Surveillance of avian paramyxoviruses (APMVs) in wild bird population in Hokkaido and characterization of the APMV isolates

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北海道に飛来する野鳥における鳥パラミクソウイルスのサーベイランスと分離ウイルスの特徴付け

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ラピワン　タムパイサーン

Contents

Abbreviation

A	aa	amino acid
	AIV	avian influenza virus
	APMV	avian paramyxovirus
B	BSA	bovine serum albumin
C	cDNA	complementary DNA
	CEFs	chick embryo fibroblasts
	CPE	cytopathic effect
D	DNA	deoxyribonucleic acid
	dpi	day post infection
E	EAA	East Asian-Australasian
	ELISA	enzyme-linked immunosorbent assay
	EM	electron microscopy
F	F	fusion
G	GE	gene-end
	GS	gene-start
H	HA	hemagglutination activity
	HI	hemagglutination-inhibition
	HN	haemagglutinin-neuraminidase
I	IBD	infectious bursal disease
	ICPI	intracerebral pathogenicity index
L	L	RNA-dependent RNA polymerase
M	M	Matrix

	mAb	monoclonal antibody
	MDBK	Madin-Darby bovine kidney
	MDCK	Madin-Darby canine kidney
	MEM	minimum essential medium
N	ND	Newcastle disease
	NDV	Newcastle disease virus
	NP	nucleocapsid protein
	nt	nucleotide
O	OD	optical density
P	P	phosphoprotein
	PBS	phosphate buffered saline
	PBS-T	phosphate buffered saline containing Tween 20
	PCR	polymerase chain reaction
R	RNA	ribonucleic acid
	RT-PCR	reverse transcription polymerase chain reaction
S	SH	small hydrophobic protein
	SPF	specific pathogen free
T	$TCID_{50}$	50% tissue culture infective dose
	TMB	3,3′,5,5′-Tetramethylbenzidine
V	Vero	African green monkey kidney
	VN	virus neutralization

General Introduction

Historical background

Avian paramyxoviruses (APMVs) are one of the important virus impacting birds around the world. In 1926, the first APMV was discovered from the outbreak of virulent APMV-1 or Newcastle disease (ND) in Java, Indonesia (Kraneveld, 1926) followed by the case in Newcastle-upon-Tyne in England in the same year (Doyle, 1927). However, there was a report showing evidence that the disease might occur before this date. Macpherson (1956) described that an epizootic among poultry in the Western Isles of Scotland in 1898 was the case of ND. The second serotype of APMV, designated APMV-2 was isolated in 1956, and then other serotypes of APMV have been reported. Among all APMVs, APMV-1 or called ND virus has been well characterized because the virulent APMV-1 strain causes severe diseases in poultry industry worldwide. Besides high morbidity and mortality, the preventive strategies such as surveillance program, and intensive vaccination program also result in significant economic cost. ND is included in OIE-Listed diseases which notification of an outbreak is required by OIE (OIE, 2016). With concern of the severity of ND, many countries invoke the highly strict statutory control measures to control the disease (Council of the Economic Community, 1992). Consequently, the outbreak of the disease affects the international trading of poultry industry immensely. Nine serotypes of APMV (serotype 1–9) had been isolated until 1978, while most of research on APMVs have been focused on APMV-1, very little is known about virological properties of serotypes of APMV, which are usually isolated from various species of wild birds. Although APMV-2 to APMV-9 were low virulent to chickens, some serotypes were reported to cause respiratory disease and egg production loss in other poultry, such as ducks and turkeys (Kim et al., 2012; Samal, 2011).

Taxonomy

APMVs belong to the family *Paramyoxvirida*e, order *Mononegavirales*. All viruses in this order are single-stranded, nonsegmented, negative-sense, enveloped RNA viruses. The family *Paramyxoviridae* contains many infectious viruses found in humans and animals, such as measles virus, human parainfluenza virus, mumps virus, bovine respiratory syncytial virus (Wang et al., 2012). In the past, it consisted of 2 subfamilies; *Paramyxovirinae* and *Pneumovirinae*, but due to the expanding of new viruses in this family over the last five years, Pneumoviruses have been split to the new genus named Orthopneumovirus of the new family *Pneumoviridae* recently (Afonso et al., 2016). The family *Paramyxoviridae* contains seven genera. All APMVs belong to the genus *Avulavirus* which includes different serotypes of APMV. All APMVs were isolated from avian species and have been classified into 13 serotypes (APMV-1 to APMV-13) antigenically and/or genetically, so far (Afonso et al., 2015; Yamamoto et al., 2015).

Virus structure, genome, and protein

APMV viral particles are pleomorphic with spike-like projections surrounding viral envelope but mostly spherical, with ranging from 100–500 nm in diameter. Variable length of filamentous form also can be observed (Suarez et al., 2013). The viral envelope is derived from the plasma membrane of the host cell. The envelope comprises of two transmembrane glycoproteins, namely fusion (F) and hemagglutinin-neuraminidase (HN) proteins. The nucleocapsid protein possesses a herringbone type pattern with a diameter 15–20 nm (Catroxo et al., 2012).

The genome of APMV is a non-segmented negative-sense single-stranded RNA. The complete genome is 15,500 nucleotides (nt) long in average, while the shortest is APMV-2

genome with the length of 14,904 nt and the longest is APMV-5 genome with the length of 17,262 nt (Samuel et al., 2010). The genome length of APMVs always follows the rule of six, which is necessary for virus replication (Kolakofsky et al., 1998). All APMV genomes encode six proteins from 5′ to 3′ as follows: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), F, HN and RNA-dependent RNA polymerase (L), except APMV-6 which has additional coding sequence between the F and HN protein called small hydrophobic protein (SH) (Suarez et al., 2013). In paramyxovirus, V and W proteins can be encoded by RNA-editing of P gene with the addition of one or two G nucleotides at this site, respectively (Steward et al., 1993). The both 3′ and 5′ ends of the genomic RNA contain extracistronic regions known as the leader and the trailer, respectively. The viral RNA polymerase starts transcription at the 3′ end and proceeds downstream in a sequential manner generating each mRNAs by terminating and reinitiating at gene-end (GE) and gene-start (GS) of each gene (Lamb and Parks, 2013).

Virus replication

F and HN proteins play key roles for APMV to enter host cells. HN protein recognizes and binds to sialic acid receptors on the host cell surface. This process triggers the fusion activity of the F protein to cell surface leading to the fusion between viral envelope and host cell membrane (McGinnes et al., 2002). Then, the assembly of RNA, NP, P, and L proteins, termed ribonucleoprotein complex can enter the cytoplasm of host cells where the negative-sense viral RNA is transcribed into the structural mRNAs and translated to viral proteins. In the host cytoplasm, transcription and replication of the viral genome occurs by the action of L. The synthesized viral components are transported and assembled to the plasma membrane by the action of the M protein. Finally, the new virus particles are released from cell by budding process (El Najjar et al., 2014).

Host range and pathogenicity

APMV-1 is known to infect more than 250 avian species in 27 orders. In poultry, chickens are the most highly susceptible to virulent strains and usually develop severe clinical signs, while the susceptibility among other poultry is variable (Wakamatsu et al., 2006). For example, the clinical signs in turkeys was less severe than in chickens, and turkeys could possibly be a subclinical carrier for some of the isolates (Piacenti et al., 2006). The severity of APMV-1 clinical signs depends on the age of the host, the immunity of the host, the species of the bird, the virus strain, and the amount of virus obtained. Low virulent or lentogenic strains usually cause mild respiratory disease only in younger chickens. Intermediate virulent or mesogenic strains can cause respiratory signs along with mild neurological signs and egg production loss. Highly virulent or velogenic strains cause severe disease in chickens with up to 100% mortality and infected chickens show watery diarrhea and respiratory signs such as cyanosis, head and neck tissue swelling. Neurological signs, such as paralysis, torticollis, circling are also commonly seen (The Center for Food Security & Public Health, 2016). Wild birds, particularly waterfowl are reservoir hosts of low virulent strains. However, cormorants were found to carry and shed virulent APMV-1 in Canada and the US (Diel et al., 2012b; Kuiken, 1999).

APMV-2 to APMV-13 were isolated from various kinds of birds. Most of them were found in wild birds, except APMV-3 in turkeys and APMV-5 in budgerigar (Suarez et al., 2013). Experimental infection of APMV-1 to APMV-9 does not produce any clinical signs in chickens and duck (Kim et al., 2012). Similarly, hamsters, mice, and Rhesus Macaques also exhibit the same results in the experimental infection except some of APMV-9 that cause mild clinical signs (weak, weight loss, rough skin coat) in hamsters (Khattar et al., 2011; Khattar et al., 2013; Samuel et al., 2011).

Transmission

Among all APMVs, virulent strains of APMV-1 are the most highly contagious virus. The infected birds can shed viruses via feces and respiratory secretions. Hence, it can be transmitted to susceptible birds via inhaling aerosolized virus, or ingesting feces or contaminated feed and water. Infectivity of APMV-1 can remain in the bird carcasses and persist for month at cold temperature (Beard and Hanson, 1984). Contaminated fomites also have potential to transmit the virus. The spread of virus from farm to farm can be caused by the movement of APMV-1-contaminateed equipment, vaccine, or litters (Burridge et al., 1975; Jorgensen et al., 2000).

As a results from infection experiments of other APMVs in avian and mammal species**,** oro-fecal and respiratory seem to be the main route of transmission the same as that of APMV-1 (Khattar et al., 2011; Kim et al., 2012; Samuel et al., 2011). However, the possible carrier or vector is still required a further study.

APMVs identification and diagnosis

To diagnose APMV infection, isolation and characterization of virus isolates are essential because the clinical signs and gross lesions are not specific sufficiently. APMVs can be isolated from feces, tracheal, oropharyngeal, and/or cloacal swabs in live infected birds, and from tissue sample (lung, brain, liver, spleen, kidney) of dead birds. Twenty % (w/v) homogenated suspension of sample is usually inoculated into the allantoic cavity of nine- to eleven-day-old embryonated chicken egg. The eggs are incubated at 37°C for 4–7 days and are candled twice daily. The dead eggs or eggs after the end of incubation, are chilled at 4°C, and allantoic fluids are collected to be tested for haemagglutination activity (HA). HA-positive samples are tested with specific antiserum against each serotype of APMVs in haemagglutination-inhibition (HI) tests. However, the limitation of this method is due to available antisera against each serotype,

especially those to against novel serotypes (10–13) are not available. In addition, APMV-5 is difficult to isolate because the inability to grow in allantoic activity and lack in HA activity (Nerome et al., 1978).

Molecular-based techniques, such as reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR are usually used to detect viral RNA directly in case of APMV-1 infection. With NDV vaccination used in commercial poultry, nucleotide sequencing of the F gene is helpful to distinguish vaccine strains from field strains (Aldous and Alexander, 2001). For other APMVs, the increase of their full genome sequence data may lead to the establishment of RT-PCR assays for APMV serotyping in the near future.

Serological assay may not be an optimal tool to diagnose APMV-1 infections because it cannot distinguish antibodies developed by natural infection or from vaccination. Instead, it is usually applied for monitoring vaccination efficiency in commercial poultry flocks. The commonly used serological assays are HI assays and enzyme-linked immunosorbent assays (ELISAs). Other tests, such as virus neutralization (VN) assay, immunoflourescent, immunohistochemistry, or immunocytochemistry, are also available, but may be applied occasionally (Cattoli et al., 2011).

Prevention and control

A high level of biosecurity and good hygiene in poultry farms is the effective strategy to prevent an introduction of virus from other infected poultry farm and/or from wild birds. In some countries, the stamping-out policy along with the quarantine and movement control is shown to succeed in controlling the disease (Animal Health Australia, 2014). In most countries, APMV-1 vaccination program is commonly used in commercial poultry. On the contrary, the vaccine for other APMVs is still not established because they do not cause serious disease in

poultry, except APMV-3 that causes disease in turkeys (Awang and Russell, 1990). Commercial inactivated oil-emulsion vaccines against APMV-3 are available for using in turkey breeder flocks in the US and Europe (Eskelund, 1988). In addition, The vaccine candidates for parakeets were evaluated to be safe and efficient by Beck et al. (2003).

Distribution of APMVs

Epizootics of virulent APMV-1 regularly occurs in Asia, Africa, Middle east but occasionally in Europe (Alexander et al., 2004; Ashraf and Shah, 2014; Gardner and Alders, 2014; Wang et al., 2015). Migratory wild birds are considered as a reservoir of many pathogens including virus, such as avian influenza virus (AIV), APMVs, alphavirus, flavivirus, nairovirus, etc. (Jourdain et al., 2007; Reed et al., 2003). With the ability to travel over long distance between intercontinental regions, wild birds potentially disperse viruses during their migration and may harbor virulent APMV-1 into poultry. In North America, highly virulent APMV-1 has been circulated in some cormorant population (Diel et al., 2012b; Kuiken, 1999; White et al., 2015). Low virulent APMV-1 from wild birds was evidenced to have a potential to become virulent. The virulent APMV-1 causing outbreak in Australia during 1998–2000 originally evolved from low virulent APMV-1 of wild birds (Gould et al., 2001). Shengqing et al. (2002) also demonstrated that low virulent APMV-1 can become virulent after multiple passaging in experimental chickens. These facts raise awareness that backyard chickens have high risk to be exposed to virus from wild birds. Traditional backyard poultry systems or small scale poultry farming is still important in the developing countries, in which the farmers may lack of financial resources and chances to get veterinary service. In Nigeria, Oman, and Uganda the presence of wild birds has been reported as one of a risk factors associated with the sporadic outbreaks of

ND and infectious bursal disease among free-roaming village chickens (Oluwayelu et al., 2014; Otim et al., 2007; Shekaili et al., 2015).

Other APMVs were reported from a variety species of birds around the world. Many of them were isolated as a part of AIV surveillance programs which have been extensively conducted in the last decade. The incidence of other APMVs circulating in wild birds population was reported in some countries; however, its incidence is still limited (Goekjian et al., 2011; Lindh et al., 2008). The distribution of APMVs in wild bird population and the genetic diversity among them are not well understood.

Scope of this thesis

Although many research works of APMVs have been conducted, the majority was on virulent APMV-1 in chickens. The information of other APMVs is still lacking in knowledge. According to the aforementioned information, virulent APMV-1 strains can be isolated from some wild birds and low virulent strains from them may turn into highly virulent and cause disease. Moreover, novel serotypes of APMV have been reported in the last few years which may imply the genetic diversity among these viruses. Therefore, to control and prevent APMV infections in poultry, understanding of the epidemiology of APMVs circulating in wild birds is still need to be conducted. To fulfill this proposal, in chapter I, the distribution of APMVs in wild birds in Eastern Hokkaido during 2009–2013 was described. Chapter II, APMV-1 isolated from wild birds were genetically and antigenically characterized and compared with virulent APMV-1 from chickens. In chapter III, a discovery of a novel serotype of APMV designated APMV serotype 14 during the surveillance was described. Here, then, the virological, serological, and genetic characterization of this virus were described.

Finally, this thesis is completed with a general discussion.

Chapter I

Surveillance of avian paramyxovirus in wild bird population in eastern Hokkaido in 2009–2013

1.1 Introduction

APMVs belongs to the genus *Avulavirus* within the family *Paramyxoviridae.* They have been classified into 9 serotypes defined by HI tests so far (Wang et al., 2012). However, cross-reactivity between different serotypes can occur (Alexander et al., 1983; Lipkind and Shihmanter, 1986; Tumova et al., 1979). Recently, the presence of four novel APMV serotypes were reported: APMV-10 from the rockhopper penguins (Miller et al., 2010), APMV-11 from the common snipe (Briand et al., 2012), APMV-12 from the Eurasian wigeon (Terregino et al., 2013), and APMV-13 from goose feces (Yamamoto et al., 2015).

The F protein of APMV-1 plays a key role of virus to enter host cells which is therefore associated with pathogenicity. The cleavage of the F protein into F1 and F2 by cellular protease is required to initiate the fusion of the viral envelope and host cell membrane (Lamb and Parks, 2013). Based on the phylogenetic analysis of the partial or complete nucleotide sequences of the F gene, the lineage or genotype classification systems were established to classify APMV-1 isolates (Aldous et al., 2003; Czegledi et al., 2006; Diel et al., 2012a). The amino acid (aa) motif of the F protein cleavage site is also used to determine virus virulence to chickens. Velogenic (highly virulent) APMV-1 strains have a multibasic aa motif (consensus sequence: ^{112}K/R-R-X-K/R-R↓F^{117}) at this site, while APMV-1 strains with monobasic aa motif

(consensus sequence: ^{112}G/E-K/R-Q-G/E-R↓L^{117}) are defined as lentogenic (low virulent) virus (OIE, 2012).

Migratory wild birds were considered as a natural reservoir for AIVs and APMVs (Deibel et al., 1985; FAO., 2007). The movement of them during seasonal migration plays an important role in the carrying and spread of virus. Migratory routes of birds are grouped together as "flyways". As shown in Fig. 1.1, Japan is located in the East Asian-Australasian (EAA) flyway which is one of the world's major flyways for migratory birds (EAAFP Secretariat, 2010). The breeding range of birds in EAA flyway overlaps with the Central Asian flyway and Pacific flyways covering the intercontinental area in North America and Europe (Bamford et al., 2008). Hokkaido is one of the important stopover sites in Japan for migratory birds including shorebirds and Anatidae species (ducks, geese and swans). The common migratory route for these birds are the Sakhalin-Kurile route, the Kamchatka route, and the Crossing of the Sea of Japan route which cover the area from Eastern Siberia to Japan (Fig. 1.2) (Food Safety and Consumer Affairs Bureau, 2007). Moreover, they also use the Korean Peninsula route to move from eastern and northeastern Asia to Hokkaido (Hoshino et al., 2012).

The infections of APMVs in both domestic and wild birds have been reported in many countries (Fornells et al., 2013; Goekjian et al., 2011; Hanson et al., 2005; Lindh et al., 2008). Some surveillances studies of APMV in wild birds have been conducted in the two districted areas of Japan (Fujimoto et al., 2010; Mikami et al., 1982; Umali et al., 2014), except one study performed by Mase and Kanehira (2015). However, this study was aimed to APMV-1 only. In Hokkaido, surveillance studies in wild birds have not been reported since Mikami et al. (1982) conducted the small-scale surveys for isolation of APMV from migratory ducks in Betsukai-

cho, Hokkaido during 1978 to 1981. Thus, the distribution of other APMVs including APMV-1 circulating in wild birds flying in Japan is poorly understood.

Besides APMV-1, the genetic diversity within serotype has been found to exist in APMV-2, APMV-3, and APMV-6 (Bui et al., 2014; Karamendin et al., 2015; Kumar et al., 2010; Subbiah et al., 2010; Xiao et al., 2010). Therefore, the surveillance is required to monitor the status and the alteration of APMVs in wild bird populations.

In this chapter, a surveillance for APMVs conducted in wild bird population in eastern Hokkaido from 2009 to 2013 was described.

1.2 Materials and methods

Sample collection

A total of 10,606 samples, either cloacal swab or fecal samples (droppings), were collected from wild birds in eastern Hokkaido, Japan, during 2009 through 2013. The samples were collected from a variety of water bird species at lakefront, seashore, pond and riverside in the Abashiri, Hidaka, Kushiro, Nemuro, and Tokachi districts. Licensed bird banders identified bird species captured using mist, and collected cloacal swab samples. The cloacal samples were placed in sterile tubes containing a virus transport medium (M4RT; Remel, Inc., Lenexa, KS) and stored at -80^{o}C until use. Fresh fecal samples were collected near the birds' habitats, and suspended in the minimum essential medium (MEM, pH 7.4; Nissui Pharmaceutical co., Ltd., Tokyo, Japan) supplemented with 0.5% bovine serum albumin (BSA), kanamycin (a final concentration: 1 mg/ml), gentamycin (100 μg/ml) and amphotericin B (10 μg/ml) to prepare 20% homogenates and stored at -80^{o}C.

Virus isolation and identification

The samples mentioned above were centrifuged at a low speed, and the supernatants were used for virus isolation. Virus isolation was carried out as previously described (Bui et al., 2011) . Briefly, the samples were treated with antibiotics and 0.1 ml of the sample supernatant was inoculated into the allantoic cavity of ten-day-old embryonated chicken egg. The inoculated eggs were incubated for 4 days at 37°C. The dead eggs or the living eggs at the end of incubation were chilled overnight at 4°C. The allantoic fluids collected from the eggs (E1) were tested for HA activity using 0.5% chicken red blood cells according to Manual for the laboratory diagnosis and virological surveillance of influenza (WHO, 2011). HA-negative E1 samples were forwarded to the second egg inoculation, and the obtained allantoic fluids (E2) were tested for HA activity. HA-positive (E1 or E2) samples that were negative for AIV were subjected to further characterization for APMVs. The presence of AIV was examined using a real-time RT-PCR for the M gene of the influenza A virus as previously described (Bui et al., 2011) (data not shown).

HI test

To identify APMVs, HI tests were conducted according to the guidelines of the OIE (OIE, 2012). Antigens of APMV-1 to APMV-9 and an antiserum against each serotype described below were used as references except for APMV-5 (not available in our laboratory). APMV-2/chicken/California/Yucaipa/56, APMV-3/turkey/Wisconsin/68, APMV-4/duck/Hong Kong/D3/75, APMV-6/Dk/HK/77, APMV-7/dove/Tennessee/4/75, APMV8/goose/Delaware/1053/76 and APMV-9/duck/New York/22/78 and specific chicken antiserum against each virus strain used as reference antigens were purchased from National Veterinary Service Labs (Ames, IA) while APMV-1/chicken/Japan/Ibaraki/85 was provided by National Institute of Animal

Heath, Japan, and antiserum against APMV-1 was obtained from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan).

RT-PCR

Total RNA was extracted from the HA-positive allantoic fluids using ISOGEN-LS (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. First-strand cDNA was produced using 2.0 µl of random hexamer primers (0.5 µg/µl) (Invitrogen, Carlsbad, CA), 1. µl of 10 mM dNTP, 4.0 µl of RNA, 6 µl of DEPC-water, 4.0 µl of 5X first-strand buffer, 2.0 µl of 0.1 M DTT, 1 µl of RNAse inhibitor and 1.0 µl of MMLV reverse transcriptase (Invitrogen) to a total volume of 21 µl. The reaction was incubated at 25°C for 10 min, 37°C for 50 min, and 65°C for 10 min and placed on ice. PCR with 20 µl of total volume of reaction was performed using 1 µl of cDNA, 1 µl of each primer (20 µM), 1.6 µl of dNTP (2.5 mM each dNTP), 2 µl of 10X Ex Taq Buffer, 13.3 µl of DEPC water and 0.1 µl Takara Ex Taq (Takara, Shiga, Japan). The PCR conditions were as follows: 94^{o}C for 5 min; 40 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 40 sec; and final extension at 72°C for 10 min. The primer sets were designed based on the F gene sequences of APMVs available in GenBank (Table 1.1) by Dr. V.N. Bui (National Institute of Veterinary Research, Vietnam) who was my co-worker in my laboratory. Sequences of each APMV serotype were aligned and the conserved region was tracked out for designing primers. Degeneracy was incorporated into the primer to account for diversity of the F gene of each serotype which sequence is available in GenBank. Obtained PCR products were

electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Nucleotide sequencing and phylogenetic analysis

The PCR products were separated by 1.5% agarose gel electrophoresis and purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified products were used as a template for sequencing reactions using a BigDye terminator ver.3.1 cycle sequencing kit (Applied Biosystems, Foster, CA) and the primers using for amplification of the PCR product were used for sequencing. Nucleotide sequencing was performed with an ABI 3500 Genetic Analyzer (Applied Biosystems). The obtained sequences were analyzed by BioEdit and compared with other available sequences using BLAST homology searches. Evolution distances were calculated using the aligned sequences and the Kitamura 2-parameter model. Phylogenetic trees were constructed using the Maximum Likelihood (ML) method supported by 500 bootstrap replicates and MEGA 6.0 software (Tamura et al., 2013) .

1.3 Results

A total of 10,606 samples were collected during 2009–2013. The samples were collected from birds in the order *Anseriformes* (8,337 samples), *Charadriiformes* (2,087 samples), and others (182 samples). Ninety three heamagglutinating non-AIV isolates were obtained. The summary of APMVs isolates obtained from wild birds was shown in Table1.2. Based on HI test and RT-PCR, they were identified as APMV-1 (18 isolates), APMV-4 (58 isolates), APMV-6 (13 isolates), APMV-9 (2 isolates), and two heamagglutinating non-AIV isolates remained unidentified.

Genetic analysis for APMV-1 isolates

As shown in Table 1.3, the nucleotides of seven out of the 18 APMV-1 isolates in 2009, 2011, and 2013 are highly related to those of Chinese strains isolated in 2008, 2009, 2011, and 2014 with >99.44% nucleotide identity, six isolates isolated in 2010 and 2013 had a high nucleotide identity (>98.88%) with the Korean strains reported in 2007, three isolates were genetically very similar to the US strains isolated in 2001 and 2009 with >95.54% nucleotide identity, and only one isolate in 2013 was very similar to the Russian strain isolated in 2007 with 99.72% nucleotide identity. Only one isolate in 2010 shared a high nucleotide identity with the strain isolated in 2009 in Tottori prefecture (99.72% nucleotide identity). As shown in Table 1.3, two patterns of aa sequences of the F protein cleavage site were determined as follows: ^{112}GKQGR↓L^{117} (12 isolates), ^{112}ERQER↓L^{117} (6).

Phylogenetic tree was generated based on partial sequences of the F gene containing the cleavage site. The tree revealed that all the isolates were classified into two genetic classes (I and II) (Fig. 1.3).

Six isolates in 2009–2011, and 2013 were classified into class I to which low virulent APMV-1 strains derived from wild birds belong. Four (●, ■, ▲) of them were closely related to Japanese and Chinese strains, while the remaining two isolates (▽) isolated in 2011, 11OG1095 (Anas sp./Japan/11OG1095/2011) and 11OG1114 (APMV-1/Anas sp./Japan/11OG1114/2011) , fell into the same genetic group as the US strains that were isolated in Alaska.

Twelve isolates were grouped into genotype I of class II, apart from genotypes II and VI consisting of the strains derived from chickens and racing pigeons. Ten isolates (■, ▲, ▽) formed a cluster with the strains isolated from wild birds in Japan, China, Korea, and Russia. The remaining two gull isolate 9KS0098 (Slaty-backed gull/Japan/9KS0098/2009, ●) and 13G109

(Gull/Japan/13G109/2013, ■) were closely related to the Chinese strain isolated in a gull in 2014 and the US strain isolated in a red knot in 2001.

Genetic analysis for APMV-4 isolates

A total of 58 nucleotide sequences of APMV-4 isolates were obtained. A BLAST search results showed that 57 APMV-4 isolates were closely related to Mallard/South Korea/YJ/2006 isolated from a mallard duck in South Korea with 95.89-99.39% nucleotide identities. Only one isolate 10KI0182 (APMV-4/Anas sp./Japan/10KI0182/2010) had the highest nucleotide identity (97.46%) with Teal/Djankoy/Ukraine/9-17-11/2010 isolated in Ukraine (Table 1.4).

As shown in Table 1.4, the aa sequence of the F protein cleavage sites (DIPQR↓F) were highly conserved among all the isolates.

According to the phylogenetic tree of the partial F genes (Fig. 1.4), 57 APMV-4 isolates formed a large cluster together, but showed the different lineage from Korean strain (Mallard/South Korea/YJ/2006) although the isolates showed the highest nucleotide identity with this strain (Table 1.4). Only the isolate 10KI0182 configured a single branch distinct from other strains of APMV-4 isolated in Asia, Europe, and the US.

Genetic analysis for APMV-6 isolates

From the results of BLAST search, 11 of APMV-6 isolates were most closely related to the subgroup APMV-6 strain Red-necked stint/Japan/8KS0813/2008 (Bui et al., 2014) with nucleotide identity from 94.54–98.18% (Table 1.5). The other two isolates, 10EY0013 (Green-winged teal/Japan/10EY0013/2010) and 11OG0078 (Anas sp./Japan/11OG0078/2011) were closely related to the Taiwan strain and Belgium strains (97.27 and 98.18% nucleotide identity, respectively). The deduced aa sequences of the F protein cleavage site were IREPR↓L in 11

APMV-6 isolates. The remaining two isolates had the different motif, APEPR↓L. However, none of them resembled the highly virulent strain of APMV-1.

The phylogenetic tree constructed based on partial F genes sequences showed that the 11 APMV-6 isolates were separated into two genetic classes (Fig. 1.5). Class II viruses were separated into two clusters: one consisted of only the nine isolates isolated in 2011 and 2013 in this study, and another one included two isolates together with the APMV-6 subgroup strains (Red-necked stint/Japan/8KS0813/2008 and Duck/Italy/4525-2/2007). The isolate 10EY0013 and 11OG0078 were grouped into class I with the prototype APMV-6 strains.

Genetic analysis for APMV-9 isolates

Only two isolates obtained in 2009 and 2011 were identified as APMV-9 in this study. Both of them were genetically related to the strain Mallard/Italy/6226/2008 with 91.25 and 90.96% nucleotide identities, respectively (Table 1.6). Comparison of aa sequences at the F protein cleavage between the isolates and other APMV-9 sequences available in GenBank revealed that the motif IREGR↓I is conserved in this serotypes.

The phylogenetic tree indicated that the two isolates were placed in the lineage distinct from the prototype, Duck/New York/22/1978. In this lineage, the isolates and the strains isolated in Italy formed two distinct clusters each other (Fig. 1.6).

1.4 Discussion

The majority of APMVs isolated in this study was serotype 4, indicating that this virus was predominantly harbored in wild birds flying in eastern Hokkaido, which was similar to the evidence reported in the previous surveillance conducted in the western part of Japan (Umali et al., 2014), New Zealand (Stanislawek et al., 2002), and Germany (Kruckenberg et al., 2011).

So far, HI tests have been traditionally used as a standard protocol for APMV serotyping. The primer sets to detect the F genes of APMV-1 to APMV-9 (except APMV-5) designed in my laboratory (Dr. V.N. Bui, manuscript in preparation) were successfully used for serotyping in this study. The results of RT-PCR accorded with those of the HI test using reference antiserum against each serotype. However, more viruses should be evaluated to confirm a reliability of the primers used, because APMVs except APMV-1 available for evaluation of the primers were limited in number.

Based on the genetic diversity of the coding region of the F gene, APMV-1 strains are classified into two classes (class I and class II) (Czegledi et al., 2006; Diel et al., 2012a). Viruses of class I were less diverse than those of class II. Class I viruses are exclusively found in wild birds with low virulent characteristics. Class II includes viruses with a broad range of virulence and the viruses can be found in both poultry and wild birds. Class II was further divided into ten genotypes. Highly virulent viruses are grouped into genotypes V–VIII, whereas the remaining genotypes include virus of low to intermediate virulence (Dimitrov et al., 2016). In the present study, 12 APMV-1 isolates were classified into genotype I of class II in which low virulence viruses derived from wild birds and poultry are mostly found.

The phylogenetic tree revealed the close relationship of the low virulent APMV-1 isolates derived from wild birds flying in eastern Hokkaido to the Eurasian-origin strains isolated in the other parts of Japan, China, Korea, Kazakhstan, Russia, and other European countries, which confirms that migratory wild birds transport and disseminate APMV-1 widely within the Eurasian continent. Interestingly, on the other hand, the intercontinental spread of APMV-1 between North America and Eurasian continent was exhibited in class II where the isolate 9KS0098 isolated in 2009 and 13G109 isolated in 2013 were closely related to the US strains that were isolated in New Jersey in 2001. More evidence was also shown in class I in which the

two isolates isolated in 2011 (11OG1095 and 11OG1114) were closely related to the strains isolated in various wild birds in the US from 1987 to 2009. These results may suggest the continuous introducing of APMV-1 into Hokkaido from North America where there exist large breeding areas of migratory wild birds, supporting the evidence obtained in the previous report on phylogenetic analysis using the Japanese isolates isolated in 2009 and 2010 (Ramey et al., 2013). Ramey et al. (2013) reported that mutation of the F genes was estimated to be faster for both class I and class II wild-bird isolates than previously reported for APMV-1 strain of low virulence. Thus, the intercontinental transport of APMV-1 by wild birds may contribute to the global dispersal of APMV-1 and to induce genetic diversity among APMV-1 strains, probably due to gene mixing and/or mutation.

Genetic analysis of APMV-4 based on partial F genes indicated that all of them had high nucleotide and aa identity to each other. Phylogenetic analysis showed no intercontinental spread of APMV-4 isolates except one APMV-4 isolated in South Africa, revealing the possibility of viral exchange between Eurasian and African continents (Muzyka et al., 2014; Reeves et al., 2016) via migration route across Black Sea/Mediterranean and West Asian−East African flyways (Wetlands International, 2012). APMV-4 isolates obtained in this study formed a large cluster with the strains derived from wild birds in Eurasian continent, except one isolate 10KI0182 (Anas sp./Japan/10KI0182/2010) that formed a single lineage distinct from the cluster.

Genetic and antigenic variations were highlighted in APMV-6. The 2 sub-genotypes within serotype 6 can be distinguished based on nucleotide sequences and the HI test, tangibly (Bui et al., 2014; Xiao et al., 2010). APMV-6 isolates obtained here were grouped into 2 classes. Most of the isolates isolated from 2010 to 2013 were placed in class II together with Red-necked stint/Japan/8KS0813/2008 that was reported previously as subgroup in the same area (Bui et

al., 2014). This result may suggest that subgroup strains, which strain in duck was first reported in 2007 in Italy (Xiao et al., 2010), is continuously circulating among wild bird population.

APMV-9 has been rarely reported so far, and there are currently only five sequences of the F genes available in GenBank. Thus, sufficient information about ecology of APMV-9 is lacking. However, the significant genetic variation was recognized between Italian strains and the prototype strain (Duck/New York/22/1978) (Dundon et al., 2010). Although, two APMV-9 isolates were obtained in this study, and shared more similarity to the Italian strains than to the prototype stain, phylogenetic analysis indicated that they form a branch distinct from Italian strains. However, further study with more viruses is required to determine whether they represent subgroup within the serotype.

The F-protein cleavage site is mainly used to determine the virulence of APMV-1 in chickens. The presence of multiple basic aa at the C-terminus of the F2 protein and a phenylalanine at the N-terminus represents the motif of virulent strains (OIE, 2012). According to pathotyping based on this criterion, all APMV isolates obtained in this study were determined to be low virulence. However, confirmation of their virulence may be required using their own susceptible hosts.

The present results have extended the genetic information and distribution of APMVs in wild birds, besides APMV-1. The circulations of APMV-1, APMV-4, APMV-6, and APMV-9 in wild bird population flying into Hokkaido was demonstrated. Exchange of virus among migratory wild birds between different geographic areas was demonstrated in the phylogenetic analyses of APMV-1 and APMV-4. Although all isolates obtained in this study were low virulent, virulent strains of APMV-1 were isolated from wild birds in India (Roy et al., 1998) and Nigeria where they were considered as a source of infection in backyard chickens (Olabode et al., 1992). Moreover, a mutation of low virulent APMV-1 to highly virulent APMV-1 was

responsible for the ND outbreaks in Australia during 1998 to 2000 (Gould et al., 2001; Westbury, 2001). Considering these incidents, the potential of migratory wild birds to harbor and introduce virus to poultry is still a threatening problem to poultry industry. As mentioned above, the surveillance of APMVs has been increased. However, it still has not been done in many regions of the world, and the sequences of APMVs except APMV-1 are still limited in number. Therefore, continued surveillance of APMVs is still essential to greatly improve our understanding of the relationship between APMVs and natural hosts, ecology, epidemiology, genetic characteristics and evolution of APMVs.

1.5 Summary

A total of 10,606 swab and fecal samples collected from wild birds in eastern Hokkaido during 2009−2013 were examined for isolation of APMV. Samples were collected from birds in the order *Anseriformes* (8,337 samples), *Charadriiforme* (2,087 samples), and others (182 samples) As a result, a total of 91 APMV isolates (0.86%) were obtained from the 10,606 samples: APMV-1 (18 isolates), APMV-4 (58), APMV-6 (13), and APMV-9 (2). Although all APMVs was identified as low virulent based on the criteria of virulence of APMV-1 to chickens, the health impact of them to other poultry is still required further study. The phylogenetic analyses of APMV-1 and APMV-4 showed the evidence of intercontinental spreading by migratory birds between the Eurasian continent and North America, which suggest that extensive exchange of virus and/or viral genes among migratory wild birds may occur, probably leading to emergence of new viral strains. To keep a vigilant watch for the emergence of diseases that may be caused by them in the future, epidemiological and genetic information of APMVs circulating in wild birds is necessary. Therefore, monitoring of virus exchange and evolution in wild bird population should be continued and the study in the geographic area of

the world should be expanded. This study is the first surveillance study of APMVs in wild bird population conducted in eastern Hokkaido.

Table 1.1.
Primers used in this study for RT-PCR to detect APMV F gene fragments

Name	Orientation	Primer sequence (5′ to 3′)[a]	Length (bp)	Position in reference sequence	Expected product size (bp)	Accession no. reference sequence
APMV-1F	Forward	TATACACCTCRTCBCARACRGG	22	4698–4720	403	JF950510
APMV-1R	Reverses	ACRAAYTGCTGCATCTTCCC	20	5100–5080		
APMV-2F	Forward	CCTGGGARYCTATCAACATG	20	4518–4537	521	EU338414
APMV-2R	Reverses	CCCATRTCSCCTCCACATA	19	5038–5020		
APMV-3F	Forward	ATYTKTCYCATTGCCAGMKT	20	4967–4987	425	EU782025
APMV-3R	Reverses	RGTMGTCATYTCAATGAGRT	20	5392–5373		
APMV-4F	Forward	CAAAGGGAAGTTGTCACAGA	20	4634–4653	858	FJ177514
APMV-4R	Reverses	CTCTGGATTAAGCCGAGAAG	20	5491–5472		
APMV-6F	Forward	KGCWGTKAGATTRATYCCCA	20	4774–4793	362	EU622637
APMV-6R	Reverses	CKAYHGCAATYTGYCCTGTK	20	5135–5116		
APMV-7F	Forward	ATTCTACCCGTAATTGGCTT	20	4620–4639	470	FJ231524
APMV-7R	Reverses	AAGTAGTTTTGGACCTTGCT	20	5089–5070		
APMV-8F	Forward	AATTGCCGTGCGATTCCTAC	20	4561–4670	540	FJ619036
APMV-8R	Reverses	CCACCACACAGGTTGTACAG	20	5190–5171		
APMV-9F	Forward	CTACCATAGTGACACAATGGGG	22	4789–4810	614	EU910942
APMV-9R	Reverses	CTAATTGTTGAGCTGCCTGTAC	22	5402–5381		

[a] Codes for mixed-base position: B, C/G/T; H, A/T/C; K, G/T; M, A/C; R, A/G; S, C/G; W, A/T; Y, C/T

Table 1.2.
APMVs isolated from migratory wild birds in Hokkaido, 2009-2013

Location (district)	Year	No. positive/no. tested (%)	Serotype	Number	Species (number)
Abashiri, Kushiro, Nemuro, Tokachi	2009	6/2,073 (0.29)	APMV-1 APMV-4 APMV-9	2 3 1	Black-tailed Gull (64), Duck (1,069), Glaucous Gull (64), Grey-rumped Sandpiper (62), Mallard (52), Northern Pintail (160), Rufous-necked Stint (221), Slaty-backed Gull (188), Tundra Swan (112), Other (81)
Kushiro, Tokachi	2010	25/1,954 (1.28)	APMV-1 APMV-4 APMV-6	6 16 3	Duck (1,178), Green-winged teal (16), Mallard (10), Norther pintail (5), Salty-backed Gull (196), Tufter duck (28), Whooper swan (518), Other (3)
Nemuro, Tokachi	2011	35/2,048 (1.71)	APMV-1 APMV-4 APMV-6 APMV-9	5 21 8 1	Duck (1,549), Green-winged teal (16), Grey-tailed tattler (148), Mallard (16), Red-necked Stint (28), Ruddy turnstone (49), Tufted duck (13), Wigeon (202), Other (27)
Tokachi	2012	6/1,043 (0.58)	APMV-4	6	Droppings collected from duck's habitats (1,039), Swan (4)
Hidaka, Nemuro, Tokachi, Kushiro, Abashiri	2013	19/3,488 (0.54)	APMV-1 APMV-4 APMV-6	5 12 2	Black-tailed gull (168), Droppings collected from duck's habitats (2,211), Gull (899), Swan (1), Wigeon (138), other (71)
Total	5 years	91/10,606 (0.86)		91	APMV-1 (18); APMV-4 (58); APMV-6 (13); APMV-9 (2)

Table 1.3.
Isolation and genetic characterization of APMV-1

Strain name	Length (bp)	Cleavage site at the F protein	Best match	
			Strain name	Nucleotide identity (%)
APMV-1/slaty-backed gull/Japan/9KS0098/2009	360	GKQGR/L	APMV-1/red knot/US(NJ)/A1011383/2001	95.54
APVM-1/green-winged teal/Japan/9KS0644/2009	360	ERQER/L	APMV-1/duck/China/NDV08-037/2008	99.44
APMV-1/northern pintail/Japan/10EY0020/2010	360	GKQGR/L	APMV-1/mallard/South Korea/CBU2374/2007	99.72
APMV-1/Anas sp./Japan/10UO0343/2010	360	ERQER/L	APMV-1/duck/Japan/Tottori/481/2009	99.72
APMV-1/Anas sp./Japan/10UO0501/2010	360	GKQGR/L	APMV-1/teal/South Korea/WB/KU22-1/2007	99.16
APMV-1/Anas sp./Japan/10UO0525/2010	360	GKQGR/L	APMV-1/teal/South Korea/WB/KU22-1/2007	99.16
APMV-1/Anas sp./Japan/10UO0527/2010	360	GKQGR/L	APMV-1/teal/South Korea/WB/KU22-1/2007	99.16
APMV-1/Anas sp./Japan/10UO0529/2010	360	GKQGR/L	APMV-1/teal/South Korea/WB/KU22-1/2007	99.16
APMV-1/Anas sp./Japan/11OG1095/2011	360	ERQER/L	APMV-1/green-winged teal/AK/44493-409/2009	99.72
APMV-1/Anas sp./Japan/11OG1114/2011	360	ERQER/L	APMV-1/green-winged teal/AK/44493-409/2009	99.72
APMV-1/Anas sp./Japan/11UO0005/2011	360	GKQGR/L	APMV-1/duck/China/Guangxi17/2009	99.44
APMV-1/Anas sp./Japan/11UO0016/2011	360	GKQGR/L	APMV-1/duck/China/Guangxi17/2009	99.44
APMV-1/Anas ap/Japan/11UO0040/2011	360	GKQGR/L	APMV-1/duck/China/Guangxi17/2009	99.44
APMV-1/Anas ap/Japan/13UO0475/2013	360	ERQER/L	APMV-1/duck/China/Jilin/239/2011	99.44
APMV-1/Anas ap/Japan/13UO0603/2013	360	GKQGR/L	APMV-1/slatybackedgull/Russia/Nikita539FFNK11/2007	99.72
APMV-1/Anas ap/Japan/13OG0977/2013	360	GKQGR/L	APMV-1/teal/South Korea/WB/KU22-1/2007	98.88
APMV-1/Anas ap/Japan/13OG1235/2013	360	ERQER/L	APMV-1/duck/China/Jilin/239/2011	99.44
APMV-1/gull/Japan/13G109/2013	360	GKQGR/L	APMV-1/black-headed_gull/China/Ningxia/2/2014	99.72

Table 1.4.
Isolation and genetic characterization of APMV-4

Strain name	Length (bp)	Cleavage site at the F protein	Best match	
			Strain name	Nucleotide identity (%)
APMV-4/Anas sp./Japan/9UO0180/2009	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.15
APMV-4/Anas sp./Japan/9UO0234/2009	800	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.12
APMV-4/Anas sp./Japan/9UO0288/2009	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Northern_pintail/Japan/10EY0008/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.15
APMV-4/green-winged_teal/Japan/10EY0011/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.15
APMV-4/green-winged_teal/Japan/10EY0036/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.39
APMV-4/Anas sp./Japan/10KI0182/2010	828	DIQPR/F	APMV-4/teal/Djankoy/Ukraine/9-17-11/2010	97.46
APMV-4/Anas sp./Japan/10KI0287/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.39
APMV-4/Anas sp./Japan/10KI0290/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.39
APMV-4/Anas sp./Japan/10KI0292/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.39
APMV-4/Anas sp./Japan/10KI0294/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.39
APMV-4/Anas sp./Japan/10KI0309/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.39
APMV-4/Anas sp./Japan/10KI0318/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.27
APMV-4/Anas sp./Japan/10KI0321/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.27
APMV-4/Anas sp./Japan/10UO0441/2010	803	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.87
APMV-4/Anas sp./Japan/10UO0481/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.39
APMV-4/Anas sp./Japan/10UO0499/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.79
APMV-4/Anas sp./Japan/10UO0510/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.39
APMV-4/Anas sp./Japan/10UO0689/2010	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.15
APMV-4/goden-eye/Japan/11EY0496/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.15
APMV-4/Anas sp/Japan/11OG0195/2011	792	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.98
APMV-4/Anas sp./Japan/11OG0196/2011	792	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.98
APMV-4/Anas sp./Japan/11OG0197/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.91

Table 1.4. (Continued)

Strain name	Length (bp)	Cleavage site at the F protein	Best match	
			Strain name	Nucleotide identity (%)
APMV-4/Anas sp./Japan/11OG0198/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.91
APMV-4/Anas sp./Japan/11OG0239/2011	792	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.98
APMV-4/Anas sp./Japan/11OG0355/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG0387/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG0439/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG0799/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG0813/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG0949/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG0962/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG0965/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG0976/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.91
APMV-4/Anas sp./Japan/11OG1007/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG1034/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG1075/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11OG1077/2011	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/11UO0072/2011	769	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.69
APMV-4/Anas sp./Japan/11UO0325/2011	771	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.18
APMV-4/Anas sp./Japan/12OG0208/2012	801	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.12
APMV-4/Anas sp./Japan/12OG0519/2012	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	97.58
APMV-4/Anas sp./Japan/12OG0536/2012	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.42
APMV-4/Anas sp./Japan/12OG0537/2012	798	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.12
APMV-4/Anas sp./Japan/12OG0543/2012	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.03
APMV-4/Anas sp./Japan/12OG0690/2012	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	96.85

Table 1.4. (Continued)

Strain name	Length (bp)	Cleavage site at the F protein	Best match	
			Strain name	Nucleotide identity (%)
APMV-4/Anas sp./Japan/13UO890/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.43
APMV-4/Anas sp./Japan/13UO892/2013	810	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	97.90
APMV-4/Anas sp./Japan/13UO919/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.15
APMV-4/Anas sp./Japan/13UO931/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	99.15
APMV-4/Anas sp./Japan/13OG0342/2013	807	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.76
APMV-4/Anas sp./Japan/13OG0407/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	97.22
APMV-4/Anas sp./Japan/13OG0455/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.55
APMV-4/Anas sp./Japan/13OG0503/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	95.89
APMV-4/Anas sp./Japan/13OG0652/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.91
APMV-4/Anas sp./Japan/13OG1105/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.67
APMV-4/Anas sp./Japan/13OG1177/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.67
APMV-4/Anas sp./Japan/13OG1470/2013	828	DIQPR/F	APMV-4/mallard/South Korea/YJ/2006	98.67

Table 1.5.
Isolation and genetic characterization of APMV-6

Strain name	Length (bp)	Cleavage site at the F protein	Best match	
			Strain	Nucleotide Identity (%)
APMV-6/green-winged teal/Japan/10EY0002/2010	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	97.57
APMV-6/green-winged teal/Japan/10EY0013/2010	330	APEPR/L	APMV-6/duck/Taiwan/Y119/1998	97.27
APMV-6/Norther pintail/Japan/10EY0062/2010	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	98.18
APMV-6/Anas sp./Japan/11OG0078/2011	330	APEPR/L	APMV-6/mallard/Belgium/12245/2007	98.18
APMV-6/Anas sp./Japan/11OG0480/2011	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	94.54
APMV-6/Anas sp./Japan/11OG0504/2011	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	94.54
APMV-6/Anas sp./Japan/11OG0560/2011	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	94.54
APMV-6/Anas sp./Japan/11OG0697/2011	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	96.06
APMV-6/Anas sp./Japan/11OG0777/2011	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	96.06
APMV-6/Anas sp./Japan/11OG0814/2011	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	96.06
APMV-6/Anas sp./Japan/11OG1028/2011	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	96.06
APMV-6/Anas sp./Japan/13UO087/2013	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	95.76
APMV-6/Anas sp./Japan/13OG0682/2013	330	IREPR/L	APMV-6/red-necked stint/Japan/8KS0813/2008	95.15

Table 1.6.
Isolation and genetic characterization of APMV-9

Strain name	Length (bp)	Cleavage site at the F protein	Best match	
			Strain	Nucleotide identity (%)
APMV-9/Anas sp./Japan/9UO0485/2009	675	IREGR/I	APMV-9/mallard/Italy/6226/2008	91.25
APMV-9/Anas sp/Japan/11OG0839/2011	675	IREGR/I	APMV-9/mallard/Italy/6226/2008	90.96

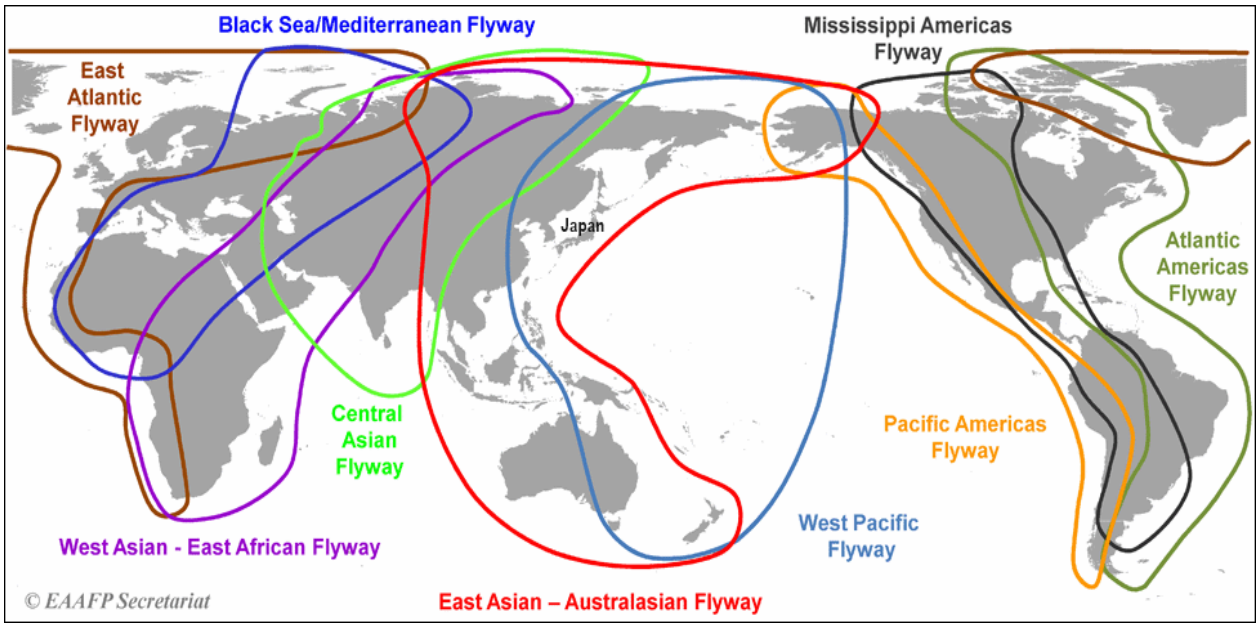

Fig. 1.1. Nine major flyways of migratory birds in the world. Japan is located in the East Asian-Australasian Flyway. (EAAFP Secretariat, 2010)

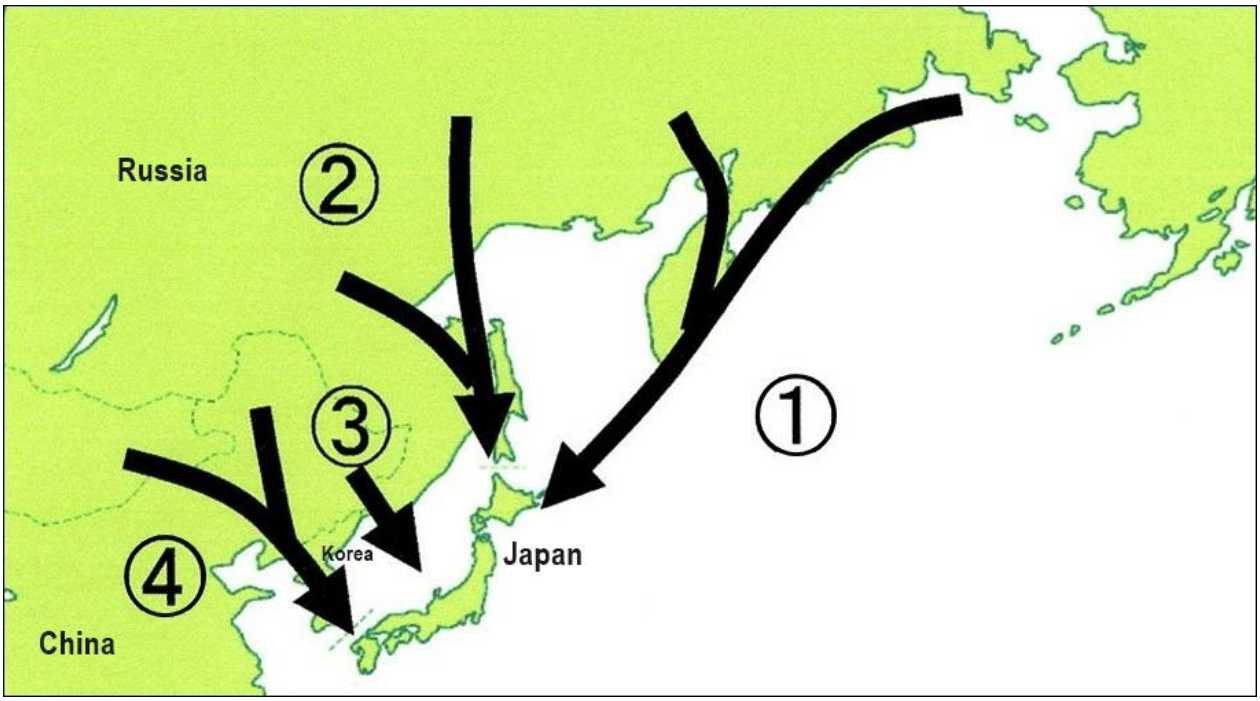

Fig. 1.2. Main migration routes of migratory winter birds in Japan: ① The Kamchatka route; ② The Sakhalin-Kurile route; ③ The crossing of the Sea of Japan route; ④ The Korean Peninsula route. (Food Safety and Consumer Affairs Bureau, 2007)

Fig. 1.3. The Phylogenetic tree of the F genes from APMV-1 isolates was generated by the Maximum Likelihood method based on the Kimura 2-parameter model. The analysis involved 57 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 197 positions in the final dataset. The symbols used in the tree are described as follows; ●: sample from 2009, ▲: sample from 2010, ▽: sample from 2011, ■: sample from 2013.

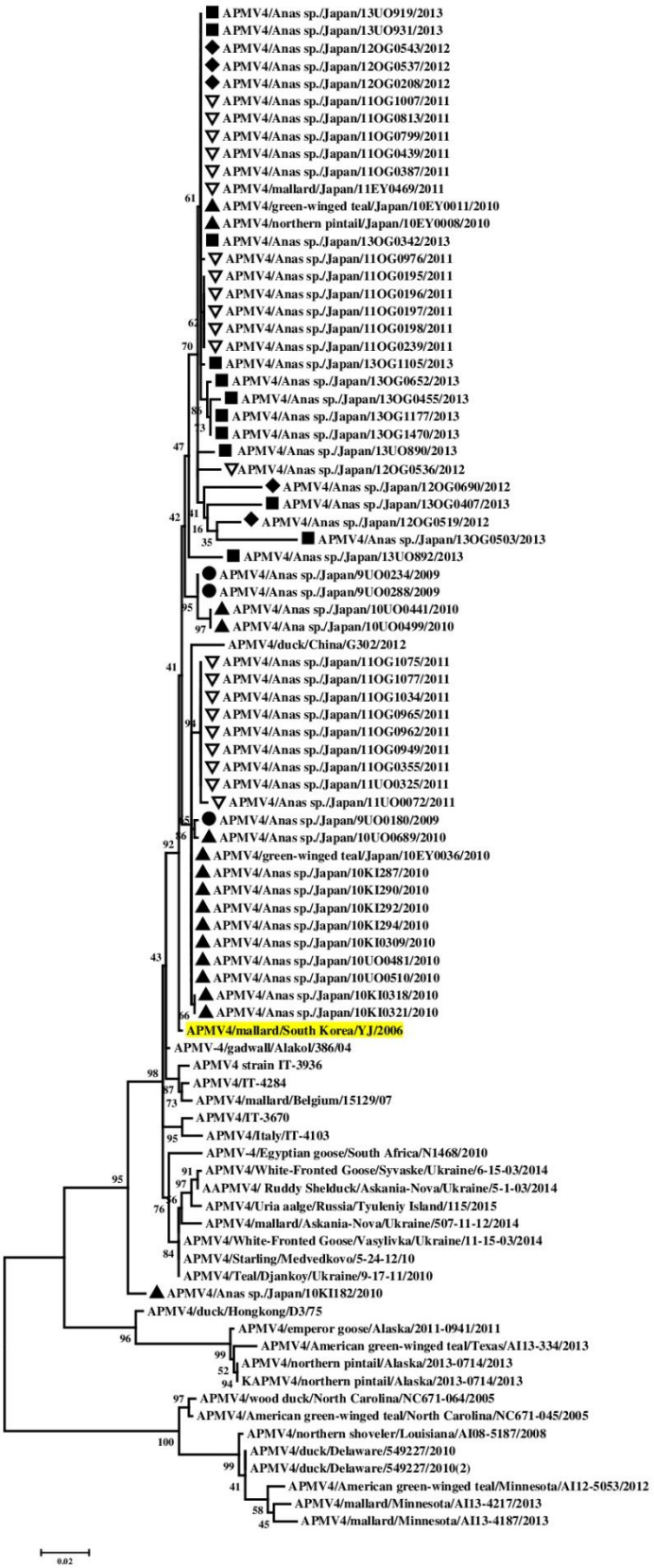

Fig. 1.4. The Phylogenetic tree of the F genes from APMV-4 isolates was generated by the Maximum Likelihood method based on the Kimura 2-parameter model. The analysis involved 87 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 762 positions in the final dataset. The symbols used in the tree are described as follows; ●: sample from 2009, ▲: sample from 2010, ▽: sample from 2011, ♦: sample from 2012, ■: sample from 2013.

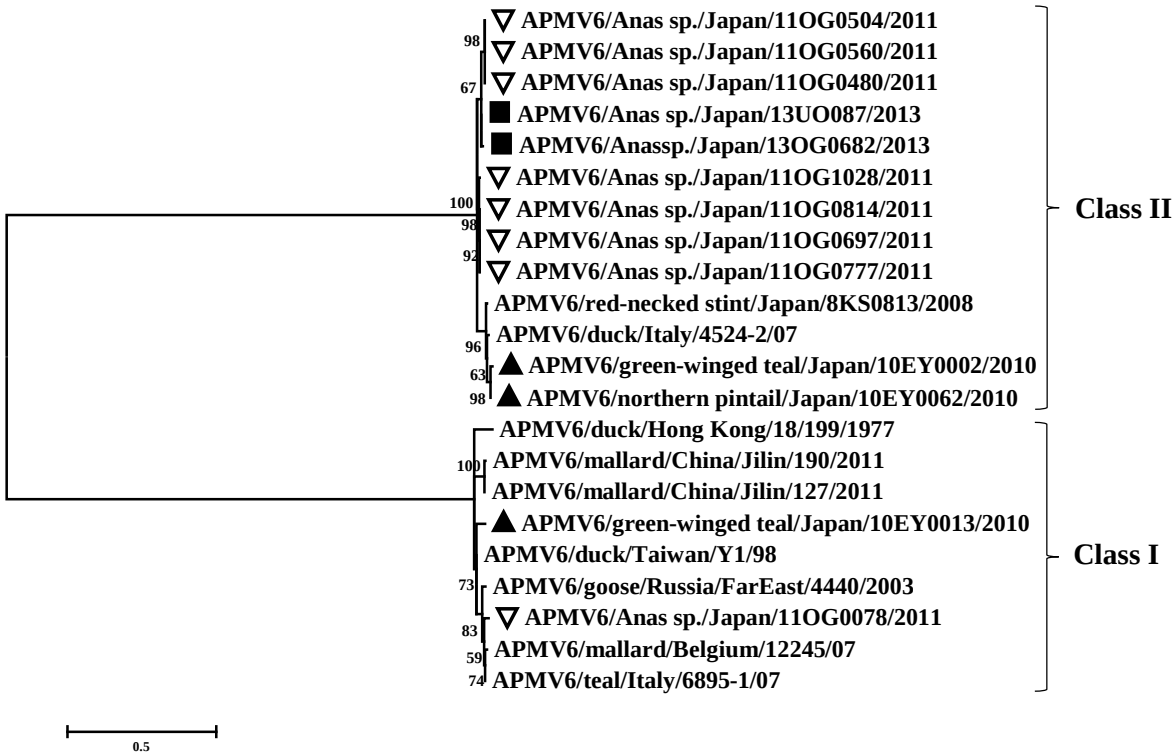

Fig. 1.5 The Phylogenetic tree of the F genes from APMV-6 isolates was generated by the Maximum Likelihood method based on the Kimura 2-parameter model. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 330 positions in the final dataset. The symbols used in the tree are described as follows; ▲: sample from 2010, ▽: sample from 2011, ■: sample from 2013.

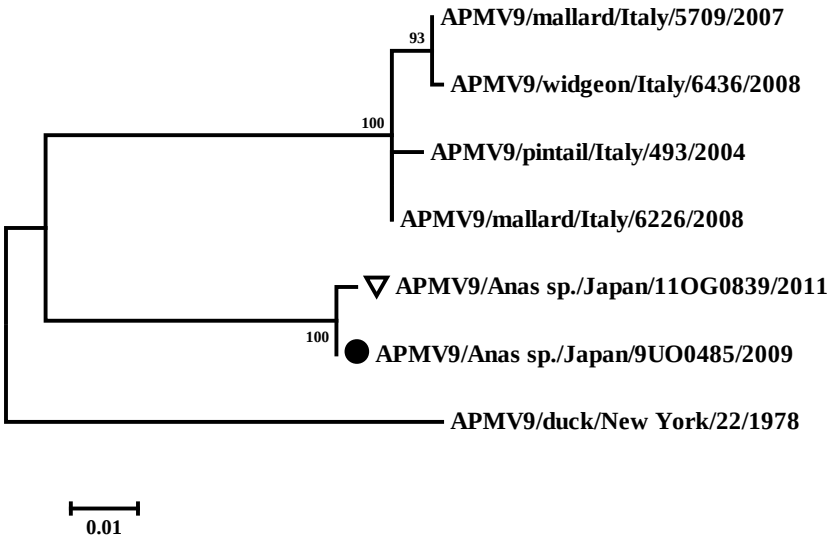

Fig. 1.6 The Phylogenetic tree of the F genes from APMV-9 isolates was generated by the Maximum Likelihood method based on the Tamura-Nei model. The analysis involved 7 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 674 positions in the final dataset. The symbols used in the tree are described as follows; ●: sample from 2009, ▽: sample from 2011.

Chapter II

Genetic and antigenic analysis of APMV-1 isolated from wild birds in Hokkaido

2.1 Introduction

ND is an important disease in poultry industries caused by virulent strains of APMV-1. APMV-1 can be grouped in to 5 pathotypes defined by clinical signs in experimentally infected chickens. The pathotypes of ND consist of viscerotropic velogenic (a highly pathogenic form characterized by high mortality and hemorrhagic intestinal lesions in any age chickens), neurotropic velogenic (usually characterized by respiratory and nervous signs, and low mortality in old chickens and high mortality in young chickens), mesogenic (lesser respiratory signs, occasional nervous signs, but low mortality in only young chickens), lentogenic (mild or subclinical respiratory infection), and asymptomatic enteric (enteric, but asymptomatic infection). However, pathotyping cannot be certainly determined, and considerable overlapping of clinical sign may be seen (Alexander and Senne, 2008). According to OIE manual, virulence of APMV-1 can be assessed by the intracerebral pathogenicity index test (ICPI) in day-old chickens, and virulent NDV is characterized by an ICPI ≥ 0.7 (OIE, 2012). OIE also suggested molecular approach to confirm virus virulence. The F protein cleavage site was used to determine virus virulence. A precursor of the F protein (F0) required host cell protease to cleave it into F1 and F2 units. Virulent APMV-1 has multiple basic aa at the C-terminus of the F2 unit and a phenylalanine at the N-terminus of the F1 unit that are cleaved by furin-like protease found in a myriad of cells and tissues throughout all systems (Morrison et al., 1993). While low virulent APMV-1 has monobasic aa at this site and a leucine at the N-terminus that are cleaved by trypsin-like protease found only in respiratory and intestinal tracts (Choi et al., 2010; Nagai

et al., 1976). However, the cleavage site of the F protein does not always correlate with pathogenicity. The APMV-1 containing the cleavage site of the virulent APMV-1 did not induce clinical disease as severe as that cause by the virulent strain with multiple basic aa at the F protein cleavage site, which means that the F protein is not the sole virulence determinant of APMV-1 (de Leeuw et al., 2005; Romer-Oberdorfer et al., 2003).

The HN protein is responsible for the attachment of virus envelop to sialic acid receptor on cell surfaces before development of fusion activity of the F protein (Lamb and Parks, 2013). Sakaguchi et al. (1989) firstly demonstrated that the HN protein of APMV-1 strains varied in length resulting in the existence of three distinct lineages. The longest HN protein of 616 aa was found only in low virulent strains, and the length of 571 aa was found only in virulent strains. This data implied that the length of the HN protein may be associated with virus virulence. However, the relation between the HN protein with an intermediate length and virulence is questioned.

Wild birds commonly harbor low virulent APMV-1. However, in the 1900, a highly virulent APMV-1 caused a large outbreak in double-crested cormorants in Canada and the US (Diel et al., 2012b). Moreover, there is an evidence indicating that the outbreak in turkey in North Dakota in 1992 was caused by virulent APMV-1 transmitted by cormorants (Seal, 1996). Though this occurrence was rare, the potential that virulent APMV-1 may be maintained in wild birds or transported by them cannot be denied. Thus, a surveillance of APMV-1 in wild bird population is still necessary.

Vaccination has been used to control NDV in poultry industries in most countries, but despite intensive vaccination, the incidences of ND has been still recognized in many countries (Ababneh et al., 2012; Cho et al., 2007; Zhang et al., 2016). Poor technique in vaccine administration was hypothesized as one of possible causes. However, the experimental

infection studies reveal that commonly used vaccines cannot completely protect virulent APMV-1 infection and virus shedding. The protective immunity induced by vaccines homologous with challenge viruses decreased viral shedding significantly more than the heterologous vaccines (Kapczynski and King, 2005; Miller et al., 2007). In addition, in South Korea and Nigeria, ND outbreaks in chicken farms with well-vaccinated practice also raised a question regarding the efficacy of conventional NDV vaccine (Cho et al., 2007; Solomon et al., 2012). That is the reason why the antigenic diversity between vaccine and field strains was of concern as the possible cause for the insufficient immunity (Hu et al., 2009; Miller et al., 2009; van Boven et al., 2008).

Although all APMV-1 isolates obtained from this surveillance had the F protein cleavage site of low virulent APMV-1, the concern of antigenic variation between vaccine strains and field strains cannot be ignored. In this study, I genetically characterized APMV-1 isolates obtained from migratory wild birds based on HN genes in comparison with F genes from chapter I. The comparison of antigenicity between APMV-1 isolated from poultry and wild birds was also described.

2.2 Materials and methods

Samples

A total of 18 APMV-1 isolates was obtained from the surveillance as shown in Chapter I. They were identified as APMV-1 antigenically and genetically using HI test and RT-PCR for detection of the F genes, respectively. Fourteen samples were selected to be sequenced for the full length of HN genes (Table 2.1).

RT-PCR

Total RNA was extracted from the HA positive allantoic fluids containing the APMV-1 isolates using ISOGEN-II (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. First-strand cDNA was produced using 2.0 µl of random hexamer primers (0.5 µg/µl) (Invitrogen, Carlsbad, CA), 1.0 µl of 10 mM dNTP, 4.0 µl of RNA, 6 µl of DEPC-water, 4.0 µl of 5× first-strand buffer, 2.0 µl of 0.1 M DTT, 1 µl of RNAse inhibitor and 1.0 µl of MMLV reverse transcriptase (Invitrogen) to a total volume of 21 µl. The reaction was incubated at 25°C for 10 min, 37°C for 50 min, and 65°C for 10 min and placed on ice. PCR with 20 µl of total volume of reaction was performed using 1 µl of cDNA, 1 µl of each primer (20 µM), 1.6 µl of dNTP (2.5 mM each dNTP), 2 µl of 10X Ex Taq Buffer, 13.3 µl of DEPC water and 0.1 µl Takara Ex Taq (Takara, Shiga, Japan). The PCR conditions were as follows: 94ºC for 5 min; 40 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 40 sec; and final extension at 72°C for 10 min. The primer sets were designed to amplify complete gene of HN gene (Table 2.2). Obtained PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

Nucleotide sequencing and phylogenetic analysis

The PCR products were purified from agarose gels using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified products were used as a template for sequencing reactions using a BigDye terminator ver. 3.1 cycle sequencing kit (Applied Biosystems, Foster, CA) and the primers using for amplification of the PCR product were used for sequencing. Nucleotide sequencing was performed with an ABI 3500 Genetic Analyzer (Applied Biosystems). The obtained sequences were analyzed by BioEdit and compared with other available sequences using BLAST homology searches. Pairwise comparison between sequences were conducted using the p-distance model. Evolution distances were calculated

using the aligned sequences and the Kitamura 2-parameter model. Phylogenetic trees were constructed using the Maximum Likelihood (ML) method supported by 500 bootstrap replicates and MEGA 6.0 software (Tamura et al., 2013).

Antigenic analysis

Monoclonal antibodies (mAbs) produced using the three strains (D26/76, Ibaraki/85, and Sato/30) of APMV-1 isolated in Japan were used. Strain D26/76 was isolated from duck in 1976. Strain Ibaraki/85 was isolated from chicken in 1985 and strain Sato/30 was isolated from chicken in 1930.

The mAbs against the HN protein used in this study are shown as follows; mAbs against Ibaraki/85 strain (M1, M2, M3, M10, M13), mAbs against D26/76 strain (HN14, HN21, HN31, HN41). The mAbs against the Ibaraki/85 strain was produced in my laboratory. The mAbs against the D26/76 strain were provided by Nagoya University Graduate School of Medicine. The comparison of antigenicity was done using HI tests as previous described (OIE, 2012). The other mAbs (70/1, 320/1, 743/1) against the F protein of APMV-1 strain Sato/30 were also used in this study. These mAbs were provided by Dr. Kida, Hokkaido University. VN tests were used to compare the antigenicity. Four-fold dilutions of mAbs against APMV-1 strain Sato/30 from 1/100 to 1/12,800 dilution, were mixed with an equal amount of 100 EID_{50}/0.1ml of the APMV1 isolates. The mixture was incubated at 37°C for 1 h and then inoculated into the allantoic cavity of ten-day-old embryonated chicken eggs. Afrer incubating at 37°C for 3 days, allantoic fluids were tested for HA activities as described above. The reciprocal of the highest diulution of the mAbs that neutralizing 50% of the virus was taken as the neutralizing titer.

Besides the homologous strains with the mAbs, other APMV-1 strains used for antigenicity comparison were as follows: the Chiba/85 strain provided by National Institute of

Animal Health, Japan, and vaccinal strains B1 and VG/GA purchased from the Chemo-Sero-Therapeutic Research Institute (Kumamoto) and MERIAL Japan (Tokyo), respectively.

2.3 Results

Phylogenetic analysis

Fourteen HN gene nucleotide sequences of the APMV-1 isolates were phylogenetically analyzed (Fig. 2.1). The phylogenetic tree revealed that the isolates were classified into 2 genetic classes similar to the F genes analyzed in Chapter I. Six isolates were classified into class I. Four isolates (9KS0644, 10UO0343, 13OG1235, and 13UO0475) of them obtained in 2009, 2010, and 2013 were genetically close to the Eurasian lineage strains including four Chinese and one German strains resulting in one cluster, while the two isolates (11OG1095, 11OG1114) obtained in 2011 formed the different cluster with the US strains. Other eight isolates were grouped into genotype I of class II which includes the APMV-1 isolates from Asia, Australia, and Europe (Fig. 2.1). Seven isolates (10UO0529, 10EY0020, 11UO0005, 11UO0016, 11UO0040, 13OG0603, and 13OG0977) obtained in 2010, 2011, and 2013 formed one cluster with the Chinese strains within genotype I. Only one isolate (9KS0098) in 2009 was more close to the Ishii strain that was isolated from a chicken in 1962, Japan, resulting in one cluster apart from the above cluster.

As shown in Table 2.3, based on the HN genes, the obtained APMV-1 isolates within the same class shared homology to each other with nucleotide sequence identity greater than 88.91%, while the nucleotide identities between the isolates from different class ranged from 70.56–72.56%. The similarity of the HN protein between APMV-1 isolates was ranged from 81.67–100.00%. The HN protein of the isolates 11UO0005 and 11UO0016 were completely identical to each other.

The HN proteins of the isolates have the length of 616 aa, except the isolate 9KS0098. The stop codon of the HN open reading frame of this isolate was found at positon 586, resulting in the length of 585 aa (Fig. 2.2).

Antigenic analysis

The HI test results with mAbs against the HN protein of APM-1 strain Ibaraki/85 showed that all mAbs reacted with the homologous virus (HI titers:160–5,120) (Table 2.4). In contrast, all wild bird APMV-1 reacted with only one mAb, M2 (HI titers:80–320), and one isolate (9KS0098) did not react with any mAbs. The three mAbs, M1, M10, M13 recognized the aa residue K263, E260, and D287 of strain Ibaraki/85. In Fig. 2.3, the aa sequences alignment of the HN protein of APMV-1 isolates showed the same aa residue at E260 and D287 but the position 263 (K263Q for 9KS0644, K263N for 9KS0098) were different.

Then, one representative wild bird isolate from each genetic class was selected (class I, 9KS00644; class II, 9KS0098) for antigenicity comparison using mAbs against the HN proteins of strain D26/76 and mAbs against the F protein of strain Sato/30.

The reactivity of mAbs against strain D26/76 with different strains of APMV-1 were shown in Table 2.5. The results showed that mAb, HN21 reacted with all isolates including the two isolates. Although the mAb HN21 recognizes the aa residue E495 of the HN protein of strain D26/76, this residue was found in the HN proteins of all the strains examined (Fig. 2.3). However, the remaining three mAbs did not recognize the isolates in contrast to the reactivity of the mAbs to the strains derived from domestic birds. The mAb HN14 and HN41 recognized the aa residues E347 and N481 of strain D26/76, respectively. As shown in Fig. 2.3, the substitution of aa residue at position 347 (E347D) in the HN proteins of 9KS0098 and 9KS0644

was found. In contrast, there was no aa substitution at position 481 (N481) recognized by HN 41 among all the APMV-1 strains including the isolates.

The reactivity of mAbs against the F protein of strain Sato/30 with the two isolates was analyzed by VN tests. As shown in Table 2.6, none of three mAbs (70/1, 320/1, 743/1) neutralized the isolate 9KS0644, whereas only the mAbs 70/1 was able to neutralize the isolate 9KS0098 growth in embryonated chicken eggs (VN titer: 400).

2.4 Discussion

The primers designed here successfully amplified the full length of HN genes derived from the viruses of both class I and class II. The phylogenetic tree generated based on the HN genes indicated that the isolates of APMV-1 were divided into the two lineage as observed in the tree based on the F genes.

Regarding the HN protein length, all the APMV-1 isolates sequenced in this study, except one isolate (9KS0098), had the HN protein length of 616 aa. The shortest length (571 aa) has been found only in virulent APMV-1 strains. The extension of the 571 aa to 616 aa (571+45 aa) of the HN protein was described to reduce pathogenicity in chickens (Kim et al., 2014). The 45 aa in the C-terminal extension present in the HN protein was a precursor that works as an auto-inhibited state to block the neuraminidase active sites and second sialic acid binding sites (Yuan et al., 2012). In this study, one isolate, 9KS0098 has the length of 585 aa and no chicken embryo infected the isolate die (data not shown). This data may imply that the length of 585 aa does not influence virus virulence. The contribution of the HN protein length to virulence of APMV-1 is still controversial. Several studies highlighted the influence of the HN protein length to virus virulence by exchanging genes between low virulent and virulent strains, or by mutating some nucleotide residue (Huang et al., 2004; Kim et al., 2014; Zhao et al., 2013). In

contrast, Jin et al. (2016) demonstrated that the length diversity of the HN protein was associated with APMV-1 replication but not the virulence. Thus, further studies are required to determine the exact role of the HN protein in virus virulence.

In APMV-1, seven antigenic sites within the HN protein have been reported; site 1, site 2, site 3, site 4, site 12, site 14, and site 23. All sites are conformational epitope, except site 14 that is a linear epitope (Iorio et al., 1991). In this study, three mAbs (M1, M10, M13) against strain Ibaraki/85 recognized aa residue K263, E260, and D287 of site 3 in the HN protein, respectively. These residues except K263 were conserved among wild bird and poultry APMV-1 (Fig. 2.3). However, even these residues were conserved in the HN protein of wild bird isolates, these mAbs did not recognize them. In other studies, APMV-1 strain Miyadera and strain Ulster also failed to react with the mAbs, although these conserved residues exist in their HN protein (Nishikawa et al., 1987). It is unclear why mAbs M10 and M13 cannot recognize E260 and D287 in the HN protein of the wild bird and poultry APMV-1 of low virulence.

The mAb, HN41 against strain D26/76 binds to the site 4 and recognized N481 of the HN protein (Fig. 2.3). All the APMV-1 isolates sequenced in this study possessed the same residues. However, these mAbs could not inhibit HA activity of the isolate 9KS0098 and 9KS0644.

No reaction between the mAbs and APMV-1 isolates may have been caused by several factors. The overlapping of the antigenic site may be one of the possible cause. Moreover, the production of mAbs against the HN protein used in this study were done in mice immunized with disrupted viral antigens. This process may change the conformation of the HN protein. Iorio and Bratt (1983) described that the antibodies against the HN protein are directed against determinants of a conformational nature rather than primary aa sequences. Additionally, these results implied that there might be more epitopes that influence the antigenicity, and the conformation of those two antigenic sites more than previous thought.

The mAb, HN14 against strain D26/76 binds to the linear epitope of the site 14, and recognized E347 (Gotoh et al., 1988). The mAb reacted with all the reference APMV-1 strains (E347), except strain Ibaraki/85 (K347). As shown in Fig. 2.3, The isolates 9KS0644 and 9KS0098 possessed aa residue D347, resulting in no reactivity with the mAb HN14. The residue 347 was a critical determinant of the antigenic site 4 on the HN protein (Hu et al., 2010). The increased prevalence of this linear epitope mutants, especially E347K was found in APMV-1 isolated from the outbreak in South Korea during 2000 to 2006, which is was probably caused by the antigenic selection under intensive NDV vaccination (Cho et al., 2008).

The epitopes recognized by the mAbs M2 and HN21 seem to be highly conserved among APMV-1 strains because all most of APMV-1 isolates reacted with these mAbs (Tables 2.4 and 2.5). Accordingly, these mAbs can be used for diagnosis of ND.

APMV-1 strain Sato/30 is a virulent strain that has been used as a standard challenge virus in evaluating NDV vaccines efficacy in Japan (Abenes et al., 1986). The mAbs against its F protein were applied to wild bird isolate 9KS0098 and 9KS0644. The mAbs against the F protein displayed the different in antigenicity between chicken and wild bird APMV-1. One mAbs (70/1) neutralized only the isolate 9KS0098 (Table 2.6).

In summary, antigenicity analysis of APMV-1 with mAbs revealed quite different antigenicity between wild bird and poultry APMV-1. The results obtained showed that mAbs against chicken APMV-1 seem to detect poultry APMV-1 preferentially. Consequently, a panel of mAbs against the HN protein used in this study can be applied to distinguish between chicken and wild bird APMV-1. However, more number of APMV-1 strains derived from different source should be examined to clarify this important point.

Protective immunity of vaccine is influenced by antigenicity of challenging viruses. The present results suggest that current NDV vaccines used in poultry may not protect APMV-1

infection derived from wild bird. Although most of wild bird APMV-1 obtained in this study are low virulent, multiple passages of low virulent APMV-1 in chicken was proved to increase pathogenicity (Islam et al., 1994; Shengqing et al., 2002). More importantly, the variant virulent APMV-1 has been reported more frequently since 2000 in spite of widely use of NDV vaccination in poultry industry (Cho et al., 2007; Hu et al., 2009; Yu et al., 2001). Considering the emergence of new variants of APMV-1, antigenic analysis of APMV-1 should be conducted along with the genetic analysis and alternative strategy to prevent NDV outbreak should be developed promptly.

2.5 Summary

From a total of 18 APMV-1 isolates obtained from wild birds, 14 isolates were selected to be sequenced for the complete codon of HN genes. The HN protein among all the isolates showed high similarity with aa identity varying from 81.67–100.00%. The delineation of phylogenetic tree based on the HN genes was consistent with the phylogenetic tree based on the F gene. The isolates were classified into 2 genetic classes (I and II) similar to the F genes analyzed in Chapter I. The HN protein length varies among APMV-1 strains. The shortest (571 aa) length has been reported to find only in virulent strains, and the longest length (616 aa) was found only in low virulent strains. All of the isolates had HN proteins length of 616 aa except the isolate 9KS0098 with 585 aa. Nonetheless, no pathogenicity of the isolate 9KS0098 to chicken embryos was observed, which means that this length may not be involved with virus virulence.

The antigenicity analysis was performed using mAbs against reference APMV-1 strains derived from APMV-1 strain Ibaraki/85, D26/76, and Sato/30. The results revealed that antigenicity of wild bird APMV-1 isolates quite differed from that of poultry APMV-1 strains

including current vaccine strain. Four mAbs (M1, M10, M13, HN41) against strain Ibaraki/85 and D26/76 did not react with the APMV-1 isolates, although the same aa residues recognized by mAbs exist in their HN protein. The discrepancy of this results cannot be elucidated, here. However, the antigenic variation of APMV-1 observed in this study may lead to the concern of the efficacy of current NDV vaccines used in poultry industry.

Table 2.1.
List of selected APMV-1 isolates to be sequenced for the full length of the HN genes

Collection Year	APMV-1 isolates	HN protein length (aa)
2009	APMV1/slaty-backed gull/Japan/9KS0098/2009	585
	APVM1/green-winged_teal/Japan/9KS0644/2009	616
2010	APMV1/northern pintail/Japan/10EY0020/2010	616
	APMV1/Anas sp./Japan/10UO0343/2010	616
	APMV1/Anas sp./Japan/10UO0529/2010	616
2011	APMV1/Anas sp./Japan/11OG1095/2011	616
	APMV1/Anas sp./Japan/11OG1114/2011	616
	APMV1/Anas sp./Japan/11UO0005/2011	616
	APMV1/Anas sp./Japan/11UO0016/2011	616
	APMV1/Anas ap./Japan/11UO0040/2011	616
2013	APMV1/Anas sp./Japan/13UO0475/2013	616
	APMV1/Anas sp./Japan/13OG0603/2013	616
	APMV1/Anas sp./Japan/13OG0977/2013	616
	APMV1/Anas sp./Japan/13OG1235/2013	616

Table 2.2.
Primers used for amplification and sequencing of the HN gene

Primer Name	Orientation	Primer Sequences (5′-3′)[a]	Length (bp)	Position[b]	Expected product size (bp)
FHN1-F	Forward	AARACHHTRHTRTGGCTYGGGAA	23	6143–6165	760
FHN1-R	Reverse	GGGATRAAATTMARRTGYTCTTG	23	6880–6902	
HN2-F	Forward	ATYGRRGGGRTAGGYAMRGA	20	6808–6827	842
HN2-R	Reverse	AAGWAATGRGABRTCCCBAYHGT	23	7627–7649	
HN3-F	Forward	AYAAYRAYACRTGCCCHGA	19	7430–7448	568
HN3-R	Reverse	GATGTYGYGTATGCTGC	17	7981–7997	
HNL4-F	Forward	ACDGGRGYBTAYACWGAYCC	20	7810–7829	708
HNL4-R	Reverse	AAGTCRCAYTCRTCDGGDAG	20	8498–8517	

[a] Codes for mixed-base position: B, C/G/T; H, A/T/C; K, G/T; M, A/C; R, A/G; S, C/G; W, A/T; Y, C/T
[b] Positions are based on the complete genome of NDV strain Lasota (GenBank accession number JF950510).

Table 2.3.
Nucleotide (upper right) and aa (lower left) sequence identities (%) among the HN gene of APMV-1 isolates

	Isolates	9KS00644	10UO0343	11OG1095	11OG1114	13OG1235	13UO0475	9KS0098	10UO0529	10EY0020	11UO0005	11UO0016	11UO0040	13OG0603	13OG0977
Class I	9KS00644	-	98.00	95.35	95.29	97.73	97.78	71.00	72.19	72.08	72.46	72.46	72.40	72.24	72.13
	10UO0343	98.53	-	95.73	95.62	99.08	99.13	71.05	72.02	72.02	72.29	72.29	72.24	72.02	71.97
	11OG1095	98.36	98.53	-	99.84	95.40	95.45	70.56	71.65	71.65	71.75	71.75	71.70	71.70	71.59
	11OG1114	98.20	98.36	99.84	-	95.29	95.35	70.62	71.70	71.70	71.81	71.81	71.75	71.75	71.65
	13OG1235	98.69	99.18	98.69	98.53	-	99.95	71.27	72.13	72.13	72.51	72.51	72.46	72.24	72.08
	13UO0475	98.86	99.35	98.86	98.69	99.84	-	71.32	72.19	72.19	72.56	72.56	72.51	72.29	72.13
Class II	9KS0098	82.06	81.67	81.87	81.67	81.87	82.06	-	89.02	89.12	89.12	89.12	89.07	89.12	88.91
	10UO0529	84.56	84.18	84.37	84.18	84.37	84.56	94.31	-	98.43	99.13	99.13	99.08	97.94	99.57
	10EY0020	84.56	84.18	84.37	84.18	84.37	84.56	94.14	99.18	-	98.32	98.32	98.27	97.56	98.11
	11UO0005	84.75	84.37	84.56	84.37	84.56	84.75	94.66	99.51	99.02	-	100.00	99.95	97.94	98.81
	11UO0016	84.75	84.37	84.56	84.37	84.56	84.75	94.66	99.51	99.02	100.00	-	99.95	97.94	98.81
	11UO0040	84.56	84.18	84.37	84.18	84.37	84.56	94.48	99.35	98.86	99.84	99.84	-	97.89	98.76
	13OG0603	84.56	84.18	84.37	84.18	84.37	84.56	94.48	99.02	98.69	99.18	99.18	99.02	-	97.62
	13OG0977	84.56	84.18	84.37	84.18	84.37	84.56	94.31	99.67	99.18	99.51	99.51	99.35	99.02	-

Table 2.4.
HI tests of APMV-1 isolate with mAbs against the HN protein of APMV-1 strain Ibaraki/85

	APMV-1 isolate	HI titer [a]				
		M1 (K263)	M2 (N[b])	M3 (D349)	M10 (E260)	M13 (D287)
	Ibaraki/85	320	160	5,120	640	1,280
Class I	9KS0644	< 10	160	< 10	< 10	< 10
	10UO0343	< 10	80	< 10	< 10	< 10
	11OG1095	< 10	160	< 10	< 10	< 10
	11OG1114	< 10	320	< 10	< 10	< 10
	13OG1235	< 10	160	< 10	< 10	< 10
	13UO475	< 10	160	< 10	< 10	< 10
Class II	9KS0098	< 10	< 10	< 10	< 10	< 10
	10UO0529	< 10	160	< 10	< 10	< 10
	10EY0020	< 10	320	< 10	< 10	< 10
	11UO0005	< 10	80	< 10	< 10	< 10
	11UO0016	< 10	160	< 10	< 10	< 10
	11UO0040	< 10	160	< 10	< 10	< 10
	13OG0603	< 10	160	< 10	< 10	< 10
	13OG0977	< 10	320	< 10	< 10	< 10

[a] The number in the parentheses represents the position of aa residue that mAbs recognized in the HN protein. The letter before the number represent the aa sequence.
[b] N: the position of aa residue that cannot be determined.

Table 2.5.
HI tests of APMV-1 isolate with mAbs against the HN protein of APMV-1 strain D26/76

Virus stain	HI titer [a]			
	HN14 (E347)	HN21 (E495)	HN31 (N[b])	HN41 (N481)
D26/76	2,560	10,240	5,120	160
Ibaraki/85	<10	10,240	<10	10,240
Chiba/85	5,120	40,960	20,480	10,240
Sato/30	2,560	2,560	320	<10
B1	5,120	320	1,0240	5,120
VG/GA	1,280	2,560	<10	320
9KS0644	<10	10,240	<10	<10
9KS0098	<10	1,280	<10	<10

[a] The number in the parentheses represents the position of aa that mAbs recognized in the HN protein. The letter before the number represent the aa sequence
[b] N: the position of aa residue that cannot be determined

Table 2.6.
Virus neutralizing (VN) titers of APMV-1 isolates and mAbs against the F protein of strain Sato/30[a]

Virus strain	VN titer		
	70/1	320/1	743/1
Sato/30	400	400	100
9KS0098	400	N	N
9KS0644	N	N	N

[a]N: Titer < 100

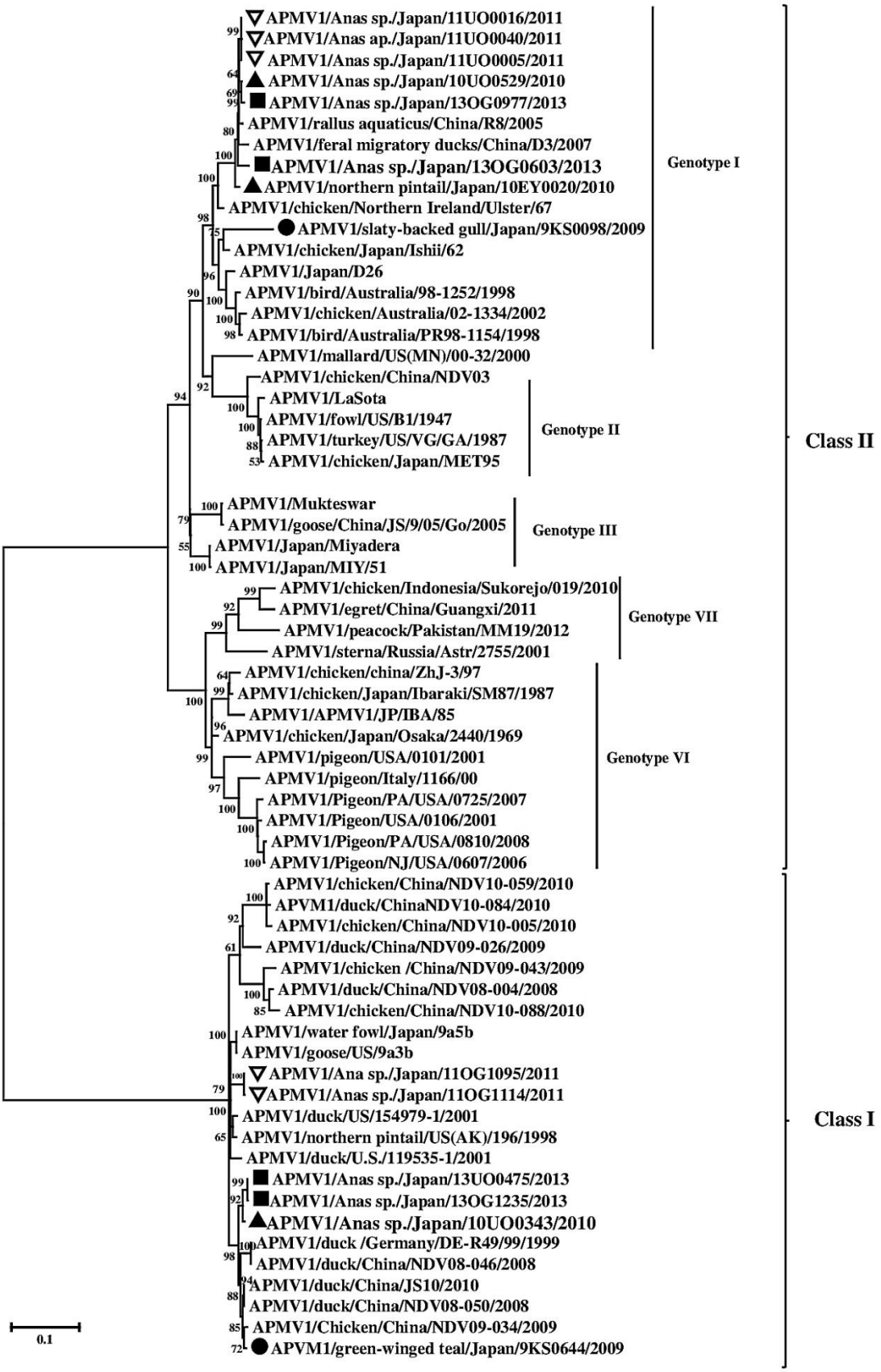

Fig. 2.1. The Phylogenetic tree of the HN genes from APMV-1 isolates was generated by the Maximum Likelihood method based on the Kimura 2-parameter model. The analysis involved 64 nucleotide sequences. There were a total of 1711 positions in the final dataset. The symbols used in the tree are described as follows; ●: sample from 2009, ▲: sample from 2010, ▽: sample from 2011, ♦: sample from 2012, ■: sample from 2013.

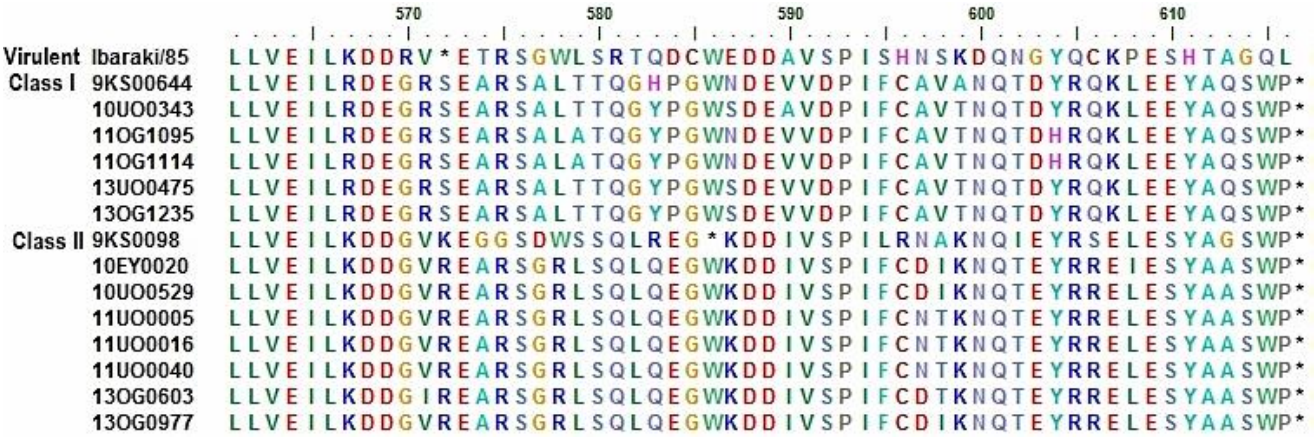

Fig. 2.2 aa sequences alignment of C-termini of the HN protein of APMV-1 isolates.
The sign * means the position of stop codon.

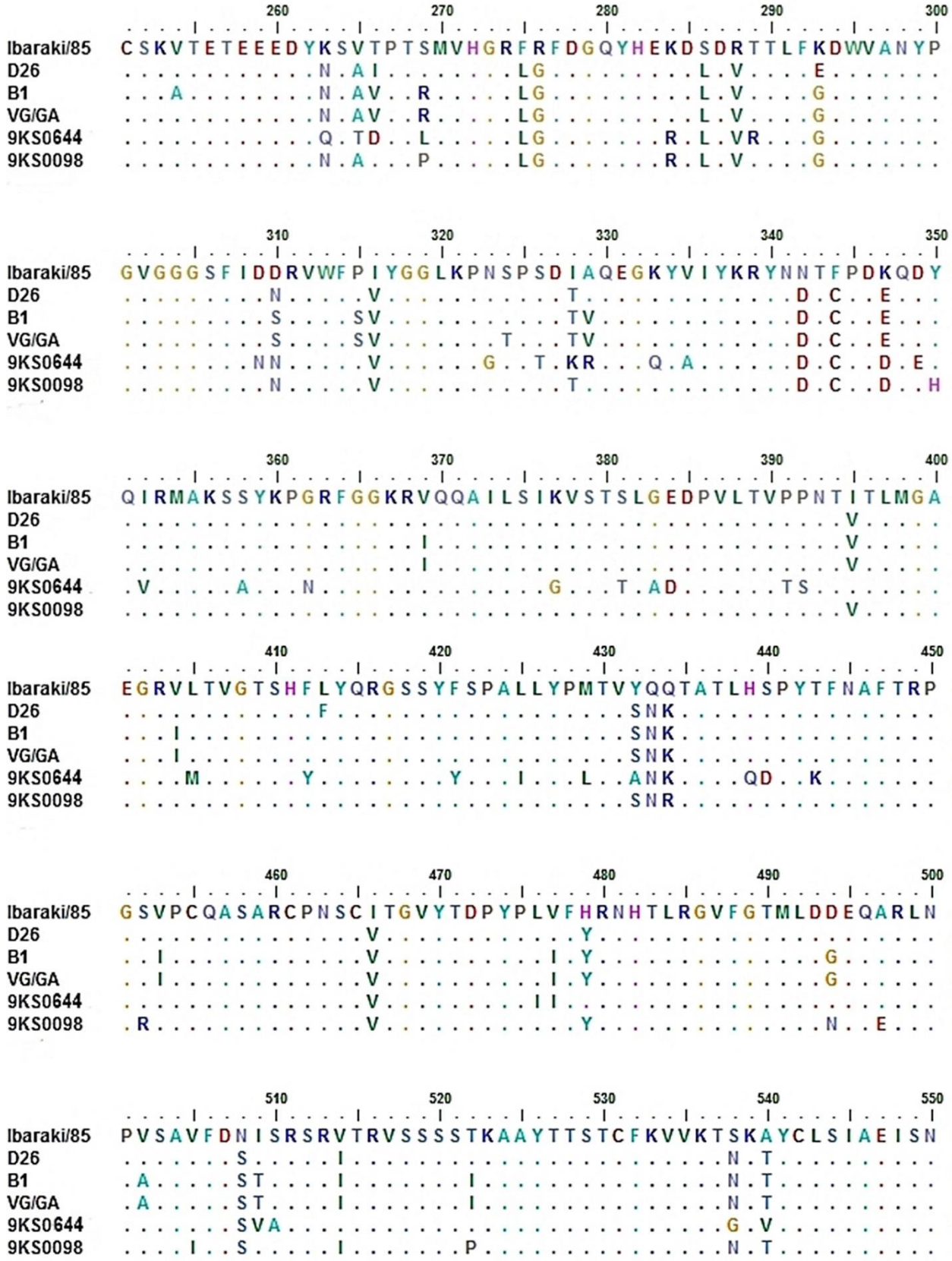

Fig. 2.3. aa sequences alignment of the HN protein of APMV-1 isolates from position 250 to 550.

Chapter III

Characterization of avian paramyxovirus serotype 14, a novel serotype, isolated from a duck fecal sample in Japan

3.1 Introduction

Nine serotypes of APMVs, APMV-1 to APMV-9 have been defined by HI tests (Wang et al., 2012). However, cross-reactivity has been noticed among different serotypes (Alexander et al., 1983; Kessler et al., 1979; Lipkind and Shihmanter, 1986; Shortridge et al., 1980; Tumova et al., 1979). In addition, antisera to APMV serotypes are not always available in many laboratories. The presence of new serotypes in wild bird population had not been long reported since the 1970s, until additional four novel serotypes, APMV-10 isolated from the rockhopper penguins (Miller et al., 2010), APMV-11 from the common snipe (Briand et al., 2012), APMV-12 from the Eurasian wigeon (Terregino et al., 2013), and APMV-13 from goose were reported (Yamamoto et al., 2015). These four viruses were differentiated from the nine known serotypes and were proposed as new serotype based on the results of HI tests with limited numbers of serotype antisera and/or genetic analyses.

Most APMV research works have focused on APMV-1 because the highly virulent strains of this serotype cause severe disease known as ND, in chickens. However, other APMVs are still lacking in knowledge. APMV-2, APMV-3, APMV-6, and APMV-7 were involved in respiratory disease and egg production drop in turkeys (Awang and Russell, 1990; Bankowski et al., 1981; Saif et al., 1997). APMV-5 isolated from the outbreak in pet birds, budgerigar, with diarrhea and mortality did not affect chickens and ducks (Kim et al., 2012; Nerome et al., 1978). APMV-12 and APMV-13 are low virulent to chickens (Terregino et al., 2013; Yamamoto et al., 2015).

Low virulent APMV-1 has been studied as a vaccine vectors due to the highly strict replication in respiratory tract of birds and other species including mouse and non-human primate (Kim and Samal, 2016). Recently, the potential of other APMVs as a vaccine vector has been evaluated against highly contagious avian viruses. For example APMV-2 to APMV-10 as a vaccine vector against NDV and APMV-3 as a vaccine vector for infectious bronchitis virus have been described (Kumar et al., 2011; Tsunekuni et al., 2014). The possibility to use other APMVs as a vaccine vector against human and mammal pathogens has also been considered since the restricted replication of APMV-2 to APMV-9 was demonstrated in mice and hamsters (Khattar et al., 2011; Samuel et al., 2011). Moreover, APMV-2, APMV-3, APMV-4, APM-7 and APMV-9 were shown to replicate in respiratory tract of rhesus monkeys (Khattar et al., 2013).

During the surveillance of APMVs in migratory wild birds from 2009 to 2013 conducted in this research, a heamagglutinating viral agent was isolated from the duck feces, which agent was not genetically and/or antigenically identified as AIV or reported APMVs (serotypes 1 to 13). Therefore, this isolate was assumed to be a novel APMV serotype. Here, I described the first isolation of APMV serotype 14, APMV/avian/Japan/11OG0352/2011 (isolate 11OG0352), and then, virological, serological, and genetic characterization of this virus are also described.

3.2 Materials and methods

Sample collection and virus isolation

Feces of migratory water birds in Obihiro City in the eastern part of Hokkaido Prefecture (GPS coordinates 44° 10′ N, 143° 40′ E) were collected during AIV active surveillance since 2009 and homogenized as described previously (Bui et al., 2011). Briefly, a 20% fecal homogenate was inoculated into the allantoic cavity of ten-day-old embryonated chicken eggs.

After incubating at 37°C for 3 days, allantoic fluids were tested for HA activity using 0.5% chicken red blood cells according to Manual for the laboratory diagnosis and virological surveillance of influenza (WHO, 2011).

Virus purification

Viruses propagated in eggs were partially purified using ultracentrifugation through 30% and 60% sucrose solution at 40,000 rpm for 2 h as previously described (Imai et al., 2012). The partially purified virus was further purified by centrifuging the preparation through a continuous sucrose density gradient (30-60%).

Electron microscopy

Samples for electron microscopy (EM) were prepared using 400 mesh-carbon-coated collodion grids (NISSHIN EM Co., Ltd., Tokyo, Japan) according to the two-step method previously described (Chrystie, 1996). Briefly, the grid was washed by ultrapure water and stained with 2% phosphotungstic acid (pH 6.5) for 2 min. The grid was examined using a Hitachi H7500 transmission EM (Hitachi High-Technologies Corporation, Tokyo, Japan).

Nucleotide sequencing

The nucleotide sequence of the whole viral genome was determined using a next-generation sequencing approach. Viral RNA was extracted from the infected allantoic fluid using Isogen II (NIPPON GENE, Toyo, Japan), followed by DNase I treatment (TaKaRa Bio Inc., Shiga, Japan). cDNA libraries were constructed using the NEB Next1 Ultra RNA Library Prep Kit for Illumina Version 2.0 (New England Biolabs, Ipswich, MA) following the manufacturer's guidelines. Purification of double-strand cDNA and size selection of 300 bp

was conducted using Agencourt AMPure XP beads (Beckman Coulter, Pasadena, CA). After measuring the quantity of sample libraries using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), DNA was loaded into the reagent cartridge. Sequencing was performed using a MiSeq bench-top sequencer (Illumina, San Diego, CA) to generate 51 bp single-end reads. To analyze data, FASTQ formatted sequence data was created using MiSeq Reporter program (Illumina). The contiguous sequence or contigs were assembled from the short sequence reads using CLC Genomic Workbench version 6.5.1 (CLC bio, Aarhus, Denmark) with de novo assembly commands. The consensus sequence of assembled contigs was determined using BLAST.

Phylogenetic analysis

Nucleotide sequences of whole genome and individual genes were aligned using the MUSCLE command. Pairwise comparison between sequences were conducted in MEGA 6.0 software using the p-distance model (Tamura et al., 2013). Phylogenetic trees were also generated using MEGA 6.0 software with the Maximum-likelihood method with 500 bootstraps.

Cross-HI test

HI tests were conducted according to the guidelines of the OIE (OIE, 2012). Antigens of APMV-1 to APMV-9 and an antiserum against each serotype described below were used as references except for APMV-5 (not available in our laboratory). APMV-2/chicken/California/Yucaipa/56, APMV-3/turkey/Wisconsin/68, APMV-4/duck/Hong Kong/D3/75, APMV-6/duck/Hong Kong/18/199/77, APMV-7/dove/Tennessee/4/75, APMV-8/goose/Delaware/1053/76, APMV-9/duck/New York/22/1978 and specific polyclonal chicken antiserum against each virus strain were purchased from National Veterinary Service

Laboratories (Ames, IA), while APMV-1/chicken/Japan/Ibaraki/85 was provided by National Institute of Animal Heath, Japan, and antiserum against APMV-1 was obtained from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan).

An antiserum against the isolate 11OG0352 was produced in six-week-old SPF white leghorn chickens. Chickens were inoculated with one subcutaneous injection of 0.2 ml of inactivated purified virus (HA, 1:10,240) emulsified with TiterMax Gold adjuvant (TiterMax USA, Inc., Norcross, GA), and the antiserum was collected two weeks later.

Virus infection of cultured cells

African green monkey kidney (Vero) cells, Madin-Darby bovine kidney (MDBK) cells, Madin-Darby canine kidney (MDCK) cells, and primary chick embryo fibroblasts (CEFs) in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and 2 mM L-glutamine were cultured in 96-well tissue culture microplates. Each culture was inoculated with the 10-fold diluted viral isolate propagated in eggs and incubated in DMEM supplemented with 25 mM HEPES, 0.01% glucose, 0.2% BSA and 6.25 μg/ml trypsin (Difco trypsin 1:250, BBD, Tokyo, Japan) or without trypsin at 37°C for four days. Based on the cytopathic effect (CPE) observed 4 days post-inoculation, followed by confirmation of HA activities in the culture fluids as described above, virus titers were quantified as the median tissue culture infectious dose ($TCID_{50}$) using the Behrens-Kärber method. APMV-1 vaccine strain clone 30 purchased from Intervet K.K. (Tokyo, Japan) was used for comparison.

Pathogenicity test in chickens

To determine virulence of the isolate 11OG0352, an ICPI test was performed using 10 one-day-old SPF white leghorn chicks as previously described (OIE, 2012).

All studies using chickens were conducted in compliance with the institutional rules for the care and use of laboratory animals, and the protocols were approved by the relevant committee of our institution.

Experimental infection of Mice

The experiment with female BALB/c mice aged 6 to 8 weeks were performed in an isolator placed in Biosafety Level-2 facility. Water and food were provided ad libitum. A total of 16 mice were used. A group of eight mice was anaesthetized by isofluorane and inoculated intranasally with 50 µl of allantoic fluids harvested from the 11OG0352-inoculated eggs. Another eight mice were mock administrated with normal fresh allantoic fluid. Mice were weighed and examined twice a day for clinical signs or behavior change. Three mice from each group were euthanized at 3 day post infection (dpi) and issues from brain, lung, and nasal turbinate were collected and homogenized into 20% (W/V) in viral transport media (M4RT; Remel, Inc., Lenexa, KS). Each tissue was fixed in 10% neutral buffered formalin and histopathological examination was done by Dr. Yoshiyasu Kobayashi, Obihiro University of Agriculture and Veterinary Medicine.

To detect the virus in tissues, 100 µl of the homogenate tissue was inoculated into allantoic cavity of two ten-day-old embryonated chicken eggs. Eggs were incubated at 37°C for 4 days. Allantoic fluids were collected and the HA test as described above was done to detect the presence of virus. RNA was extracted to amplify the F gene of the isolate using the following primers: 352Forward: 5′-ATGGAGAAGGGAACTGTGCT-3′ and 352Reverse: 5′-ATCTTTAACCCCACAATCTCGC -3′. The PCR amplification was carried out using TaKaRa Ex Taq (Takara Bio Inc) following cycling profile: initial denaturation of 95°C for 5 min, followed by 40 cycles of denaturation, annealing and extension at 95°C for 30 sec, 50°C for 30

sec and 72°C for 30 sec, respectively, and the final extension was carried out at 72°C for 10 min. The PCR products were then analyzed by 1.5% agarose gel electrophoresis and imaged with UV light.

Establishment of ELISA to detect antibody against the 11OG0352 isolate in chickens

Antigen used in ELISA was the purified virus of the isolate inactivated by incubating at 50°C, 30 min. To optimize the concentration of antigens, inactivated purified viruses were diluted in carbonate-bicarbonate coating buffer (pH 9.6); 0.25, 0.5, 1, and 2 µg/50 µl). One % BSA and 1% alkaline-soluble casein purchased from Novagen (Darmstadt, Germany) were evaluated as blocking buffer.

Diluted antigens were coated to microplate wells (Nunc MaxiSorp F96, Denmark) by adding 50 µl/well. The plate was incubated at 4°C overnight and washed with PBS 3 times. In blocking step, 2 types of blocking buffer were used for comparison. One-hundred µl of each blocking buffer was added to each well and incubated at 37°C for 2 h, follow by washing 3 times with 0.05% PBS-T. Sera from the pre-immunized SPF chicken and 11OG0352-immunized SPF chicken were used as a negative control serum and positive control serum, respectively. Fifty µl of each serum diluted at 1:50, 1:100, 1:200, and 1:400 in blocking buffer were added to the wells and incubated at 37°C for 1 h, then washed 3 times with 0.05% PBS-T. The conjugated antibody used in this study was F(ab')2 anti-chicken IgG (H&L) antibody peroxidase conjugated purchased from Rockland Immunochemicals Inc. (Limerick, PA). The substrate reagent, 3,3′,5,5′-Tetramethylbenzidine (TMB) (BD Biosciences, San Diego, CA) was dispensed 50 µl to each well. After incubation at room temperature for 10 min, the reaction was stop by adding 50 µl of 2N H_2SO_4 to each well. The absorbance was read as an optical density

(OD) value using Tecan GENios Pro Multifunction Multimode Microplate Reader (Tecan Group Ltd. Switzerland) at wavelengths of 450 nm.

To determine condition of blocking buffer and washing, after antigen coating, I applied 1% alkaline-soluble casein as a blocking buffer and incubate at 37°C for 2 h and 4°C overnight. In this condition, serum from 55 SPF chickens were tested. After each incubation, the plate was washed with 0.1% PBS-T, 4 times. Further steps were done followed the ELISA protocol as mentioned above.

The cut-off value was calculated using this formula, cut-off value = Mean of OD value from serum of SPF chickens + (3 x Standard Deviation).

According to the optimized conditions, a total of 189 field sera collected from chicken farms were tested. The age of chickens varied from 19 days to 57 weeks.

VN test

Two-fold dilutions of antiserum against the isolate 11OG0352 and field chicken sera that gave high OD value (>0.9) in ELISA, from 1:40 to 1:320 dilution, were mixed with an equal amount of 100 $TCID_{50}$/0.1ml of the isolate. The mixture was incubated at 37°C for 1 h and then inoculated into CEFs grown on the 96-well plate. After incubation for 4 days, the culture fluids were collected to confirm HA activities as described above. The reciprocal of the highest dilution of the serum that neutralizing 50% of the virus was taken as the neutralizing titer.

Nucleotide sequence accession numbers

GenBank accession number of APMV-14/duck/Japan/11OG0352/2011 is KX258200. Accession numbers of APMVs used to compare nucleotide sequences are as follow: APMV-1,

Y18898; APMV-2, EU338414; APMV-3, EU782025; APMV-4, FJ177514; APMV-5, GU206351; APMV-6, EU622637; APMV-7, FJ231524; APMV-8, FJ215863; APMV-9, NC025390; APMV-10, NC025349; APMV-11, NC025407; APMV-12, NC025363; and APMV-13, NC030231.

3.3 Results

Virus isolation

A virus with HA activity isolated from a duck fecal sample was tentatively designated isolate 11OG0352. Conventional RT-PCR was used to detect the M gene of influenza A virus and the F gene of APMV-1, and each analysis was negative (data not shown).

EM

EM analysis revealed that the morphology of the isolate 11OG0352 resembled that of members of the family *Paramyxoviridae*. The majority of virus particles had spike-like projections surrounding the envelope, were spherical, and ranged in diameter from 120–200 nm in diameter (Fig. 3.1a). Pleomorphic shapes and "herringbone-shaped" nucleocapsids ranging approximately 20 nm in diameter were also seen (Fig. 3.1b).

Genomic analysis

The complete genome of the isolate 11OG0352 was determined (length = 15,444 nt, GenBank accession number KX258200). The isolate 11OG0352 sequence comprised six genes, which is the same as that of members of the genus *Avulavirus* with genomes comprising N, P, M, F, HN, and L genes, including intergenic regions of 2‑36 nt. The 3′-leader region of isolate 11OG0352 was 55 nt, typical of members of the genus *Avulavirus* and the length of 5′-trailer

region was 277 nt (Fig. 3.2). The first 12 nt of the 3′-leader region were highly conserved compared with those of other APMVs and 100% identical to those of APMV-2, -5, -6, -7 and -8 (Fig. 3.3a). Similarly, the last 12 nt of the 5′-trailer region were conserved compared with those of other APMVs (Fig. 3.3b). The first 13 nts of the 3′-leader region of isolate 11OG0352 are complementary to those of the 5′-trailer region (84.62%) (Fig. 3.3c). The GS and GE sequences of six genes were well conserved (Table 3.1). Comparison of full genome sequences among APMVs are shown in Table 3.2; results reveal that APMV-5 and APMV-6 are most closely related to isolate 11OG0352 (56.1% and 55.6% nucleotide identities, respectively) and differ most from APMV-4 (46.0%).

The nucleotide sequence identities of the isolate 11OG0352 gene to those of other APMVs ranged from 43.8% (M) to 61.4% (N) nucleotide identity (Table 3.3). The sequences of all genes of APMV-5 and APMV-6 were >50% identical compared with those of isolate 11OG0352. APMV-5 genes were most similar to isolate 11OG0352 in the sequence N (61.4%), M (55.9%), HN (61.2%), and L (56.7%). The highest identities of the P and F genes were those of APMV-6 (52.5% and 56.7%, respectively). The aa and nucleotide sequence identities were consistent (Table 3.4).

The P gene of isolate 11OG0352 contains a putative RNA editing site, 3′-AUUUUCCC-5′ (negative sense) at nucleotide positions 2,097–2,104 in the complete genome sequence. The sequence of the isolate 11OG0352 editing site is similar but not identical to those of APMV-1, -2, -5, -6, -7, -8, 9, -10, -12, -13 (3′-UUUUUCCC-5′), APMV-3 and -4 (3′-AAUUUCCC-5′), and APMV-11 (3′-UCUUAGUC-5′) (Table 3.5).

The F gene of isolate 11OG0352 encoded 541 aa. The F-protein cleavage site of isolate 11OG0352 is R-E-G-K ↓ L, which resembles the aa motif of the low virulent APMV-1, confirming the ICPI data. The aa sequence of isolate 11OG0352 contained lysine (K) at position

−1 (underlined), instead of arginine (R) present in all members of the genus *Avulavirus* (Table 3.6).

Phylogenetic analysis

As shown in Fig. 3.4, a phylogenetic tree of complete genomes indicated that isolate 110G0352 is a member of the genus *Avulavirus* and distinct from other APMVs. Based on F gene sequence, the isolate 11OG0352 forms a cluster with APMV-5 and APMV-6 with a bootstrap value of 99 (Fig. 3.5). Moreover, a cluster of these three viruses in the phylogenetic tree of the HN genes had a bootstrap value of 99 (Fig. 3.6).

Cross-HI tests

As shown in Table 3.7, the titers of antisera against each representative APMV serotype were highest with the homologous virus. The HI titer of the antiserum against isolate 11G0352 had the highest HI with the homologous virus (1:256) and reacted with APMV-6 (APMV-6/duck/Hong Kong/18/199/77) with a low HI titer (64-fold lower). In addition, the HI titer for isolate 11OG0352 with the antiserum against APMV-6 was low (1:8) (32-fold lower compared with the homologous virus).

Virus infection of cultured cells

Infectivities of isolate 11OG0352 and the APMV-1 vaccine strain clone 30 for four different types of cultured cells are shown in Table 3.8. In the absence of trypsin, the isolate 11OG0352 replicate in CEFs but not in MDBK, MDCK, and Vero cells. In contrast, APMV-1 strain clone 30 replicated in all cell cultures except MDCK. In the presence of trypsin, the strain clone 30 replicated more efficiently in all cell cultures, while the highest titer of isolate 11OG0352 was $10^{4.0}$ $TCID_{50}$/ml in CEFs compared with the titer in the absence of trypsin ($10^{2.0}$

$TCID_{50}$/ml). Further, the extent of syncytium formation in CEFs was similar to that of clone 30 (Fig. 3.7).

Pathogenicity test in chickens

The ICPI of isolate 11OG0352 was 0.0, indicating that it can be classified as low virulence according to the categorization of the virulence of APMV-1.

Experimental infection in mice

None of the mice infected with the isolate 11OG0352 exhibited any clinical signs through the experimental period. However, the infected mice lost more weight than the control mice. The pronounced loss in weight was observed in infected mice at 1 dpi and then the weight increased afterward. At 4 dpi, all the inoculated mice recovered their weight (Fig. 3.8). At 3 dpi, virus was isolated from the lung of the three scarified mice. In only one mouse, virus was isolated from lung, nasal turbinate, and brain. The RT-PCR results were consistent with the virus isolation from eggs (Table 3.9).

The histopathological examinations showed no significant lesions in the brain sections. In contrast, interstitial pneumonia was observed in the lung sections from the 3 infected mice (Fig. 3.9). The severity of the lung lesions varied from mild to severe.

ELISA

The optimized conditions that gave the highest positive/negative ratio were found as follows; antigen concentration: 0.5 µg/50 µl/well, serum dilution: 1/400, and blocking buffer: 1% alkaline-soluble casein at 37°C for 2 h (Fig. 3.10).

The conditions were applied to the ELISA to detect antibodies against the isolate in chickens, and SPF chicken sera were tested to determine the cut-off value. In Fig. 3.11, the

result from blocking condition at 37°C for 2 h gave higher mean OD (0.146) value and SD (0.144) than the blocking condition at 4°C, overnight. After the blocking condition at 4°C, overnight and washing with 0.1% PBS-T 4 times were applied to the ELISA, none of the high background was observed and the cut-off value was determined as follows; mean OD value (0.105) + 3 Standard Deviation (0.095). Accordingly, the cut-off value of ELISA established is calculated as 0.390.

The result revealed that 32.3% of the fields samples (61/189) had OD values greater than 0.390. Fourteen sera with the OD value greater than 0.9 were applied to VN tests to confirm that the sera with high OD values contain antibody against the isolate or not. The result showed that none of them cannot inhibit the replication of the isolate in CEFs (Table 3.10).

3.4 Discussion

In this study, I show that the complete genome sequence of the isolate 11OG0352 from the duck feces was similar to those of members of the family *Paramyxoviridae*, and the genome size follows the "rule of six" (Kolakofsky et al., 1998). Thus, the genome of isolate 11OG0352 was structured into six open reading frames, 3′-N-P-M-F-HN-L-5′, identical to members of the genus *Avulavirus*, except APMV-6 that harbors SH gene between F and HN (Suarez et al., 2013). This high level of complementarity between the leader and trailer regions suggests that the conserved sequences in the promoter region of the genome and antigenome are essential for RNA replication (Lamb and Parks, 2013). Analysis of the P genes of APMVs including isolate 11OG0352 showed that four different sequence patterns of the P-gene editing site exist among them, and the sequence of the isolate 11OG0352 editing site (3′-AUUUUCCC-5′, negative sense) has not been discovered in other APMVs so far (Table 3.5). The addition of single or double G residues to the P-gene editing site of the isolate 11OG0352 would yield a V and W

proteins, respectively as described in other APMVs (Samuel et al., 2010). V proteins were reported to be involved in interrupting host response to NDV infection (Alamares et al., 2010). Further studies are required to understand the function of V and W proteins in isolate 11OG0352 replication cycle.

The HI test is commonly and successfully used for APMV grouping or serotyping (nine serotypes). However, APMV serotypes cross-react (Alexander and Collins, 1984; Lipkind and Shihmanter, 1986; Nerome et al., 1978), and there is significant diversity among strains within certain serotypes (Alexander and Collins, 1981; Alexander and Collins, 1984). Thus, cross-reactivities detected using the HI test may cause misleading grouping or serotyping of APMV. The novel APMV serotypes 10, 12, 13 exhibited cross-reactivities with other serotypes (Miller et al., 2010; Terregino et al., 2013; Yamamoto et al., 2015).

These cross-reactions may be explained by the source of the antiserum used in the HI test, including hyperimmune sera raised using multiple immunizations. Miller et al. (2010) reported that the cross-reactivity between APMV-8 and APMV-10 was not detected when anti-APMV-10 was produced after only one virus injection. Further, a hyperimmune serum against APMV-1 exhibited a high level of cross-reactivity with APMV-13 (4-fold difference in HI titers), although a hyperimmune serum against APMV-13 did not react significantly with other serotypes such as APMV-1 (Yamamoto et al., 2015). Similar findings were reported for APMV-1 and APMV-12 (Terregino et al., 2013). Thus, a high level of one-way antigenic cross-reactivity among serotypes detected using the HI test may cause confusion in the identification of serotypes, particularly when antisera for all serotypes are not available. Although I immunized chickens using only one injection to avoid this problem, very low cross-reactivity was detected only between isolate 11OG0352 and APMV-6 (64-fold difference in HI titers) (Table 3.7), which might reflect the relatively high aa sequence similarity (52.9%) of the HN

protein between them. However, cross-reactivity between the serotypes does not always depend on the similarity of HN aa sequences, because APMV-3 is significantly cross-reactive and induces cross-protective immunity against virulent APMV-1, which shares only 32.6% aa sequence identity with the HN protein (Nayak et al., 2012). I was unable to use antisera against APMV-5, and APMV-10 to APMV-13 in this study. Because of obstacles imposed by the cross-reactivity as mentioned above and the limitation of available reference antiserum against each APMV serotype, analysis of complete genome sequences should be conducted to identify new APMV serotypes as suggested previously (Miller et al., 2010). Although definitive new classification guidelines for serotyping have not yet been established, Terregino et al. (2013) proposed a classification based on nucleotide sequence identities of the whole genome as one simple method.

In this study, I determined that the lowest and highest nucleotide sequence identities of the whole genomes of two different serotypes are 45.9% (APMV-4 and APMV-5) and 64.0% (APMV-12 and APMV-13). The nucleotide sequence identities of the whole genomes of isolate 11OG0352 and other APMVs were in the range from 46.3% to 56.1% (Table 3.2) and genetic variation between subgroups within the serotype are in the sequence APMV-2 (69.4%) (Subbiah et al., 2010), APMV-3 (67%) (Kumar et al., 2010), APMV-6 (70%) (Bui et al., 2014; Xiao et al., 2010). Accordingly, the nucleotide sequence identities of the whole genome between different serotypes ≤60% seem reasonable as a criterion for differentiating a group of APMVs. Nevertheless, I show here that APMV-12 is 64.0% identical to APMV-13, which was isolated in Japan. Recently, (Karamendin et al., 2016) reported that APMV-13 detected in Kazakhstan is most closely related to APMV-12 (69% nucleotide sequence identity). The relatively high nucleotide value between APMV-12 and APMV-13 was nearly identical to or higher compared with the lowest nucleotide sequence identity calculated for subgroups within

the serotype 3 (67%) (Kumar et al., 2010). Unfortunately, APMV-13 was not antigenically compared with APMV-12 (Karamendin et al., 2016; Yamamoto et al., 2015). This problematic result indicates that researchers in the field should establish a consensus genetic classification system for APMVs.

Thus, to improve the reliability of the genetic classification system, more of viruses representing each serotype should be analyzed. The use of virus-specific antisera, particularly these against a new group or serotype, or generating mAbs will likely facilitate APMV grouping or serotyping based on genetic analyses. Exchanging antisera between laboratories should also be considered.

Of the four different types of cells derived from avian and mammal species, isolate 11OG0352 replicated only in CEFs. This result showed that replication of isolate 11OG0352 is highly restricted to avian species, which indicated that the virus differs from APMV-1 to APMV-9 and APMV-13 that can replicate in cultured cells derived from avian and mammalian species (Kim et al., 2012; Yamamoto et al., 2015).

APMV-1 (NDV) can replicate in both avian species and non-avian species including humans, although the virus replication is very restricted in non-avian species with very mild or no symptom. From these characteristics, APMV-1 is considered to be available as a potential vaccine vector to deliver foreign antigens of animal and human pathogens (Khattar et al., 2011). As a vaccine vector, APMV-1 induced local and systematic responses against foreign antigens of human pathogen and was protective against pathogen challenge (Bukreyev and Collins, 2008). Khattar et al. (2011) evaluated whether other APMV serotypes 2 to 9 in addition to APMV-1 can be used as potential vaccine vectors in experimental infection of mice with these viruses. As a result, five serotypes (1, 2, 6, 7 and 9) produced clinical disease and weight loss in all of the three inoculated mice, but no mortality was seen. Replication of all of the serotypes

used except APMV-5 was observed in the nasal turbinate and lungs. Similarly, in this study, although the virus titers were very low, isolate 11OG0352 replicated in the lungs of all the inoculated mice without inducing any clinical signs, but in the nasal turbinate of only one inoculated mouse. The present result may imply that the new APMV serotype isolated in this study could also be a potential candidate as an alternative vaccine vector for human and non-human primate pathogens. However, the virus was detected from the brain in only one inoculated mouse, although the virus titers were very low. Similar findings were observed in some mice inoculated with APMV-4 and APMV-9 (Khattar et al., 2011). Therefore, the ability of virus to replicate in various organs and tissues including brain needs to be evaluated further using non-human primate study model.

ELISA to detect antibody against isolate 11OG0352 established in this study was able to differentiate positive serum from negative serum in SPF chickens in the optimized condition. In contrast, many sera of older field chickens gave high OD values. However, these sera did not inhibit the virus replication in VN test. These false positive results might come from hyperimmune serum associated with extensive NDV vaccination in commercial chickens (Maldonado et al., 1994). Similar phenomenon was also found in the study to examine the prevalence of antibodies to APMV serotypes 1 to 9 except APMV-5 in commercial poultry in the US (Warke et al., 2008). Warke et al. (2008) reported that positive HI results shown for APMV-2, APMV-3, APMV-4, APMV-6, APMV-7, and APMV-8 were found in the flocks with high titers of APMV-1 (≥1,024), and suggested that cross-reactivity against those APMVs might be induced by extensive APMV-1 vaccination. Interestingly, most of positive samples in this study were from chickens older than 13 weeks old. In commercial chicken farm, multiple APMV-1 vaccinations had been administrated to them. However, it is unclear why antibody responses against most of the other APMV serotypes were observed only in the presence of the

high antibody titers for APMV-1. More sensitive and sophisticated techniques to differentiate antibodies against different serotypes in the field conditions are required.

The F-protein cleavage site is mainly used to determine the virulence of APMV-1. Highly virulent strains of APMV-1 have a furin cleavage site motif that contains multiple basic aa (R-X-K/R-R ↓ F) that is cleaved by an intracellular furin-like protease. Conversely, the F-protein cleavage site of low virulent strains of APMV-1 includes a monobasic aa residue, which is cleaved by an extracellular trypsin-like protease (Samal, 2011). In the presence of trypsin, isolate 11OG0352 multiplied more efficiently and caused syncytium formation, indicating that this virus required the activity of an extracellular protease to cleave the F protein. Two basic aa residues in the F protein cleavage site of 11OG0352 (R-E-G-K ↓ L) resemble those of the low virulent APMV-1 (R-Q-G-R ↓ L), associated with a very low ICPI value in day-old chicks, and interestingly, I reveal here, for the first time to our knowledge, the detection of a K residue at the position −1, in contrast to other APMVs. The function of the K residue at this position in APMVs is unknown. This motif occurs only in Hendra virus (V-D-G-V-K ↓ L), genus *Henipavirus*, although a specific aa at this position is not required for the cleavage of the Henipavirus F protein (Craft Jr and Dutch, 2005; Moll et al., 2004). The F-protein cleavage site of certain APMVs paradoxically shows virological properties similar to that of highly virulent APMV-1. For example, APMV-5 does not cause disease in chickens despite of containing the multiple basic aa motif (K-R-K-K-R ↓ F) in its F protein (Samuel et al., 2010). Further, modification of the F-protein cleavage site of APMV-2, APMV-4, and APMV -7 to become multiple basic aa does not increase pathogenicity in chickens (Kim et al., 2013; Subbiah et al., 2011; Xiao et al., 2012), indicating that infection mechanisms of other APVMs may differ from that of APMV-1. Therefore, the F protein cleavage site may not be the optimum tool to determine the virulence of other APMVs except APMV-1.

Shihmanter et al. (1997) raised the question of whether the vaccination failure in poultry may be caused by mixed infection with APMVs. Thus, the health impact of the new APMV including isolate 11OG0352 on other poultry should be studied further.

1.5 Summary

The hemagglutinating isolate 11OG0352 was isolated from a fecal sample of a migratory duck. Available whole genome sequences of recently reported APMVs (serotypes 11–13) as well as the known APMV serotypes, except APMV-1, are limited in number. Phylogenetic analysis, low nucleotide sequence identity between isolate 11OG0352 and other APMVs, together with the results of cross-HI tests, support the conclusion that isolate 11OG0352 represents the prototype strain of a new APMV serotype, APMV-14. I therefore assigned APMV-14/duck/Japan/11OG0352/2011 as the full name of this strain. In the ELISA system to detect APMV-14 antibodies established in this study, positive reactions were observed in only older commercial chickens extensively vaccinated with APMV-1. However, I could not conclude whether these reactions observed were nonspecific or due to cross-reactivity with APMV-1. The replication of virus in the respiratory tract of the infected mice without clinical signs may imply the possibility of virus to become a vaccine vector candidate in mammalian species including humans.

Table 3.1.

Gene-start and gene-end sequences of isolate 11OG0352[a].

Genes	Gene-start	Gene-end
N	CUCCCCCUUA	AAUUAUUUUUU
P	CUCCCCCUUC	AAUUAUUUUUUU
M	CUCCCCCUUG	AAUACUUUUUU
F	CUCCCCCCUU	AAUUAUUUUUUU
HN	CUCCCCCUUG	AAUUAUUUUUU
L	CUCCCCCUGG	AAUUCCUUUAU

[a] Sequences are in the negative sense.

Table 3.2.
Nucleotide sequence identities (%) among the complete genomes of the isolate 11OG0352 and other APMVs.

Virus	APMV-1	APMV-2	APMV-3	APMV-4	APMV-5	APMV-6	APMV-7	APMV-8	APMV-9	APMV-10	APMV-11	APMV-12	APMV-13
APMV-2	49.0												
APMV-3	46.6	46.1											
APMV-4	46.3	46.4	49.5										
APMV-5	49.2	52.1	46.6	45.9									
APMV-6	48.6	52.4	46.3	46.3	56.9								
APMV-7	48.9	52.0	47.1	46.3	52.9	52.3							
APMV-8	48.9	59.9	47.1	46.1	52.3	51.9	53.1						
APMV-9	61.5	48.3	46.3	46.0	47.9	47.8	48.2	49.1					
APMV-10	48.7	60.6	46.5	46.2	53.0	52.6	52.3	60.8	48.8				
APMV-11	48.3	53.3	48.0	46.6	53.5	52.5	53.8	53.7	48.7	53.5			
APMV-12	58.8	48.2	46.1	46.5	48.5	48.2	48.4	48.8	57.9	48.6	48.6		
APMV-13	58.6	48.6	47.2	46.5	48.8	48.8	49.3	49.4	57.3	48.7	48.8	64.0	
11OG0352	48.1	51.2	46.7	46.0	56.1	55.6	51.7	52.9	48.0	52.4	52.5	48.5	48.6

Table 3.3.
Comparison of the nucleotide sequences of the individual genes of isolate with those of other APMVs.

Virus	% Nucleotide identities with individual genes of the isolate 11OG0352					
	N	P	M	F	HN	L
APMV-1	48.9	44.1	44.5	48.3	48.8	49.8
APMV-2	57.6	46.3	50.8	51.6	53.3	51.5
APMV-3	49.5	45.4	44.3	45.9	47.9	47.3
APMV-4	46.9	44.3	44.7	45.8	48.2	46.1
APMV-5	61.4	50.1	55.9	54.2	61.2	56.7
APMV-6	56.9	52.5	54.6	56.7	59.1	55.3
APMV-7	57.0	45.0	51.3	49.3	53.1	52.1
APMV-8	57.2	49.3	50.2	51.6	53.9	53.1
APMV-9	48.5	47.9	45.1	45.8	49.4	49.5
APMV-10	58.7	48.0	51.0	53.0	55.1	52.2
APMV-11	55.0	46.1	49.3	48.8	55.0	53.7
APMV-12	50.8	45.4	43.8	50.3	47.8	49.6
APMV-13	51.5	45.8	44.3	48.1	49.1	50.0

Table 3.4.
Comparison of the aa sequences of the proteins of the isolate 11OG0352 with those of other APMVs.

Virus	% aa sequence identity compared with isolate 11OG0352					
	N	P	M	F	HN	L
APMV-1	40.0	24.8	33.2	40.1	35.8	37.7
APMV-2	53.5	28.2	42.9	46.5	44.5	42.8
APMV-3	37.6	24.2	29.6	30.9	37.1	34.8
APMV-4	36.2	23.6	31.9	34.6	34.0	32.6
APMV-5	60.5	31.3	49.6	49.6	53.8	50.1
APMV-6	56.8	33.4	52.1	54.5	52.9	50.2
APMV-7	50.9	26.1	43.2	40.7	43.9	43.8
APMV-8	51.6	29.1	41.3	45.3	44.3	45.3
APMV-9	39.0	24.5	32.7	35.4	35.6	39.0
APMV-10	51.9	29.1	40.7	45.3	42.6	44.3
APMV-11	49.5	28.2	39.9	40.7	45.8	46.1
APMV-12	39.7	27.0	32.1	39.5	36.6	38.3
APMV-13	41.1	26.7	32.1	38.3	37.3	38.6

Table 3.5.
Sequences of the P gene editing site of isolate 11OG0352 and other APMVs[a].

Virus	Sequence
11OG0352	AUUUUCCC
APMV-1	UUUUUCCC
APMV-2	UUUUUCCC
APMV-3	AAUUUCCC
APMV-4	AAUUUCCC
APMV-5	UUUUUCCC
APMV-6	UUUUUCCC
APMV-7	UUUUUCCC
APMV-8	UUUUUCCC
APMV-9	UUUUUCCC
APMV-10	UUUUUCCC
APMV-11	UCUUAGUC
APMV-12	UUUUUCCC
APMV-13	UUUUUCCC

[a]Sequences are in the negative sense.

Table 3.6.
aa sequence of the F protein cleavage site of isolate 11OG0352 and those of other APMVs.

Virus	Cleavage site[a]						
11OG0352	^{98}T	**R**	E	G	**K**	↓	L^{103}
APMV-1 (Low virulent)	^{112}G	**R**	Q	G	**R**	↓	L^{117}
APMV-1 (highly virulent)	^{112}**R**	**R**	Q	**R**	**R**	↓	F^{117}
APMV-2	^{93}**K**	P	A	S	**R**	↓	F^{98}
APMV-3	^{96}**R**	P	S	G	**R**	↓	L^{101}
APMV-4	^{115}D	I	Q	P	**R**	↓	F^{110}
APMV-5	^{104}**K**	**R**	**K**	**K**	**R**	↓	F^{109}
APMV-6	^{113}A	P	E	P	**R**	↓	L^{118}
APMV-7	^{101}L	P	S	S	**R**	↓	F^{106}
APMV-8	^{98}Y	P	Q	I	**R**	↓	L^{103}
APMV-9	^{104}I	**R**	E	G	**R**	↓	I^{109}
APMV-10	^{158}**K**	P	S	Q	**R**	↓	I^{163}
APMV-11	^{113}S	G	T	**K**	**R**	↓	F^{118}
APMV-12	^{105}G	**R**	E	P	**R**	↓	L^{110}
APMV-13	^{102}V	**R**	E	N	**R**	↓	L^{107}

[a]Basic aa (R:arginine, K:lysine) are in bold. Numbers show aa position and ↓ indicates the predicted cleavage site.

Table 3.7.
Cross-HI test of isolate 11OG0352 and APMVs

Virus	Antisera								
	APMV-1	APMV-2	APMV-3	APMV-4	APMV-6	APMV-7	APMV-8	APMV-9	11OG0352
APMV-1	1024	N[a]	8	N	N	N	N	16	N
APMV-2	16	512	8	N	N	16	N	N	N
APMV-3	32	N	1024	N	N	16	N	32	N
APMV-4	N	N	N	128	N	N	N	N	N
APMV-6	N	N	N	N	256	N	N	N	4
APMV-7	N	N	8	N	N	256	N	16	N
APMV-8	N	N	N	N	N	N	128	N	N
APMV-9	128	N	8	N	N	8	N	512	N
11OG0352	N	N	N	N	8	N	N	N	256

[a] N: Titer < 4

Table 3.8.
Infectivity of isolate 11OG0352 and APMV-1 (Clone 30) in cell cultures

	Virus titer ($TCID_{50}$/ml)			
	11OG0352		APMV-1 (Clone 30)	
Cells	Trypsin -	Trypsin +	Trypsin -	Trypsin +
MDBK	$<10^{1.5}$	$<10^{1.5}$	$10^{4.5}$	$10^{8.5}$
MDCK	$<10^{1.5}$	$<10^{1.5}$	$<10^{1.5}$	$10^{2.5}$
Vero	$<10^{1.5}$	$<10^{1.5}$	$10^{3.5}$	$10^{7.0}$
CEF	$10^{2.0}$	$10^{4.0}$	$10^{6.0}$	$10^{10.0}$

Table 3.9.

Virus detection in the tissue collected from three infected mice 3 at dpi.

Methods	Tissue		
	Lung	Nasal turbinate	brain
Virus isolation	+++	+ – –	+ – –
RT-PCR	+++	+ – –	+ – –

+ indicates the presence of virus from one mice. – indicates that no virus was detected.

Table 3.10.

Virus neutralizing titers in field chicken sera showing the high OD values (>0.9) in ELISA

Sample number	Age of chicken	OD value	Virus neutralization titer[a]
438	13 weeks	1.047	N
468	14 weeks	0.948	N
476	14 weeks	1.097	N
478	14 weeks	1.094	N
482	14 weeks	1.11	N
485	15 weeks	0.900	N
490	15 weeks	1.074	N
608	unknown	0.961	N
634	unknown	0.998	N
635	unknown	1.343	N
636	unknown	1.106	N
3487	37 weeks	1.117	N
3463	37 weeks	0.971	N
3732	63 weeks	1.029	N
Positive serum control	-	1.542	1:40

[a] N: Titer < 40

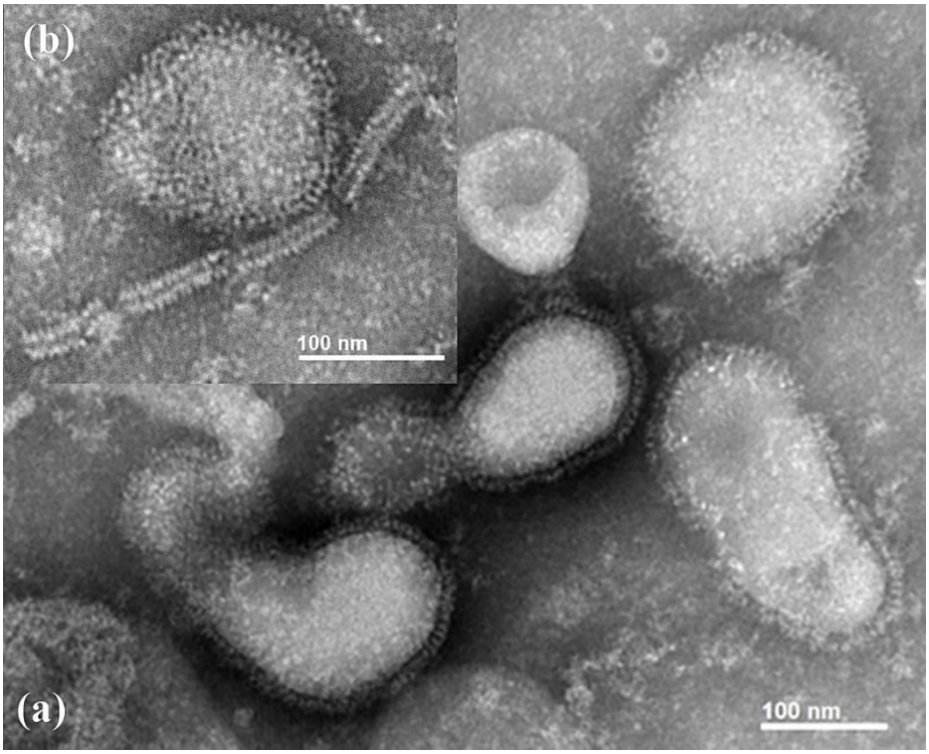

Fig. 3.1. EM of negatively stained isolate 11OG0352 particles showing (a) spherical and pleomorphic forms with spike-like projections surrounding the envelope of the particle, and (b) the "herringbone-shaped" nucleocapsid.

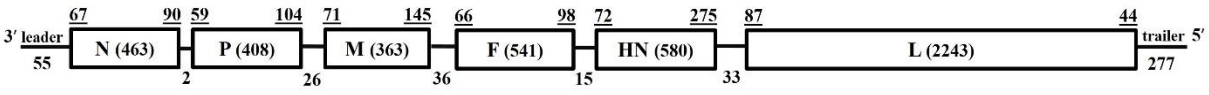

Fig. 3.2. Diagram of the isolate 11OG0352 genome. Each rectangle represents an individual gene. The aa lengths of encoded proteins are shown in the boxes. The lengths of the non-translated upstream and downstream regions are underlined. Intergenic regions locate between each box.

Fig 3.3. Alignment of the 3′-leader (a) and 5′-trailer regions (b) of isolate 11OG0352 with other APMVs. Dots indicate identities. (c) Complementarity between the 3′-leader and 5′-trailer regions of the isolate 11OG0352. All sequences are negative-sense. Leader and trailer regions of APMV-10 were not available.

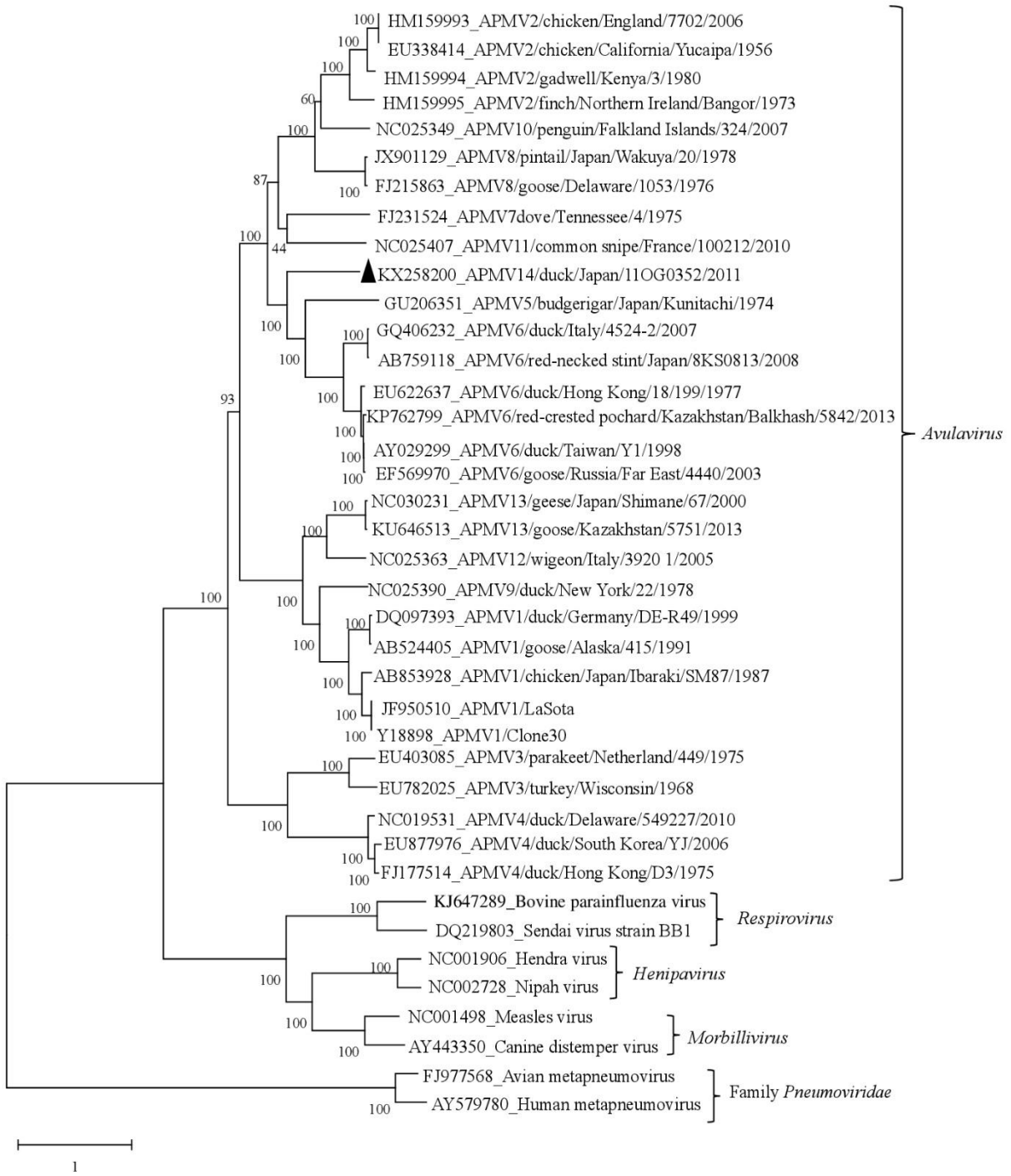

Fig. 3.4. Phylogenetic tree of the complete genome with the highest log-likelihood (-305157.6255) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are shown at the nodes. All positions containing gaps and missing data were eliminated.

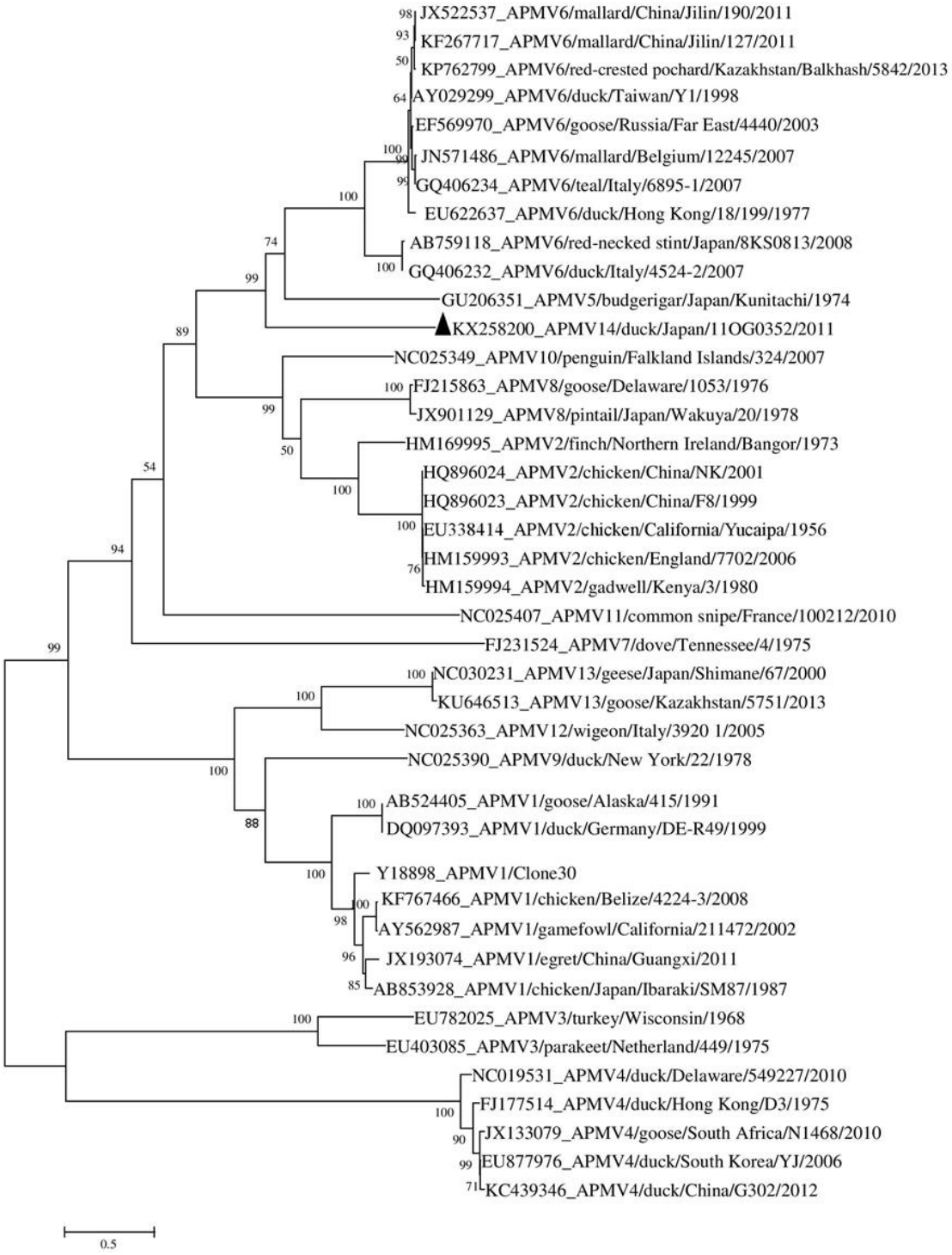

Fig. 3.5. Phylogenetic tree of the F genes with the highest log-likelihood (-30627.9893) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are shown at the nodes. All positions containing gaps and missing data were eliminated.

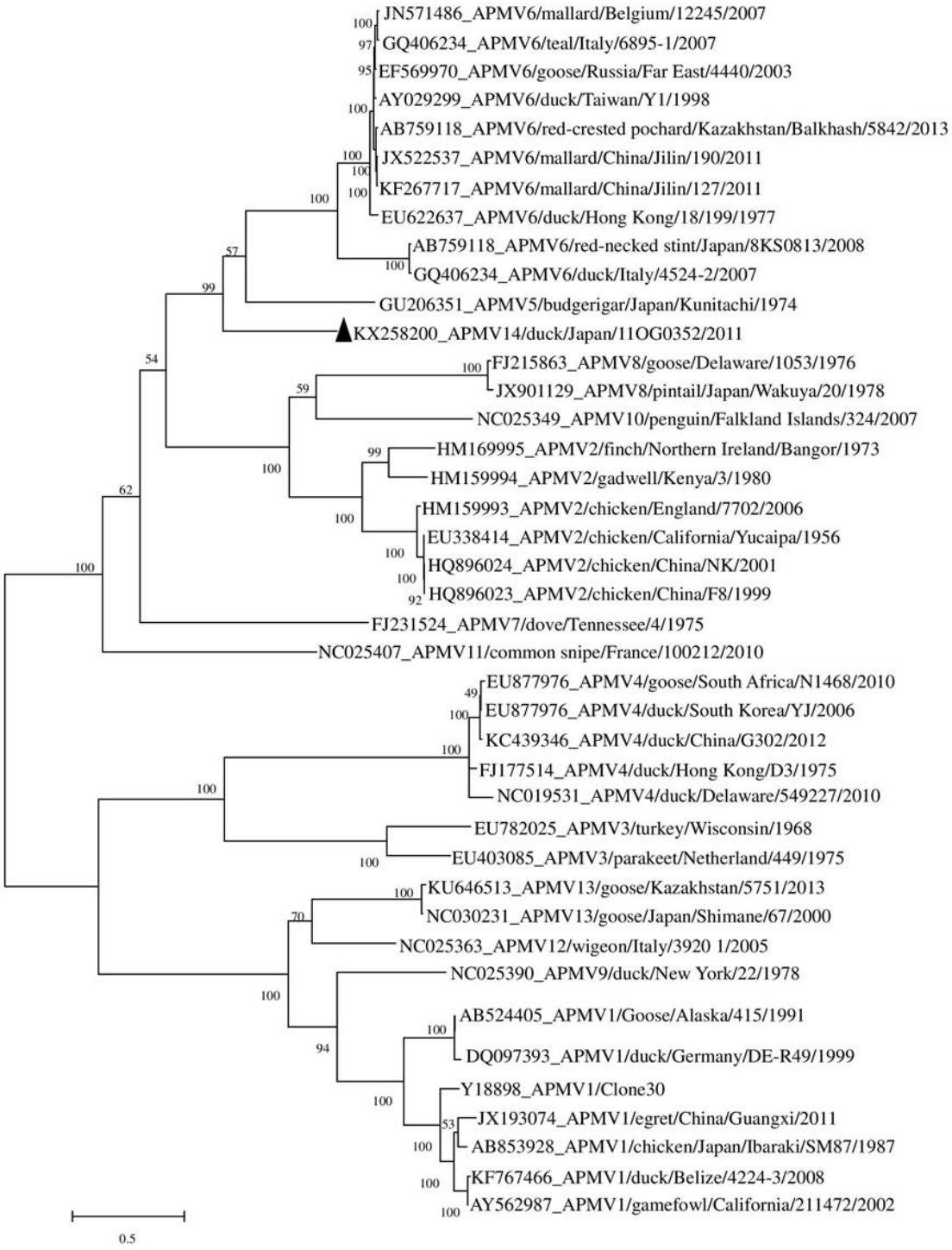

Fig. 3.6. Phylogenetic Tree of the HN genes with the highest log-likelihood (-34960.9496) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are shown at the nodes. All positions containing gaps and missing data were eliminated.

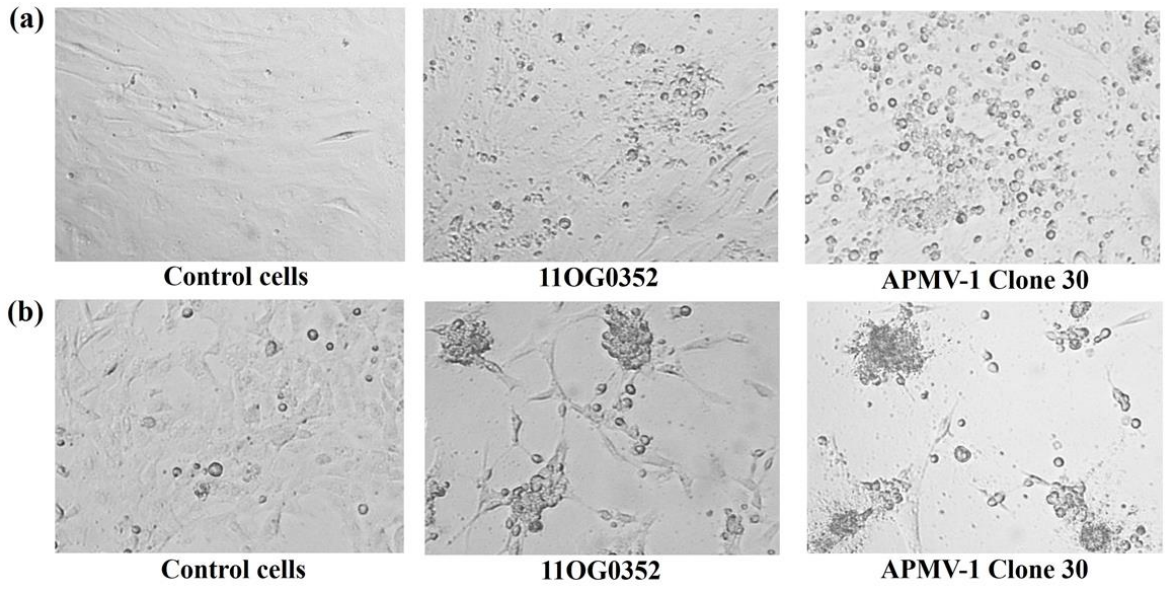

Fig. 3.7. Cytopathic effects of CEFs caused by infection with 11OG0352 and APMV-1 clone 30 in the absence (a) or presence (b) of trypsin.

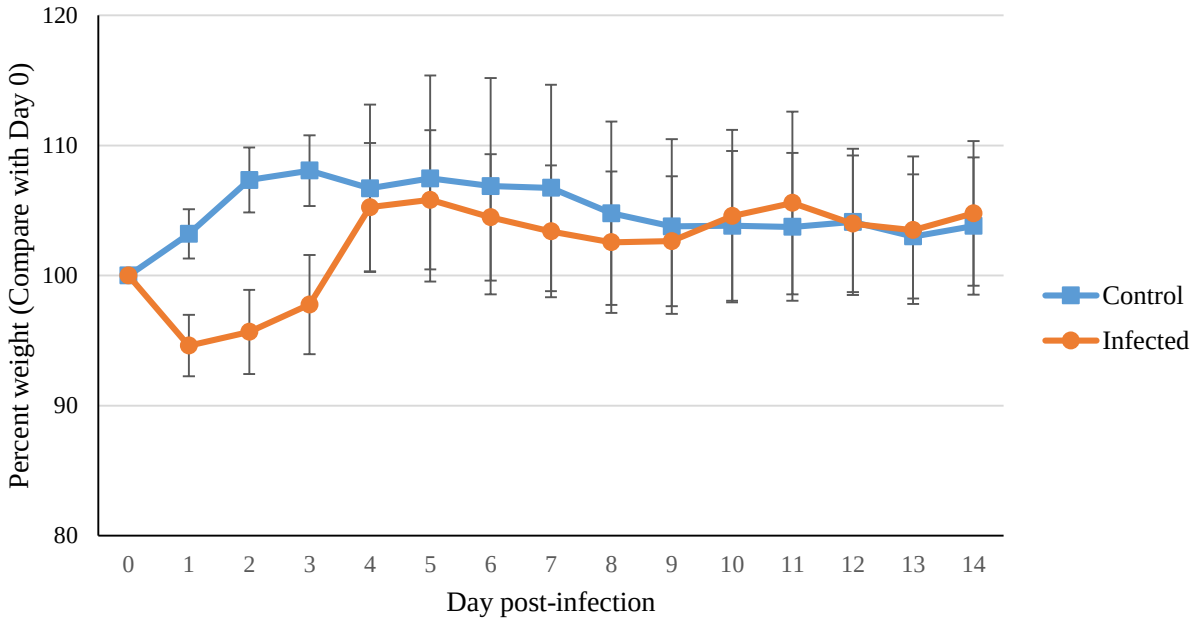

Fig. 3.8. Weight loss in mice infected with isolate 11OG0352. The mice were weighed daily and weight lost was calculated as a percent of the weight on day 0. Data depict the mean ± SD. (n = 8)

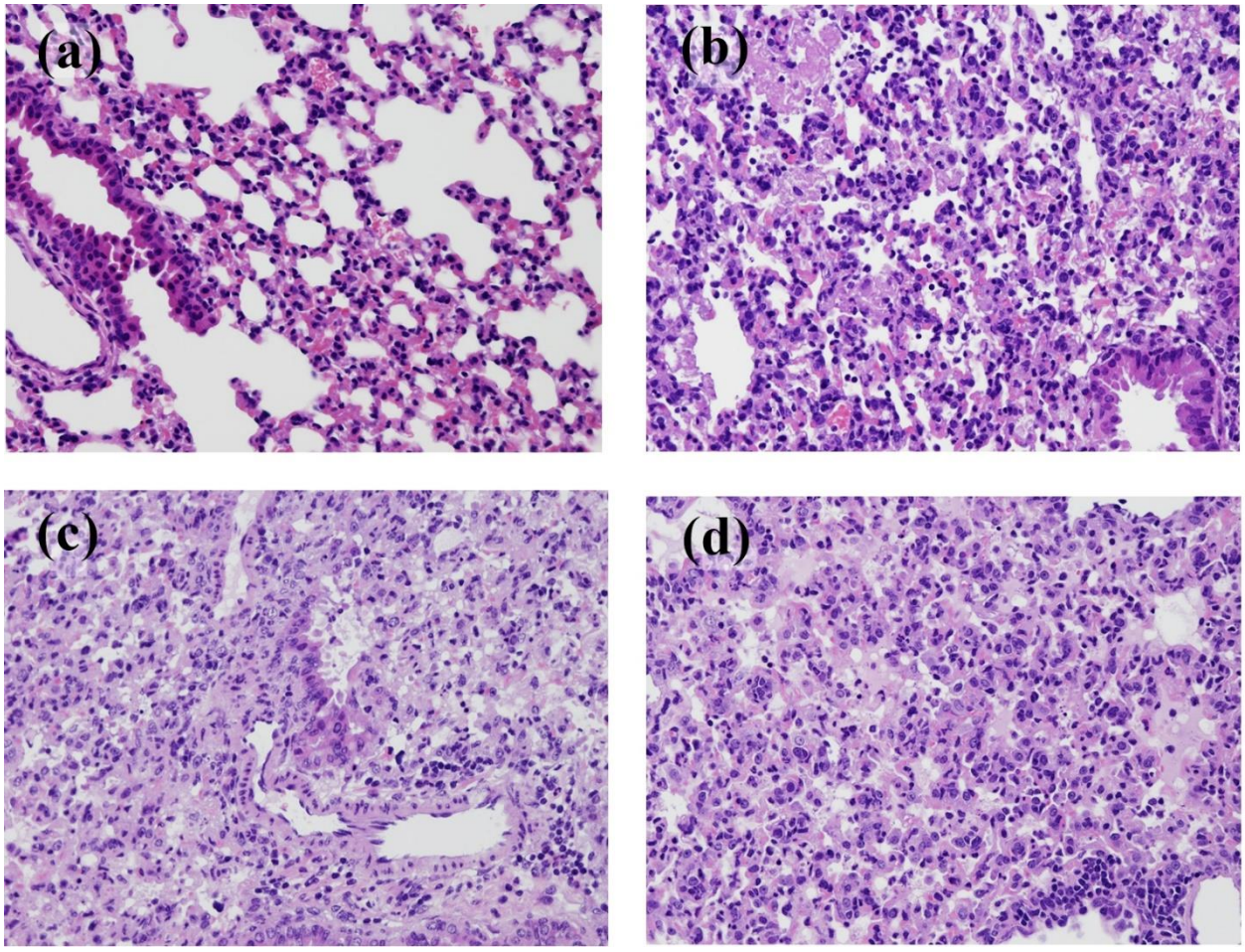

Fig. 3.9. Histopathological findings in lung sections collected from mice at 3 dpi. No significant lesions were found in mice inoculated with fresh allantoic fluid (a). Interstitial pneumonia were found in 3 mice with mild (b), moderate (c), and severe (d) lesions. Alveolar wall was thickened due to inflammatory cell infiltration. Swelling of alveolar epithelium with the presence of numerous karyorrhectic debris and perivascular mononuclear cell infiltration were observed. Alveolar space was filled with serous and fibrin exudates.

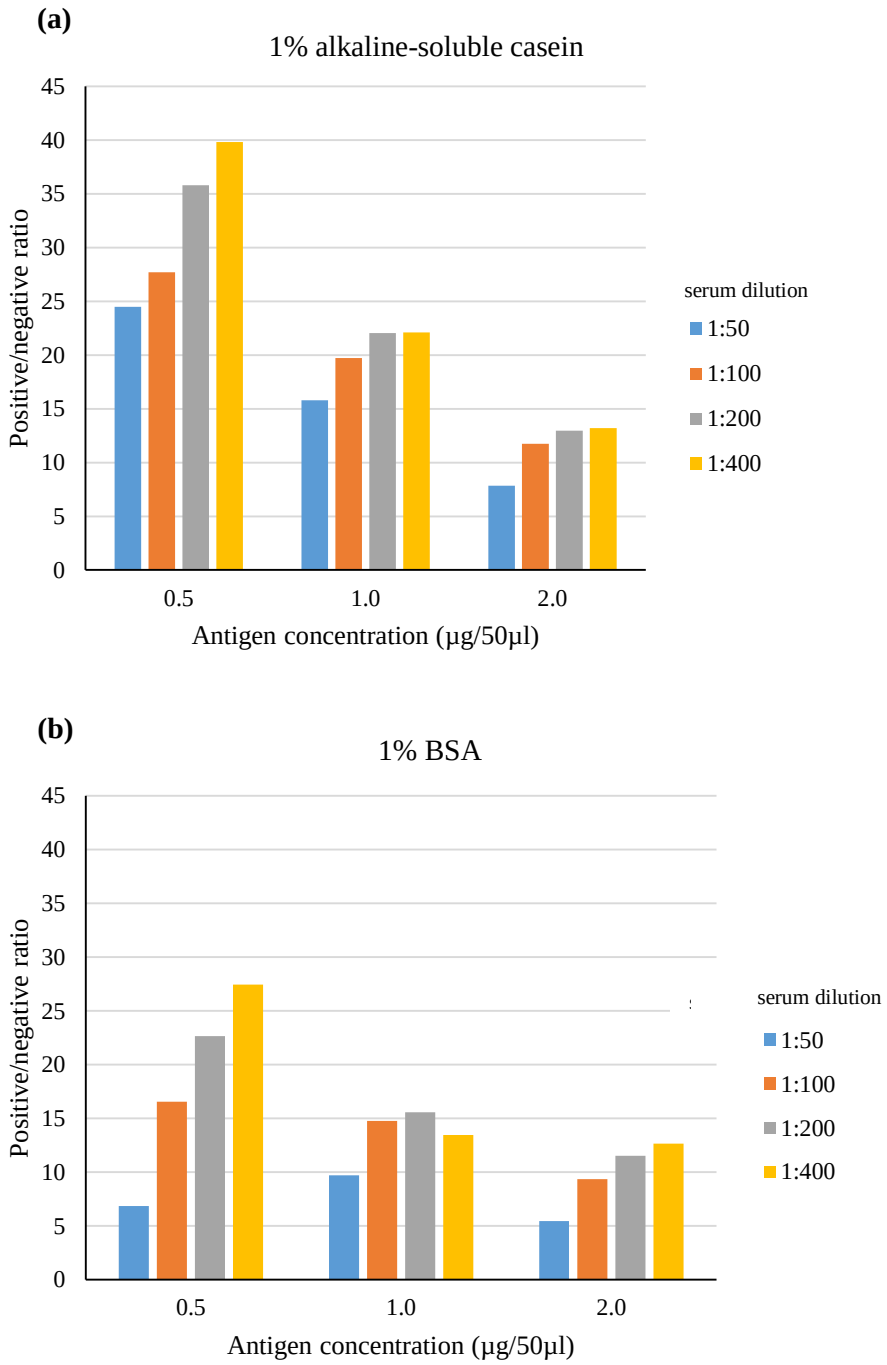

Fig. 3.10. The positive/negative ratio from different serum dilution was shown. The blocking buffers used were 1% alkaline-soluble casein (a), and 1% BSA (b).

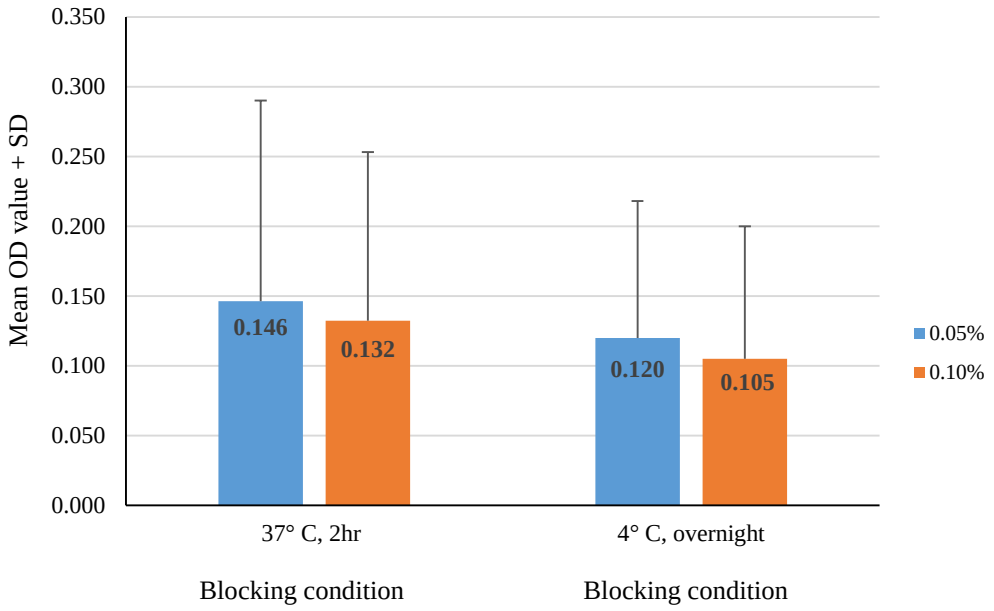

Fig. 3.11. Mean OD values of SPF chicken serums in the ELISA system with 2 different blocking conditions (37° C, 2hr and 4° C, overnight). Two different concentration of Tween20 in PBS (0.05% and 0.10%) as washing solution were also evaluated.

General discussion

Avian paramyxoviruses (APMVs) is an important virus in bird species. They belong to the genus *Avulavirus* which is now classified into 13 serotypes (APMV-1 to APMV-13) based on antigenic and/or genetic analysis (Afonso et al., 2015; Yamamoto et al., 2015). APMV-1 was well characterized because the highly virulent strain of this serotype can cause severe disease known as ND, in chickens (Suarez et al., 2013). In contrast, very little is known about virological and ecological characteristics for other serotypes of APMV. In the last five years, four novel APMV serotypes (APMV-10 to APMV-13), have been reported, indicating that APMVs are under genetic change (Briand et al., 2012; Miller et al., 2010; Terregino et al., 2013; Yamamoto et al., 2015). Migratory wild bird was well known as a natural reservoir for APMVs. Japan is in the East Asian Australia (EAA) flyway which is one of the world's major flyways for migratory birds. Although, many epidemiological studies in APMVs have been conducted, the majority was done on virulent APMV-1 in chickens. The distribution of other APMVs is still poorly understood. In this study, the genetic and antigenic diversity of APMVs have been evidenced.

In this surveillance studies, cloacal swab and fecal samples were collected from a variety of birds; however, the majority of samples was droppings collected from ducks' habitat. Genetic analysis was first performed based on the F genes. The aa motif at the cleavage site of the F protein is commonly used to indicate virus virulence of APMV-1 (OIE, 2012). The predominance of APMVs isolated from this study was APMV-4 (58 isolates), followed by APMV-1 (18), APMV-6 (13), and APMV-9 (2). Although APMV-4 was the most found in this study, its genetic diversity was less than other serotypes. Genetic analysis of the APMV-4 isolates indicated that they all were highly similar to each other and they were all clustered

together with the APMV-4 isolates isolated in Eurasian continent including Ukraine, Kazakhstan, and European countries (Fig. 1.4). However, one APMV-4 isolate in South Africa was related to this cluster, indicating the possibility of viral exchange between Eurasia and African continent (Muzyka et al., 2014; Reeves et al., 2016). Genetic variation also presented in other serotypes. APMV-1 and APMV-6 isolates were classified into 2 genetic classes within each serotype (Fig. 1.3 and Fig. 1.5). The phylogenetic tree of the APMV-9 isolates revealed that Japanese isolates formed a lineage distinct from the prototype and the previously reported Italian isolates (Dundon et al., 2010). However, more numbers of APMV-9 need to be examined to conclude, since only a few sequences were available (Fig. 1.6).

So far, APMV-1 genotyping has been done based on the F genes, which divides APMV-1 into two classes (I and II). Class I viruses are low virulent strains that were found in wild birds. Class II viruses had broad range of virulence and can be found in both poultry and wild birds. Class II was further divided into ten genotypes. Genotypes V–VIII contain highly virulent viruses, whereas the remaining genotypes include viruses of low to intermediate virulence (Dimitrov et al., 2016). Beside the F protein, the HN protein was considered as a co-determinant to virus virulence because it also has a major role for the entry of virus to host cells. At least three different lengths of the HN protein have been described and have gained more attention for its relation to virus properties such as virus virulence, virus tropism, virus replication (Jin et al., 2016; Sakaguchi et al., 1989; Zhao et al., 2013). In this study, the phylogenetic tree generated from the HN genes indicated that the APMV-1 isolates were also classified into two genetic classes (I and II) and 12 of APMV-1 isolates from class II were also placed into genotype I as observed in the tree from the F genes. The shortest length (571 aa) of the HN proteins has been found only in virulent APMV-1 strains. In this study, one isolate 9KS0098 has the length of 585 aa, although the other isolates have the length of 616 aa which is found in

low virulent strains (Table 2.1). However, the shorter length (585 aa) of the HN protein seen in isolate 9KS0098 was not associated with the virus virulence.

The considerable antigenicity variations were found between wild bird APMV-1 and poultry strains including current vaccine strains. Most of mAbs against the reference APMV-1 strains used in this study did not recognize wild bird strains. Interestingly, four mAbs (M1, M10, M13, HN41) could not react with all the wild bird isolates, although the aa residues recognized by these mAbs were also found in the HN protein of the isolates. The reason for this phenomenon is still ambiguous and need more investigation. The APMV-1 strains that possessed the mutation of the aa residue E347 on the HN protein, especially E347K, were associated with ND outbreaks in China and South Korea (Cho et al., 2008; Hu et al., 2010). In this study, the mAbs HN14 recognizing the aa residue E347 on the HN protein could not recognize the isolates 9KS0098 and 9KS0664 possessing K or D at position 347, although the mAb bound to the current ND vaccine strains without the mutation at this position. It is well known that protective immunity was related to antigenicity; therefore, this finding may imply that current vaccines may not protect APMV-1 infection from wild birds. While intensive vaccination program is commonly used as a control strategy, high immunity against NDV was demonstrated to cause viral gene mutation. The HN gene mutations in the residue related to the antigenic site was found under antibody immune selective pressure in cell culture (Gong and Cui, 2011). Although the length of HN protein cannot be concluded whether it has effect to virus virulence or not, the role of ongoing genetic drift in the HN gene to protective immunity should be concerned and monitored.

Since nine serotypes of APMVs had been recognized by 1900. Four novel APVM serotypes have been reported in the last five years (Briand et al., 2012; Miller et al., 2010; Terregino et al., 2013; Yamamoto et al., 2015). The newest serotype, APMV-14 was discovered

in this study. Identification of this virus was performed by both antigenic and genetic analyses. The cross-reactivity and limitation of available reference antiserum against each APMV serotype were the hinder for antigenic analysis. Here, I supported the concept to use complete genome sequences for identifying new APMV serotypes as suggested by Miller et al. (2010). However, the relatively high nucleotide value between APMV-12 and APMV-13 was nearly identical to or higher compared with the lowest nucleotide sequence identity calculated for subgroups within the serotype 3 (67%) (Kumar et al., 2010). I suggest that a consensus genetic classification system for APMVs should be established. Most of other serotypes except APMV-1 show no virulence in chickens. To know the presence of different APMVs in poultry will elucidate our knowledge in APMV distribution. However, ELISA system to detect APMV-14 antibody created in this study could not differentiate high reactions from natural infection, nonspecific reaction, and/or NDV vaccination. Therefore, the specificity of this method and/or other detection method should be evaluated further.

In the present study, the phylogenetic results supported the evident of intercontinental spreading of APMVs by migratory birds including Eurasia, America, and Africa. The genetic variation within the same serotype, and the discovery of novel APMV serotypes highlighted the continuation in virus evolution. In addition to genetic diversity, antigenic variation among APMV-1 strains also existed, which raised the concern of the efficacy of current commercial NDV vaccines. For this reason, the surveillance of APMVs in wild birds should be continued with the expansion to more variety of regions and bird species. Taken together, both genetic and antigenic analysis should be considered for characterizing APMV isolates. Besides the F genes, the HN genes analysis should be also included more in characterization due to its role to antigenicity and protective immunity.

SUMMARY

Surveillance of avian paramyxoviruses (APMVs) in wild bird population in Hokkaido and characterization of the APMV isolates

（北海道に飛来する野鳥における鳥パラミクソウイルスのサーベイランスと分離ウイルスの特徴付け）

Avian paramyxoviruses (APMVs) are single-stranded, nonsegmented, negative-sense, enveloped RNA viruses belonging to the family *Paramyxoviridae.* All APMVs belong to the genus *Avulavirus* that are now classified into 13 serotypes (APMV-1 to APMV-13) based on antigenic and/or genetic analysis, so far. APMV is one of the important viruses in bird species. Research on APMVs has been exclusively focused on APMV-1, because virulent APMV-1 causes serious economic loss in poultry industry. However, very little is known about virological properties or ecology of other APMV serotypes in nature. Migratory wild birds are well known as natural reservoir for APMVs and may introduce virus to poultry. Therefore, to understand the ecology of APMVs circulating in wild birds, I conducted a surveillance of APMVs in wild birds flying in Hokkaido, and the characterization of APMV isolates were also described.

Chapter I describes the surveillance of APMVs in wild birds flying in eastern Hokkaido. Wild birds are of concern as a carrier of APMVs. Japan is in the East Asian Australia flyway, and Hokkaido is one of the important stopover sites for migratory birds in Japan. A total of 10,606 cloacal swab or fecal samples were collected form wild birds. The predominance of APMVs isolated from this study was APMV-4 (58 isolates), followed by APMV-1 (18), APMV-6 (13), and APMV-9 (2). The phylogenetic analysis was performed based on partial F

genes covering the F protein cleavage site motif. The aa sequence at this site in all the isolates resembled low virulent APMV-1. The genetic diversity within serotypes was observed in APMV-1, APMV-6, and APMV-9, while the high similarity was found among all APVM-4 isolates. On the other hand, the geographical connection between different continents were found due to the phylogenetic analysis of APMV-1 and APMV-4, because the virus exchange between Africa, America, Asia, and Europe were noticed. This evidence emphasized the important role of wild birds in virus transmission. The results obtained in this study provided evidences of ongoing genetic changes in APMVs. Therefore, a surveillance of APMVs in wild bird populations should be continued. This is the first large−scaled surveillance study of APMV performed in Hokkaido

Chapter II describes genetic characterization of APMV-1 isolates derived from wild birds based on the hemagglutinin-neuraminidase (HN) genes and antigenic comparison between chicken and wild bird APMV-1 using monoclonal antibodies (mAbs) against the F and HN proteins of reference strains of APMV-1. So far, the F protein cleavage site of APMV-1 has been used to determine virus virulence, since the presence of multibasic amino acid (aa) motif at the cleavage site is associated with the virulence in chickens. However, it has been reported that the pathogenicity of virus in chickens did not always correlate with the aa sequence at this site. Beside the F genes, the HN genes were also considered as a virus virulence determinant. The phylogenetic tree generated from the HN genes classified APMV-1 isolates into two genetic classes, I and II as observed in the tree based on the F genes. The various length (585 and 616 aa) of the HN proteins were found in the APMV-1 isolates in this study. Although the short length (571 aa) of the HN protein was reported to be related to the high virulence of APMV-1, the short length (585 aa) of the HN protein of the APMV-1 isolate found in this study seemed not to be related to the high virulence. The antigenicity comparison study using nine

mAbs against reference APMV-1 strains showed the considerable antigenic difference between wild bird APMV-1 and poultry strains including vaccine strains. Most of the mAbs did not recognize the APMV-1 isolates in this study. Thus, the antigenic difference between field and vaccine strains may affect the efficacy of vaccination in poultry. Therefore, to monitor antigenic changes in APMV-1, the HN genes should be analyzed together with the F genes, because immune response is raised against the F and HN proteins on the viral envelope.

Chapter III describes the identification and virological characterization of a novel serotype of APMV. A hemagglutinating virus isolate designated 11OG0352, was obtained from a duck fecal sample. Genetic and virological analyses indicated that it might represent a novel serotype of APMV. Electron micrographs showed that the morphology of the virus particle was similar to that of APMV. The phylogenetic analysis of the whole genome revealed that the virus was a member of the genus *Avulavirus*, but that it was distinct from APMV-1 to APMV-13. Although the F-protein cleavage site was TREGK↓L, which resembles a low virulent strain of APMV-1, the K residue at position −1 of the cleavage site was first discovered in APMV members. The intracerebral pathogenicity index test did not detect virulence in infected chicks. The virus replicate in the respiratory tract of infected mice. In hemagglutination inhibition (HI) tests, an antiserum against this virus did not detectably react with other APMVs (serotypes 1–4, 6–9) except for a low cross-reactivity with APMV-6. From these results, I designated this isolate, as APMV-14/duck/Japan/11OG0352/2011 and propose that it is a novel APMV serotype. This is the first report on a new APMV serotype, APMV-14. The HI test may not be widely applicable for the classification of a new serotype because of the limited availability of reference antisera against all serotypes and cross-reactivity data. The nucleotide sequence identities of the whole genome of 11OG0352 and other APMVs ranged from 46.3% to 56.1%. Such comparison may provide a useful tool for classifying new APMV isolates. However, the nucleotide sequence

identity between APMV-12 and APMV-13 was higher (64%), which was nearly identical to the lowest nucleotide identity (67%) reported in subgroups within the serotype. Therefore, consensus criteria for using whole genome analysis should be established.

In conclusion, this study provides the informative information of APMVs circulating in wild birds. The results supported the evidence of intercontinental dispersal of APMV by migratory birds between different continents. The genetic and antigenic variation among each serotype and the discovery of a novel serotype, APMV-14 indicated that APMVs are undergoing evolution. The finding obtained in this study highlights the importance to continue an extensive surveillance of APMVs in wild bird populations to understand the global dispersal, genetic diversity and evolution of APMVs.

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References

Ababneh, M.M., Dalab, A.E., Alsaad, S.R., Al-Zghoul, M.B., Al-Natour, M.Q., 2012. Molecular characterization of a recent Newcastle disease virus outbreak in Jordan. Res. Vet. Sci. 93, 1512-1514.

Abenes, G.B., Kida, H., Yanagawa, R., 1986. Biological activities of monoclonal antibodies to the hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus. Nihon Juigaku Zasshi 48, 353-362.

Afonso, C.L., Amarasinghe, G.K., Banyai, K., Bao, Y., Basler, C.F., Bavari, S., Bejerman, N., Blasdell, K.R., Briand, F.X., Briese, T., Bukreyev, A., Calisher, C.H., et al, 2016. Taxonomy of the order Mononegavirales: update 2016. Arch. Virol. 161, 2351-2360.

Afonso, C.L., Briand, F.X., Terregino, C., Rima, B.K., Collins, P.L., Easton, A.J., Fouchier, R., Kurath, G., Lamb, R., Maisner, A., Randall, R., Rota, P., Wang, L.F., 2015. ICTV taxonomic proposal 2015.012aM.A.v3.Avulavirus_3sp. Create 3 species in the genus Avulavirus, subfamily Paramyxovirinae, family Paramyxoviridae, order Mononegavirales.

Alamares, J.G., Elankumaran, S., Samal, S.K., Iorio, R.M., 2010. The interferon antagonistic activities of the V proteins from two strains of Newcastle disease virus correlate with their known virulence properties. Virus Res. 147, 153-157.

Aldous, E.W., Alexander, D.J., 2001. Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). Avian Pathol. 30, 117-128.

Aldous, E.W., Mynn, J.K., Banks, J., Alexander, D.J., 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol. 32, 239-256.

Alexander, D.J., Bell, J.G., Alders, R.G., 2004. A technology review: Newcastle disease. Food and Agriculture of Organization of the United Nations, Rome.

Alexander, D.J., Collins, M.S., 1981. The structural polypeptides of avian paramyxoviruses. Arch. Virol. 67, 309-323.

Alexander, D.J., Collins, M.S., 1984. Characterisation of avian paramyxoviruses of serotype PMV-3 isolated from commercial turkeys in Great Britain. Avian Pathol. 13, 215-221.

Alexander, D.J., Hinshaw, V.S., Collins, M.S., Yamane, N., 1983. Characterization of viruses which represent further distinct serotypes (PMV-8 and PMV-9) of avian paramyxoviruses. Arch. Virol. 78, 29-36.

Alexander, D.J., Senne, D.A., 2008. Newcastle disease and other avian paramyxoviruses. In: Dufour-Zavala, L. (Ed.), A laboratory manual for the isolation an dharacterization of Avian Pathogens, 4 ed. American Association of Avian Pathologists, Athens, GA, pp. 135-141.

Animal Health Australia, 2014. Disease strategy: Newcastle disease (Version 3.3). Australian Veterinary Emergency Plan (AUSVETPLAN). 3 ed. Agriculture Ministers' Forum, Canberra.

Ashraf, A., Shah, M.S., 2014. Newcastle Disease: Present status and future challenges for developing countries. Afr. J. Microbiol. Res. 8, 411-416.

Awang, I.P.R., Russell, P.H., 1990. Turkey avian paramyxovirus type 3 infections in poultry. Worlds Poult. Sci. J. 46, 211-218.

Bamford, M., Watkins, D., Bancroft, W., Tischler, G., Wahl, J., 2008. Migratory Shorebirds of the East Asian - Australasian Flyway; Population Estimates and Internationally Important Sites. Wetlands International - Oceania, Canberra, Australia.

Bankowski, R.A., Almquist, J., Dombrucki, J., 1981. Effect of paramyxovirus Yucaipa on fertility, hatchability, and poult yield of turkeys. Avian Dis. 25, 517-520.

Beard, C.W., Hanson, R.P., 1984. Newcastle Disease. In: Hofstad, M.S., Barnes, H.J., Calneck, B.W., Reid, W.M., Yorder, H.Y. (Eds.), Diseases of Poultry 8ed. Iowa State University Press, Ames, Iowa, pp. 452-470.

Beck, I., Gerlach, H., Burkhardt, E., Kaleta, E.F., 2003. Investigation of several selected adjuvants regarding their efficacy and side effects for the production of a vaccine for parakeets to prevent a disease caused by a paramyxovirus type 3. Vaccine 21, 1006-1022.

Briand, F.X., Henry, A., Massin, P., Jestin, V., 2012. Complete genome sequence of a novel avian paramyxovirus. J. Virol. 86, 7710.

Bui, V.N., Mizutani, T., Nguyen, T.H., Trinh, D.Q., Awad, S.S., Minoungou, G.L., Yamamoto, Y., Nakamura, K., Saito, K., Watanabe, Y., Runstadler, J., Huettmann, F., Ogawa, H., Imai, K., 2014. Characterization of a genetic and antigenic variant of avian

paramyxovirus 6 isolated from a migratory wild bird, the red-necked stint (Calidris ruficollis). Arch. Virol. 159, 3101-3105.

Bui, V.N., Ogawa, H., Karibe, K., Matsuo, K., Nguyen, T.H., Awad, S.S., Minoungou, G.L., Xininigen, Saito, K., Watanabe, Y., Runstadler, J.A., Happ, G.M., Imai, K., 2011. Surveillance of avian influenza virus in migratory water birds in eastern Hokkaido, Japan. J. Vet. Med. Sci. 73, 209-215.

Bukreyev, A., Collins, P.L., 2008. Newcastle disease virus as a vaccine vector for humans. Curr. Opin. Mol. Ther. 10, 46-55.

Burridge, M.J., Riemann, H.P., Utterback, W.W., 1975. Methods of spread of velogenic viscerotropic Newcastle disease virus in the southern Californian epidemic of 1971-1973. Avian Dis. 19, 666-678.

Catroxo, M.H.B., Martins, A.M.C.P.F., Petrella, S., Milanelo, L., Aschar, M., Souza, F., Nastari, B.D.B., Souza, R.B., 2012. Avian paramyxoviruses. detection by transmission electron microscopy techniques. Int. J. Morphol. 30, 723-730.

Cattoli, G., Susta, L., Terregino, C., Brown, C., 2011. Newcastle disease: a review of field recognition and current methods of laboratory detection. J. Vet. Diagn. Invest. 23, 637-656.

Cho, S.-H., Kwon, H.-J., Kim, T.-E., Kim, J.-H., Yoo, H.-S., Kim, S.-J., 2008. Variation of a Newcastle disease virus hemagglutinin-neuraminidase linear epitope. J. Clin. Microbiol. 46, 1541-1544.

Cho, S.H., Kim, S.J., Kwon, H.J., 2007. Genomic sequence of an antigenic variant Newcastle disease virus isolated in Korea. Virus Genes 35, 293-302.

Choi, K.-S., Lee, E.-K., Jeon, W.-J., Kwon, J.-H., 2010. Antigenic and immunogenic investigation of the virulence motif of the Newcastle disease virus fusion protein. J. Vet. Sci. 11, 205-211.

Chrystie, I.L., 1996. 5 - Electron microscopy A2 - Kangro, Brian WJ MahyHillar O, Virology Methods Manual. Academic Press, London, pp. 91-106.

Council of the Economic Community, 1992. Council Directive 92/66/EEC of 14 July 1992 introducing Community measures for the control of Newcastle disease. Official Journal of the European Communitites L260, 1-20.

Craft Jr, W.W., Dutch, R.E., 2005. Sequence motif upstream of the Hendra virus fusion protein cleavage site is not sufficient to promote efficient proteolytic processing. Virology 341, 130-140.

Czegledi, A., Ujvari, D., Somogyi, E., Wehmann, E., Werner, O., Lomniczi, B., 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. 120, 36-48.

de Leeuw, O.S., Koch, G., Hartog, L., Ravenshorst, N., Peeters, B.P., 2005. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. J. Gen. Virol. 86, 1759-1769.

Deibel, R., Emord, D.E., Dukelow, W., Hinshaw, V.S., Wood, J.M., 1985. Influenza viruses and paramyxoviruses in ducks in the Atlantic flyway, 1977-1983, including an H5N2 isolate related to the virulent chicken virus. Avian Dis. 29, 970-985.

Diel, D.G., da Silva, L.H.A., Liu, H., Wang, Z., Miller, P.J., Afonso, C.L., 2012a. Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect. Genet. Evol. 12, 1770-1779.

Diel, D.G., Miller, P.J., Wolf, P.C., Mickley, R.M., Musante, A.R., Emanueli, D.C., Shively, K.J., Pedersen, K., Afonso, C.L., 2012b. Characterization of Newcastle disease viruses isolated from cormorant and gull species in the United States in 2010. Avian Dis. 56, 128-133.

Dimitrov, K.M., Ramey, A.M., Qiu, X., Bahl, J., Afonso, C.L., 2016. Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). Infect. Genet. Evol. 39, 22-34.

Doyle, T.M., 1927. A hitherto unrecorded disease of fowls due to a filter-passing virus. J. Comp. Pathol. Ther. 40, 144-169.

Dundon, W.G., Heidari, A., De Nardi, R., Terregino, C., Capua, I., Cattoli, G., 2010. Genetic variation of Italian avian paramyxoviruses serotype 9. Virus Genes 41, 43-46.

EAAFP Secretariat, 2010. The flyways. East Asian-Australasian Flyway Partnership, Korea.

El Najjar, F., Schmitt, A.P., Dutch, R.E., 2014. Paramyxovirus glycoprotein incorporation, assembly and budding: a three way dance for infectious particle production. Viruses 6, 3019-3054.

Eskelund, K.H., 1988. Vaccination of Turkey breeder hens against paramyxovirus type 3 infection. Proc. West. Poult. Dis. Conf. 37, 43-45.

FAO., 2007. Wild Birds and Avian Influenza: an introduction to applied field research and disease sampling techniques. FAO Animal Production and Health Manual, edited by Whitworth, D., Newman, S., Mundkur, T., Harris, P. FAO., Rome.

Food Safety and Consumer Affairs Bureau, 2007. Report of the Investigation Team on the Infection Route of Highly Pathogenic Avian Influenza. Ministry of Agriculture, Forestry and Fisheries, Tokyo

Fornells, L.A., Travassos, C.E., Costa, C.M., Novelli, R., Petrucci, M.P., Soffiati, F.L., Bianchi, I., de Souza, L.F., Veiga, V.F., Liberal, M.H., Couceiro, J.N., 2013. Detection of avian paramyxoviruses in migratory and resident birds in the state of Rio de Janeiro, Brazil. Avian Dis. 57, 780-784.

Fujimoto, Y., Ito, H., Shivakoti, S., Nakamori, J., Tsunekuni, R., Otsuki, K., Ito, T., 2010. Avian influenza virus and paramyxovirus isolation from migratory waterfowl and shorebirds in San-in district of western Japan from 2001 to 2008. J. Vet. Med. Sci. 72, 963-967.

Gardner, E., Alders, R., 2014. Livesto risks and opportunities: Newcastle disease and Avian Influenza in Africa. GRF Davos Planet@Risk 2, 208-211.

Goekjian, V.H., Smith, J.T., Howell, D.L., Senne, D.A., Swayne, D.E., Stallknecht, D.E., 2011. Avian influenza viruses and avian paramyxoviruses in wintering and breeding waterfowl populations in North Carolina, USA. J. Wildl. Dis. 47, 240-245.

Gong, Y., Cui, Z., 2011. Epitope variation in the Newcastle disease virus HN gene under antibody immune selective pressure in cell culture. Sci. China Life Sci. 54, 474-479.

Gotoh, B., Sakaguchi, T., Nishikawa, K., Inocencio, N.M., Hamaguchi, M., Toyoda, T., Nagai, Y., 1988. Structural features unique to each of the three antigenic sites on the hemagglutinin-neuraminidase protein of newcastle disease virus. Virology 163, 174-182.

Gould, A.R., Kattenbelt, J.A., Selleck, P., Hansson, E., Della-Porta, A., Westbury, H.A., 2001. Virulent Newcastle disease in Australia: molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998-2000. Virus Res. 77, 51-60.

Hanson, B.A., Swayne, D.E., Senne, D.A., Lobpries, D.S., Hurst, J., Stallknecht, D.E., 2005. Avian influenza viruses and paramyxoviruses in wintering and resident ducks in Texas. J. Wildl. Dis. 41, 624-628.

Hoshino, K., Takahashi-Nakaguchi, A., Isawa, H., Sasaki, T., Higa, Y., Kasai, S., Tsuda, Y., Sawabe, K., Kobayashi, M., 2012. Entomological surveillance for flaviviruses at migratory bird stopover sites in Hokkaido, Japan, and a new insect flavivirus detected in Aedes galloisi (Diptera: Culicidae). J. Med. Entomol. 49, 175-182.

Hu, S., Ma, H., Wu, Y., Liu, W., Wang, X., Liu, Y., Liu, X., 2009. A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. Vaccine 27, 904-910.

Hu, S., Wang, T., Liu, Y., Meng, C., Wang, X., Wu, Y., Liu, X., 2010. Identification of a variable epitope on the Newcastle disease virus hemagglutinin-neuraminidase protein. Vet. Microbiol. 140, 92-97.

Huang, Z., Panda, A., Elankumaran, S., Govindarajan, D., Rockemann, D.D., Samal, S.K., 2004. The Hemagglutinin-Neuraminidase Protein of Newcastle Disease Virus Determines Tropism and Virulence. J. Virol. 78, 4176-4184.

Imai, K., Ogawa, H., Bui, V.N., Inoue, H., Fukuda, J., Ohba, M., Yamamoto, Y., Nakamura, K., 2012. Inactivation of high and low pathogenic avian influenza virus H5 subtypes by copper ions incorporated in zeolite-textile materials. Antiviral Res. 93, 225-233.

Iorio, R.M., Bratt, M.A., 1983. Monoclonal antibodies to newcastle disease virus: delineation of four epitopes on the HN glycoprotein. J. Virol. 48, 440-450.

Iorio, R.M., Syddall, R.J., Sheehan, J.P., Bratt, M.A., Glickman, R.L., Riel, A.M., 1991. Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition. J. Virol. 65, 4999-5006.

Islam, M.A., Ito, T., Takakuwa, H., Takada, A., Itakura, C., Kida, H., 1994. Acquisition of pathogenicity of a Newcastle disease virus isolated from a Japanese quail by intracerebral passage in chickens. Jpn. J. Vet. Res. 42, 147-156.

Jin, J., Zhao, J., Ren, Y., Zhong, Q., Zhang, G., 2016. Contribution of HN protein length diversity to Newcastle disease virus virulence, replication and biological activities. Sci. Rep. 6, 36890.

Jorgensen, P.H., Handberg, K.J., Ahrens, P., Manvell, R.J., Frost, K.M., Alexander, D.J., 2000. Similarity of avian paramyxovirus serotype 1 isolates of low virulence for chickens obtained from contaminated poultry vaccines and from poultry flocks. Vet. Rec. 146, 665-668.

Jourdain, E., Gauthier-Clerc, M., Bicout, D., Sabatier, P., 2007. Bird Migration Routes and Risk for Pathogen Dispersion into Western Mediterranean Wetlands. Emerg. Infect. Dis. 13, 365-372.

Kapczynski, D.R., King, D.J., 2005. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. Vaccine 23, 3424-3433.

Karamendin, K., Kydyrmanov, A., Seidalina, A., Asanova, S., Sayatov, M., Kasymbekov, E., Khan, E., Daulbayeva, K., Harrison, S.M., Carr, I.M., Goodman, S.J., Zhumatov, K., 2016. Complete genome sequence of a novel avian paramyxovirus (APMV-13) isolated from a wild bird in Kazakhstan. Genome Announc. 4.

Karamendin, K., Kydyrmanov, A., Seidalina, A., Jenckel, M., Starick, E., Grund, C., Asanova, S., Khan, E., Daulbayeva, K., Kasymbekov, Y., Zhumatov, K., Sayatov, M., Beer, M., Fereidouni, S., 2015. Complete genome sequence of avian paramyxovirus strain APMV-6/red-crested pochard/Balkhash/5842/2013 from Kazakhstan. Genome Announc. 3.

Kessler, N., Aymard, M., Calvet, A., 1979. Study of a new strain of paramyxoviruses isolated from wild ducks: antigenic and biological properties. J. Gen. Virol. 43, 273-282.

Khattar, S.K., Kumar, S., Xiao, S., Collins, P.L., Samal, S.K., 2011. Experimental infection of mice with avian paramyxovirus serotypes 1 to 9. PLoS One 6, e16776.

Khattar, S.K., Nayak, B., Kim, S.-H., Xiao, S., Samal, S., Paldurai, A., Buchholz, U.J., Collins, P.L., Samal, S.K., 2013. Evaluation of the replication, pathogenicity, and immunogenicity of avian paramyxovirus (APMV) serotypes 2, 3, 4, 5, 7, and 9 in Rhesus Macaques. PLoS One 8, e75456.

Kim, S.-H., Samal, S.K., 2016. Newcastle disease virus as a vaccine vector for development of human and veterinary vaccines. Viruses 8, 183.

Kim, S.-H., Xiao, S., Paldurai, A., Collins, P.L., Samal, S.K., 2014. Role of C596 in the C-terminal extension of the haemagglutinin–neuraminidase protein in replication and pathogenicity of a highly virulent Indonesian strain of Newcastle disease virus. J. Gen. Virol. 95, 331-336.

Kim, S.-H., Xiao, S., Shive, H., Collins, P.L., Samal, S.K., 2012. Replication, neurotropism, and pathogenicity of avian paramyxovirus serotypes 1-9 in chickens and ducks. PLoS One 7, e34927.

Kim, S.-H., Xiao, S., Shive, H., Collins, P.L., Samal, S.K., 2013. Mutations in the fusion protein cleavage site of avian paramyxovirus serotype 4 confer increased replication and syncytium formation in vitro but not increased replication and pathogenicity in chickens and ducks. PLoS One 8, e50598.

Kolakofsky, D., Pelet, T., Garcin, D., Hausmann, S., Curran, J., Roux, L., 1998. Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. J. Virol. 72, 891-899.

Kraneveld, F.C., 1926. A poultry disease in the Dutch East Indies. Ned Indisch 1 Diergeneeskd. 38, 448-450.

Kruckenberg, H., Müller, T., Freuling, C., Mühle, R.-U., Globig, A., Schirrmeier, H., Buss, M., Harder, T., Kramer, M., Teske, K., Polderdijk, K., Wallschläger, D., Hlinak, A., 2011. Serological and virological survey and resighting of marked wild geese in Germany. Eur. J. Wildl. Res. 57, 1025-1032.

Kuiken, T., 1999. Review of Newcastle Disease in Cormorants. Waterbirds. 22, 333-347.

Kumar, S., Nayak, B., Collins, P.L., Samal, S.K., 2011. Evaluation of the Newcastle disease virus F and HN proteins in protective immunity by using a recombinant avian paramyxovirus type 3 vector in chickens. J. Virol. 85, 6521-6534.

Kumar, S., Nayak, B., Samuel, A.S., Xiao, S., Collins, P.L., Samal, S.K., 2010. Complete genome sequence of avian paramyxovirus-3 strain Wisconsin: Evidence for the existence of subgroups within the serotype. Virus Res. 149, 78-85.

Lamb, R.A., Parks, G.D., 2013. Paramyxoviridae. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology 6ed. Vol. 1. 2 vols. Lippincott Williams & Wilkins, Philadelphia, pp. 957-996.

Lindh, E., Huovilainen, A., Rätti, O., Ek-Kommonen, C., Sironen, T., Huhtamo, E., Pöysä, H., Vaheri, A., Vapalahti, O., 2008. Orthomyxo-, paramyxo- and flavivirus infections in wild waterfowl in Finland. Virol. J. 5, 35-35.

Lipkind, M., Shihmanter, E., 1986. Antigenic relationships between avian paramyxoviruses. Arch. Virol. 89, 89-111.

Macpherson, L.W., 1956. Some Observations On The Epizootiology Of NewCastle Disease. Can. J. Comp. Med. Vet. Sci. 20, 155-168.

Maldonado, A., Arenas, A., Tarradas, M.C., Carranza, J., Luque, I., Miranda, A., Perea, A., 1994. Prevalence of antibodies to avian paramyxoviruses 1, 2 and 3 in wild and domestic birds in southern Spain. Avian Pathol. 23, 145-152.

Mase, M., Kanehira, K., 2015. Surveillance of avian paramyxovirus serotype-1 in migratory waterfowls in Japan between 2011 and 2013. J. Vet. Med. Sci. 77, 381-385.

McGinnes, L.W., Gravel, K., Morrison, T.G., 2002. Newcastle disease virus HN protein alters the conformation of the F protein at cell surfaces. J. Virol. 76, 12622-12633.

Mikami, T., Izawa, H., Kodama, H., Onuma, M., Sato, A., Kobayashi, S., Ishida, M., Nerome, K., 1982. Isolation of ortho- and paramyxoviruses from migrating feral ducks in Hokkaido. Brief Report. Arch. Virol. 74, 211-217.

Miller, P.J., Afonso, C.L., Spackman, E., Scott, M.A., Pedersen, J.C., Senne, D.A., Brown, J.D., Fuller, C.M., Uhart, M.M., Karesh, W.B., Brown, I.H., Alexander, D.J., Swayne, D.E., 2010. Evidence for a new avian paramyxovirus serotype 10 detected in rockhopper penguins from the Falkland Islands. J. Virol. 84, 11496-11504.

Miller, P.J., Estevez, C., Yu, Q., Suarez, D.L., King, D.J., 2009. Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. Avian Dis. 53, 39-49.

Miller, P.J., King, D.J., Afonso, C.L., Suarez, D.L., 2007. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine 25, 7238-7246.

Moll, M., Diederich, S., Klenk, H.D., Czub, M., Maisner, A., 2004. Ubiquitous activation of the Nipah virus fusion protein does not require a basic amino acid at the cleavage site. J. Virol. 78, 9705-9712.

Morrison, T., McQuain, C., Sergel, T., McGinnes, L., Reitter, J., 1993. The role of the amino terminus of F1 of the Newcastle disease virus fusion protein in cleavage and fusion. Virology 193, 997-1000.

Muzyka, D., Pantin-Jackwood, M., Stegniy, B., Rula, O., Bolotin, V., Stegniy, A., Gerilovych, A., Shutchenko, P., Stegniy, M., Koshelev, V., Maiorova, K., Tkachenko, S., Muzyka, N., Usova, L., Afonso, C.L., 2014. Wild bird surveillance for avian paramyxoviruses in the Azov-black sea region of Ukraine (2006 to 2011) reveals epidemiological connections with Europe and Africa. Appl. Environ. Microbiol. 80, 5427-5438.

Nagai, Y., Klenk, H.D., Rott, R., 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology 72, 494-508.

Nayak, B., Dias, F.M., Kumar, S., Paldurai, A., Collins, P.L., Samal, S.K., 2012. Avian paramyxovirus serotypes 2-9 (APMV-2-9) vary in the ability to induce protective immunity in chickens against challenge with virulent Newcastle disease virus (APMV-1). Vaccine 30, 2220-2227.

Nerome, K., Nakayama, M., Ishida, M., Fukumi, H., Morita, A., 1978. Isolation of a New Avian Paramyxovirus from Budgerigar (Melopsittacus undulatus). J. Gen. Virol. 38, 293-301.

Nishikawa, K., Hanada, N., Morishima, T., Yoshida, T., Hamaguchi, M., Toyoda, T., Nagai, Y., 1987. Antigenic characterization of the internal proteins of Newcastle disease virus by monoclonal antibodies. Virus Res. 7, 83-92.

OIE, 2012. Newcastle disease (Infection with Newcastle disease virus), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris, pp. 555-574.

OIE, 2016. Diseases, infections and infestations listed by the OIE, Terrestrial Animal Health Code (2016), 3/06/2016 ed. Vol. I. OIE.

Olabode, A., Lamorde, A., Shidali, N., Chukwuedo, A., 1992. Village chickens and Newcastle disease in Nigeria. In: Spradbrrow, P. (Ed.), Proceedings, International Workshop held in Kuala Lumpur, Malaysia, 6-10 October 1991. Centre for International Agricultural Research (ACIAR), Canberra, pp. 159-160.

Oluwayelu, D.O., Adebiyi, A.I., Olaniyan, I., Ezewele, P., Aina, O., 2014. Occurrence of Newcastle disease and infectious bursal disease virus antibodies in double-spurred francolins in Nigeria. J. Vet. Med. 2014, 106898.

Otim, M.O., Kabagambe, E.K., Mukiibi, G.M., Christensen, H., Bisgaard, M., 2007. A study of risk factors associated with Newcastle disease epidemics in village free-range chickens in Uganda. Trop. Anim. Health Prod. 39, 27-35.

Piacenti, A.M., King, D.J., Seal, B.S., Zhang, J., Brown, C.C., 2006. Pathogenesis of Newcastle disease in commercial and specific pathogen-free turkeys experimentally infected with isolates of different virulence. Vet. Pathol. 43, 168-178.

Ramey, A.M., Reeves, A.B., Ogawa, H., Ip, H.S., Imai, K., Bui, V.N., Yamaguchi, E., Silko, N.Y., Afonso, C.L., 2013. Genetic diversity and mutation of avian paramyxovirus serotype 1 (Newcastle disease virus) in wild birds and evidence for intercontinental spread. Arch. Virol. 158, 2495-2503.

Reed, K.D., Meece, J.K., Henkel, J.S., Shukla, S.K., 2003. Birds, migration and emerging zoonoses: west nile virus, lyme disease, influenza A and enteropathogens. Clin. Med. Res. 1, 5-12.

Reeves, A.B., Poulson, R.L., Muzyka, D., Ogawa, H., Imai, K., Bui, V.N., Hall, J.S., Pantin-Jackwood, M., Stallknecht, D.E., Ramey, A.M., 2016. Limited evidence of intercontinental dispersal of avian paramyxovirus serotype 4 by migratory birds. Infect. Genet. Evol. 40, 104-108.

Romer-Oberdorfer, A., Werner, O., Veits, J., Mebatsion, T., Mettenleiter, T.C., 2003. Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. J. Gen. Virol. 84, 3121-3129.

Roy, P., Venugopalan, A.T., Selvarangam, R., Ramaswamy, V., 1998. Velogenic Newcastle disease virus in captive wild birds. Trop. Anim. Health Prod. 30, 299-303.

Saif, Y.M., Mohan, R., Ward, L., Senne, D.A., Panigrahy, B., Dearth, R.N., 1997. Natural and experimental infection of turkeys with avian paramyxovirus-7. Avian Dis. 41, 326-329.

Sakaguchi, T., Toyoda, T., Gotoh, B., Inocencio, N.M., Kuma, K., Miyata, T., Nagai, Y., 1989. Newcastle disease virus evolution. I. Multiple lineages defined by sequence variability of the hemagglutinin-neuraminidase gene. Virology 169, 260-272.

Samal, S.K., 2011. Newcastle Disease and Related Avian Paramyxoviruses. In: Siba K., S. (Ed.), The Biology of Paramyxovirueses. Caister Academic Press, Norfolk, UK, pp. 69-114.

Samuel, A.S., Paldurai, A., Kumar, S., Collins, P.L., Samal, S.K., 2010. Complete genome sequence of avian paramyxovirus (APMV) serotype 5 completes the analysis of nine APMV serotypes and reveals the longest APMV genome. PLoS One 5, e9269.

Samuel, A.S., Subbiah, M., Shive, H., Collins, P.L., Samal, S.K., 2011. Experimental infection of hamsters with avian paramyxovirus serotypes 1 to 9. Vet. Res. 42, 38-38.

Seal, B.S., 1996. Analysis of Matrix Protein Gene Nucleotide Sequence Diversity Among Newcastle Disease Virus Isolates Demonstrates that Recent Disease Outbreaks Are Caused by Viruses of Psittacine Origin. In: Becker, Y. (Ed.), Molecular Evolution of Viruses — Past and Present. Springer US, Boston, MA, pp. 145-152.

Shekaili, T.A., Clough, H., Ganapathy, K., Baylis, M., 2015. Sero-surveillance and risk factors for avian influenza and Newcastle disease virus in backyard poultry in Oman. Prev. Vet. Med. 122, 145-153.

Shengqing, Y., Kishida, N., Ito, H., Kida, H., Otsuki, K., Kawaoka, Y., 2002. Generation of velogenic Newcastle disease viruses from a nonpathogenic waterfowl isolate by passaging in chickens. Virology 301, 206-211.

Shihmanter, E., Weisman, Y., Manwell, R., Alexander, D., Lipkind, M., 1997. Mixed paramyxovirus infection of wild and domestic birds in Israel. Vet. Microbiol. 58, 73-78.

Shortridge, K.F., Alexander, D.J., Collins, M.S., 1980. Isolation and pproperties of viruses from poultry in Hong Kong which represent a new (sixth) distinct group of avian paramyxoviruses. J. Gen. Virol. 49, 255-262.

Solomon, P., Abolnik, C., Joannis, T.M., Bisschop, S., 2012. Virulent Newcastle disease virus in Nigeria: identification of a new clade of sub-lineage 5f from livebird markets. Virus Genes 44, 98-103.

Stanislawek, W.L., Wilks, C.R., Meers, J., Horner, G.W., Alexander, D.J., Manvell, R.J., Kattenbelt, J.A., Gould, A.R., 2002. Avian paramyxoviruses and influenza viruses isolated from mallard ducks (Anas platyrhynchos) in New Zealand. Arch. Virol. 147, 1287-1302.

Steward, M., Vipond, I.B., Millar, N.S., Emmerson, P.T., 1993. RNA editing in Newcastle disease virus. J. Gen. Virol. 74, 2539-2547.

Suarez, D.L., Miller, P.J., Koch, G., Mundt, E., R.C., J., Rautenschlein, S., 2013. Newcastle disease, other avian paramyxoviruses, and avian metapneumovirus infecctions. In: Swyne, D.E. (Ed.), Disease of Poultry, 13 ed. Wiley-Blackwell, Ames, pp. 89–138.

Subbiah, M., Khattar, S.K., Collins, P.L., Samal, S.K., 2011. Mutations in the fusion protein cleavage site of avian paramyxovirus serotype 2 increase cleavability and syncytium formation but do not increase viral virulence in chickens. J. Virol. 85, 5394-5405.

Subbiah, M., Nayak, S., Collins, P.L., Samal, S.K., 2010. Complete genome sequences of avian paramyxovirus serotype 2 (APMV-2) strains Bangor, England and Kenya: evidence for the existence of subgroups within serotype 2. Virus Res. 152, 85-95.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.

Terregino, C., Aldous, E.W., Heidari, A., Fuller, C.M., Nardi, R., Manvell, R.J., Beato, M.S., Shell, W.M., Monne, I., Brown, I.H., Alexander, D.J., Capua, I., 2013. Antigenic and

genetic analyses of isolate APMV/wigeon/Italy/3920-1/2005 indicate that it represents a new avian paramyxovirus (APMV-12). Arch. Virol. 158, 2233-2243.

The Center for Food Security & Public Health, 2016. Newcastle Disease. Iowa state University, Ames, Iowa, pp. 1-9.

Tsunekuni, R., Hikono, H., Saito, T., 2014. Evaluation of avian paramyxovirus serotypes 2 to 10 as vaccine vectors in chickens previously immunized against Newcastle disease virus. Vet. Immunol. Immunopathol. 160, 184-191.

Tumova, B., Robinson, J.H., Easterday, B.C., 1979. A hitherto unreported paramyxovirus of turkeys. Res. Vet. Sci. 27, 135-140.

Umali, D.V., Ito, H., Katoh, H., Ito, T., 2014. Surveillance of avian paramyxovirus in migratory waterfowls in the San-in region of western Japan from 2006 to 2012. J. Vet. Med. Sci. 76, 423-430.

van Boven, M., Bouma, A., Fabri, T.H.F., Katsma, E., Hartog, L., Koch, G., 2008. Herd immunity to Newcastle disease virus in poultry by vaccination. Avian Pathol. 37, 1-5.

Wakamatsu, N., King, D.J., Kapczynski, D.R., Seal, B.S., Brown, C.C., 2006. Experimental pathogenesis for chickens, turkeys, and pigeons of exotic Newcastle disease virus from an outbreak in California during 2002-2003. Vet. Pathol. 43, 925-933.

Wang, J.-Y., Liu, W.-H., Ren, J.-J., Tang, P., Wu, N., Wu, H.-Y., Ching, C.-D., Liu, H.-J., 2015. Characterization of emerging Newcastle disease virus isolates in China. Virol J. 12, 119.

Wang, L.F., Collins, P.L., Fouchier, R.A.M., Kurath, G., Lamb, R.A., Randall, R.E., Rima, B.K., 2012. Family - Paramyxoviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, pp. 672-685.

Warke, A., Appleby, L., Mundt, E., 2008. Prevalence of antibodies to different avian paramyxoviruses in commercial poultry in the United States. Avian Dis. 52, 694-697.

Westbury, H., 2001. Newcastle disease virus: an evolving pathogen? Avian Pathol. 30, 5-11.

Wetlands International, 2012. Waterbird Population Estimates: Summary Report. 5 ed. Wetlands International, Wageningen, The Netherlands.

White, C.L., Ip, H.S., Meteyer, C.U., Walsh, D.P., Hall, J.S., Carstensen, M., Wolf, P.C., 2015. Spatial and temporal patterns of avian paramyxovirus-1 outbreaks in double-crested cormorants (Phalacrocorax auritus) in the USA. J. Wildl. Dis. 51, 101-112.

WHO, 2011. Manual for the laboratory diagnosis and virological surveillance of influenza. WHO Press, Geneva.

Xiao, S., Khattar, S.K., Subbiah, M., Collins, P.L., Samal, S.K., 2012. Mutation of the f-protein cleavage site of avian paramyxovirus type 7 results in furin cleavage, fusion promotion, and increased replication in vitro but not increased replication, tissue tropism, or virulence in chickens. J. Virol. 86, 3828-3838.

Xiao, S., Subbiah, M., Kumar, S., De Nardi, R., Terregino, C., Collins, P.L., Samal, S.K., 2010. Complete genome sequences of avian paramyxovirus serotype 6 prototype strain Hong Kong and a recent novel strain from Italy: evidence for the existence of subgroups within the serotype. Virus Res. 150, 61-72.

Yamamoto, E., Ito, H., Tomioka, Y., Ito, T., 2015. Characterization of novel avian paramyxovirus strain APMV/Shimane67 isolated from migratory wild geese in Japan. J. Vet. Med. Sci. 77, 1079-1085.

Yu, L., Wang, Z., Jiang, Y., Chang, L., Kwang, J., 2001. Characterization of Newly Emerging Newcastle Disease Virus Isolates from the People's Republic of China and Taiwan. J. Clin. Microbiol. 39, 3512-3519.

Yuan, P., Paterson, R.G., Leser, G.P., Lamb, R.A., Jardetzky, T.S., 2012. Structure of the ulster strain newcastle disease virus hemagglutinin-neuraminidase reveals auto-inhibitory interactions associated with low virulence. PLoS Pathog. 8, e1002855.

Zhang, P., Xie, G., Liu, X., Ai, L., Chen, Y., Meng, X., Bi, Y., Chen, J., Sun, Y., Stoeger, T., Ding, Z., Yin, R., 2016. High Genetic Diversity of Newcastle Disease Virus in Wild and Domestic Birds in Northeastern China from 2013 to 2015 Reveals Potential Epidemic Trends. Appl. Environ. Microbiol. 82, 1530-1536.

Zhao, W., Zhang, Z., Zsak, L., Yu, Q., 2013. Effects of the HN gene C-terminal extensions on the Newcastle disease virus virulence. Virus Genes 47, 498-504.