane67	1280 ^{a)}	1,280	<40	<40	640	<40	320
NDV/AK/415	<40	5,120					
APMV-2/Yucaipa	<40		160				
APMV-3/WI	<40			160			
APMV-4/MI	<40				5,120		
APMV-6/HK	<40					640	
APMV-7/TN	<40						2,560

a) HI titer represents the reciprocal of the serum dilution inhibiting the activity of 4 hemagglutinating units of virus antigen. Blank: not tested.



Mock infected



APMV/Shimane67 (48 hr post infection)

Fig. 2. MDBK cells infected with APMV/Shimane67.

tory, HI tests against these 6 APMVs were not performed. Antiserum against APMV/Shimane67 showed the highest HI titer (1:1,280) with the homologous antigen, but little or no HI activity against other APMV serotypes. Reactivity of antisera against NDV/AK/415, APMV-2/Yucaipa, APMV-3/Wisconsin, APMV-4/Mississippi, APMV-6/Hong Kong and APMV-7/Tennessee on HI test was highest with each homologous antigen, while these antisera showed lower activity against APMV/Shimane67 (1:<40 – 1:1,280).

Growth in cell culture and pathogenicity of APMV/Shimane67: Growth of APMV/Shimane67 in avian and mammalian origin cell lines (DF-1, MDCK, MDBK, BHK, Vero and 293 cells) was measured. In the presence of trypsin, regardless of cell lines, APMV/Shimane67 efficiently grew and reached approximately $10^{5.0}$ EID₅₀/0.1 ml at 72 hr post-infection. Syncytium formation, a typical characteristic of paramyxovirus cytopathic effects, was clearly observed, particularly in MDBK cells (Fig. 2). In DF-1 cells without trypsin, titers of APMV/Shimane67 reached about $10^{3.5}$ EID₅₀/0.1 ml at 72 hr post-infection. These results indicate that external proteases are necessary for efficient cleavage of the F protein.

The MDT and ICPI of APMV/Shimane67 were 120 hr < and 0.0, respectively.

Nucleotide sequencing and phylogenetic analysis: A total

of 1,878 nucleotides of the genome sequence of APMV/Shimane67 were determined. The putative initiation codon, which better matched the Kozak consensus sequence ((G/A) NNATGG) [8], was the third ATG from 5'-end of the F10 primer binding site (data not shown). It contained a long open reading frame (ORF) composed of 1,638 nucleotides and encoded 545 amino acids. The nucleotide sequence of this ORF showed similarities with the F genes of NDVs and APMV-12/wigeon/Italy/3920_1/2005 (APMV-12/ITA) by discontinuous MegaBLAST search [24], indicating that this ORF was the F gene of APMV/Shimane67. The nucleotide and deduced amino acid identities between the F gene of APMV/Shimane67 and other APMV serotypes ranged from 42.9% with APMV-3/NLD to 62.7% with APMV-12/ITA, and from 28.9% with APMV-3/NLD to 67.3% with APMV-12/ITA, respectively (Table 3). The deduced amino acid sequence of the putative cleavage site of the APMV/Shimane F gene was QVRENRLVG (Fig. 3). This resembled the motif, a pair of single basic residues, of the lentogenic NDV. The phylogenetic tree of the F gene constructed with APMVs and members of subfamily *Paramyxovirinae* revealed that APMV/Shimane67 is a member of genus *Avulavirus* and is grouped with NDV, APMV-9 and APMV-12, while distinct from these APMV serotypes (Fig. 4).

Table 3. Nucleotide (upper right) and deduced amino acid (lower left) identities (%) among the fusion protein gene of avian paramyxoviruses

Virus	Shimane 67	NDV		APMV-2		APMV-3		APMV-4		APMV-5		APMV-6		APMV-7		APMV-8		APMV-9		APMV-10		APMV-11		APMV-12	
		AK/ 415	La- Sota	Yu- caipa	Ban- gor	NLD WI	HK/ D3	WI D3	YJ	Kunitachi	TWN/ Y1	ITA/ 4524-2	TN	DE/1053	Wakuya	NY/22	ITA/5709	FLK/324	FRA/ 100212	ITA/ 3920_1					
APMV/ Shimane67	-	53.6	56.1	54.3	45.6	44.6	42.9	43.0	43.7	43.3	46.2	44.6	45.3	43.5	46.6	46.8	46.8	53.3	53.4	46.3	43.8	43.8	62.7		
NDV/AK/415	52.8	-	71.8	71.5	46.4	46.8	43.3	41.0	42.8	42.0	54.4	44.6	45.0	44.7	47.0	47.4	58.5	58.4	58.4	45.6	43.1	43.1	55.1		
NDV/LaSota	53.7	87.3	-	84.4	47.5	46.6	42.3	41.9	41.6	41.4	46.8	44.4	45.0	43.5	46.7	46.9	58.2	60.3	60.3	46.1	42.1	42.1	55.5		
NDV/SF02	52.6	84.8	88.6	-	47.5	48.0	42.5	42.2	42.7	41.7	45.3	44.9	44.4	44.6	47.3	47.3	58.4	58.7	58.7	45.5	43.2	43.2	54.8		
APMV-2/ Yucaipa	37.3	40.4	40.6	40.4	-	69.8	40.7	41.1	42.8	41.8	50.2	50.9	51.8	47.2	60.5	60.9	46.3	47.2	47.2	59.2	48.5	48.5	46.7		
APMV-2/ Bangor	36.5	40.7	41.3	41.3	79.1	-	41.0	41.5	42.4	41.8	50.4	51.1	52.3	48.3	60.5	60.0	46.1	46.5	46.5	59.8	50.0	50.0	46.1		
APMV-3/ NLD	28.9	31.2	31.2	30.7	30.8	31.5	-	66.8	47.8	47.4	42.3	42.7	42.4	41.3	43.4	43.2	40.7	40.1	40.1	40.8	42.0	42.0	43.2		
APMV-3/WI	29.4	30.0	30.2	30.0	30.0	30.3	70.1	-	46.8	47.1	43.2	42.3	42.6	42.5	41.6	41.7	41.4	41.2	41.2	41.5	44.3	44.3	41.6		
APMV-4/HK/ D3	32.3	31.0	31.3	31.0	33.6	34.0	32.0	33.5	-	92.3	43.5	42.9	43.5	40.8	44.2	44.1	41.9	42.4	42.4	43.3	41.9	41.9	40.9		
APMV-4/KR/ YJ	32.1	30.8	31.3	30.6	33.6	34.2	32.2	33.1	97.7	-	42.5	43.1	44.0	40.6	44.1	43.7	41.1	42.4	42.4	42.4	41.0	41.0	41.3		
APMV-5/ Kunitachi	39.3	40.1	41.0	41.4	46.5	45.7	31.0	30.9	33.9	33.5	-	56.5	57.0	47.9	50.5	50.7	44.9	45.0	45.0	52.1	46.9	46.9	44.6		
APMV-6/ TWN/Y1	36.9	36.8	37.8	37.8	48.8	49.4	31.6	29.8	33.6	33.0	53.7	-	72.6	47.3	49.3	49.4	45.1	44.9	44.9	52.3	48.9	48.9	45.6		
APMV-6/ ITA/4524-2	37.3	37.1	37.4	37.6	49.2	48.6	31.2	29.5	33.8	33.6	54.1	85.7	-	47.0	50.4	50.0	44.8	45	45	52.2	49.2	49.2	45.1		
APMV-7/TN	35.7	38.1	39	38.8	38.1	38.8	28.5	30.1	30.1	29.3	38.0	37.0	36.9	-	49.2	49.0	42.8	43.7	43.7	48.8	53.0	53.0	44.4		
APMV-8/ DE/1053	36.4	40.1	41.1	41.1	63.7	63.7	31.8	30.6	35.9	35.9	45.5	48.2	47.3	38.3	-	97.1	47.3	47.6	47.6	59.5	48.2	48.2	46.8		
APMV-8/ Wakuya	36.2	40.1	41.1	41.1	63.7	63.7	31.8	30.6	35.9	35.7	45.5	48.2	47.3	38.1	99.4	-	47.5	48.0	48.0	59.5	48.4	48.4	46.4		
APMV-9/ NY/22	48.4	56.4	55.9	56.4	37.8	39.1	28.2	28.3	28.4	28.6	36.5	37.4	36.6	35.6	37.9	37.9	-	89.2	89.2	45.5	42.8	42.8	54.3		
APMV-9/ ITA/5709	50.1	58.1	58.6	58.4	37.8	39.6	29.3	29.2	28.6	28.8	37.8	37.9	37.5	36.6	39.4	39.4	92.7	-	-	46.5	42.6	42.6	55.2		
APMV-10/ FLK/324	37.8	39.5	39.9	39.5	61.7	60.3	29.0	28.1	32.4	32.2	46.3	48.1	48.0	38.2	61.4	61.4	38.9	39.1	39.1	-	48.0	48.0	46.5		
APMV-11/ FRA/100212	31.6	32.6	33.9	33.9	42.4	41.8	31.0	33.0	32.5	32.2	39.9	39.7	41.7	36.0	40.4	40.4	32.7	33.3	33.3	39.4	-	-	43.9		
APMV-12/ ITA/3920_1	67.3	55.1	54.6	53.8	38.1	37.3	29.5	28.5	32.1	31.9	37.4	37.7	37.9	36.5	36.1	36.1	52.3	53.2	53.2	37.8	32.7	32.7	-		

	F2←	→F1
APMV/Shimane67	Q	V R E N R L V G
NDV (lentogenic)	G	G R Q G R L I G
NDV (velogenic)	G	R R Q R R F I G
APMV-2/Yucaipa	D	K P A S R F V G
APMV-3/WI	P	R P S G R L F G
APMV-4/HK/D3	V	D I Q P R F I G
APMV-5/Kunitachi	G	K R K K R F V G
APMV-6/TWN/Y1	P	A P E P R L I G
APMV-7/TN	T	L P S S R L I G
APMV-8/DE	T	Y P Q T R L I G
APMV-9/NY	R	I R E G R I F G
APMV-10/FLK/324	D	K P S Q R I I G
APMV-11/FRA/100212	D	S G T K R F V G
APMV-12/ITA/3920_1	R	G R E P R L V G

Fig. 3. Amino acid sequences of F protein cleavage site from APMVs. NDV (lentogenic); strain LaSota, NDV (velogenic); strain Herts 33, APMV-4/HK/D3; APMV-4/duck/Hong Kong/D3/1975, APMV-5/Kunitachi; APMV-5/budgerigar/ Japan/ Kunitachi/1974, APMV-6/TWN/Y1; APMV-6/duck/Taiwan/Y1/1998, APMV-8/DE; APMV-8/goose/Delaware/1053/1976, APMV-9/NY; duck/New York/22/1978, APMV-10/FLK/324; APMV10/penguin/Falkland Islands/324/2007, APMV-11/FRA/100212; APMV-11/common_snipe/France/100212/2010. Abbreviations of other APMV are given in the text.

DISCUSSION

The typing of APMVs has mainly been conducted by HI test [1]. In addition, because the nucleotide sequence data from various APMVs have been collected in DNA databases, genetic analysis has also been attempted to classify new APMV isolates, and the results obtained have demonstrated the effectiveness of this approach [4, 12, 19]. On HI test, the anti-APMV/Shimane67 serum reacted strongly (HI titer of 1:1,280) with homologous virus and did not react with the other APMVs (HI titer of 1: <40). In addition, sera against other APMVs (NDV, APMV-2, 3, 4, 6 and 7) showed weaker reactivity with APMV/Shimane67 than with homologous viruses (Table 2). These results indicate that APMV/Shimane67 is serologically distinct from NDV, APMV-2, 3, 4, 6 and 7. Anti-NDV, APMV-4 and 7 sera showed some cross reactivity (HI titer of 1:320–1:1,280) with APMV/Shimane67. It is possible that this cross reactivity is caused by high titers of hyperimmune sera (HI titer of more than 1:2,560 against homologous viruses). The sera used in this study were prepared by multiple immunization of APMV antigen. Miller *et al.* [12] also reported cross reactivity on HI test of APMVs using hyperimmune serum.

Analysis of the F gene of APMV/Shimane67 also revealed the relationship of this virus with other APMVs. The ORF of the APMV/Shimane67 F gene was 1,638 nucleotides long and encoded 545 amino acids. Although these nucleotide and amino acid lengths for the F gene were the same as those of APMV-6/duck/Italy/4524-2/07 [23], the identities of nucleo-

tide and amino acid sequences between APMV/Shimane67 and APMV-6/duck/Italy/4524-2/07 were low (45.3% and 37.3%, respectively) (Table 3). The amino acid sequence at the F protein cleavage site of APMV/Shimane67 was deduced as being QVRENRLVG, which resembles the motif of lentogenic NDV (Fig. 3). This motif was observed in the F proteins of APMV/Shimane and NDV, APMV-9 and 12, and interestingly, these four APMVs containing conserved amino acid sequence motifs at the F protein cleavage site also formed one phylogenetic cluster (Fig. 4). The phylogenetic tree showed that APMV/Shimane67 is a member of genus *Avulavirus*, is distinct from other APMVs and showed close relationship with APMV/wigeon/Italy/3920-1/2005 (APMV-12/ITA/3920-1). APMV-2, -3 and -6 viruses have antigenic differences constituting the antigenic subgroup within each serotype [3, 9, 18]. Similar situations in phylogenetic analysis were also observed in the nucleotide and amino acid identities of the F gene (Table 3). APMV/Shimane67 exhibited the highest nucleotide and amino acid identities (62.7% and 67.3%, respectively) with APMV-12. These identities were lower than those observed between the subgroups, APMV-2/Yucaipa and APMV-2/Bangor (69.8% in nucleotide and 79.1% in amino acid sequence), and APMV-3/NLD and APMV-3/WI (66.8% in nucleotide and 70.1% in amino acid sequence) (Table 3). These observations confirm a distant relationship corresponding to APMV serotype between APMV/Shimane67 and APMV-12/ITA/3920-1.

The pathogenicity of APMV/Shimane67 to chickens was

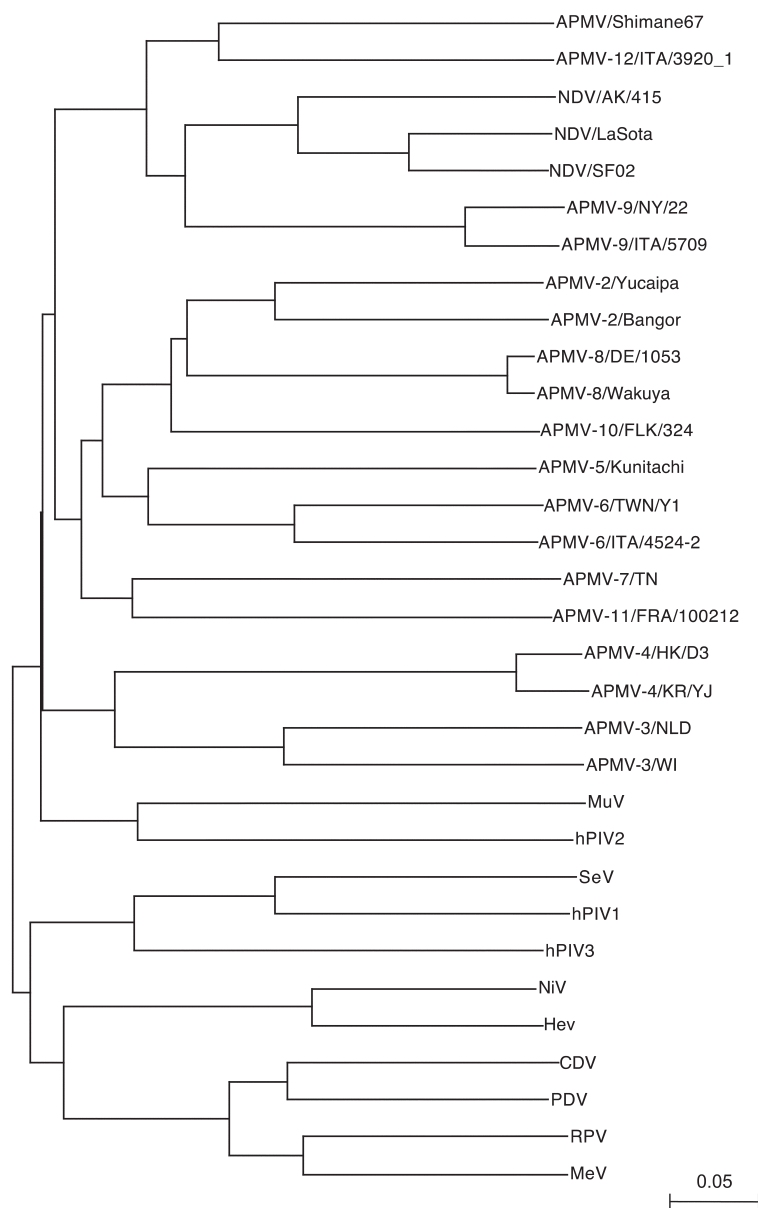


Fig. 4. Phylogenetic tree of F gene sequences from subfamily *Paramyxovirinae*. The phylogenetic tree was generated using the neighbor-joining algorithm with 1,000 bootstrap replicates in Clustal X.

evaluated by MDT and ICPI tests. MDT (120 hr <) and ICPI (0.0) of APMV/Shimane67 were applicable to the lentogenic NDV and revealed that the virus had no virulence in chickens. The amino acid sequence at the F protein cleavage site is the major determinant of NDV pathogenicity to chickens. As described above, the putative cleavage site of the APMV/Shimane F protein is QVRENK/LVG, which resembles lentogenic NDV. Non-virulence of APMV/Shimane67 may be affected. The introduction of an amino acid sequence of velogenic NDV at the F protein cleavage site by reverse genetics did not increase the virulence of APMV-2, 4 and

7 in chickens [7, 17, 22]. However, it has been reported that lentogenic NDV isolated from wild geese changed to velogenic virus by amino acid substitutions in the F protein cleavage site during serial passage in chickens [15]. Whether the virulent variant of APMV/Shimane67 will emerge with multiple passaging of APMV/Shimane67 in chickens is of interest.

Recently, whole genome sequences from various APMV serotypes have been reported, and the classification of APMVs using those whole genome sequence data has been proposed [4, 12, 14, 19]. In this study, only the F gene of

APMV/Shimane67 was identified; however, data obtained have suggested that APMV/Shimane67 is a novel APMV serotype, APMV-13. To emphasize this idea, the full-length genome sequence of APMV/Shimane67 needs to be determined.

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