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Massachusetts Institute of Technology

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BRIEF REPORT

Characterization of a genetic and antigenic variant of avian paramyxovirus 6 isolated from a migratory wild bird, the red-necked stint (*Calidris ruficollis*)

Vuong Nghia Bui · Tetsuya Mizutani · Tung Hoang Nguyen · Dai Quang Trinh · Sanaa S. A. Awad · Germaine L. Minoungou · Yu Yamamoto · Kikuyasu Nakamura · Keisuke Saito · Yukiko Watanabe · Jonathan Runstadler · Falk Huettmann · Haruko Ogawa · Kunitoshi Imai

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Abstract A hemagglutinating virus (8KS0813) was isolated from a red-necked stint. Hemagglutination inhibition and neutralization tests indicated that 8KS0813 was antigenically related to a prototype strain, APMV-6/duck/Hong Kong/18/199/77, but with an 8- and 16-fold difference, respectively, in their titers. The full genome sequence of 8KS0813 showed 98.6 % nucleotide sequence identity to that of APMV-6/duck/Italy/4524-2/07, which has been reported to belong to an APMV-6 subgroup, and showed less similarity to that of the prototype strain (70.6 % similarity). The growth of 8KS0813 and the prototype strain in four different cell cultures was greatly enhanced by adding trypsin. Interestingly, this virus induced syncytia only in Vero cells. 8KS0813 was identified as APMV-6/red-necked stint/Japan/8KS0813/08, but it is antigenically and genetically distinguishable from the prototype strain, suggesting that variant APMV-6 is circulating in migratory birds.

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Paramyxoviruses are pleomorphic enveloped viruses containing a non-segmented ssRNA genome that replicate in the cytoplasm of host cells. Members of a great variety of mammalian and avian species can be infected with these viruses [12]. Paramyxoviruses isolated from birds are divided into two distinct groups: avian paramyxovirus (APMV) and avian metapneumovirus. APMVs belong to the genus *Avulavirus* of the subfamily *Paramyxoviridae* and are divided into nine serotypes, from APMV-1 to -9 [1]. Recently, viruses of three new serotypes, APMV-10, APMV-11 and APMV-12, were isolated from rockhopper penguins, common snipes and wigeons, respectively [2, 10, 17]. Subgrouping within the APMV serotype has also been

V. N. Bui · T. H. Nguyen · D. Q. Trinh ·
S. S. A. Awad · G. L. Minoungou · H. Ogawa · K. Imai (✉)
Research Center for Animal Hygiene and Food Safety,
Obihiro University of Agriculture and Veterinary Medicine,
2-11 Inada, Obihiro, Hokkaido 080-8555, Japan
e-mail: imaiku@obihiro.ac.jp

T. Mizutani
Virology 1, National Institute of Infectious Diseases,
Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan

Present Address:
T. Mizutani
Tokyo University of Agriculture and Technology,
Saiwaicho, Fuchu, Tokyo 183-8509, Japan

Y. Yamamoto · K. Nakamura
National Institute of Animal Health, Tsukuba,
Ibaraki 305-0856, Japan

K. Saito · Y. Watanabe
Institute for Raptor Biomedicine, Kushiro,
Hokkaido 084-0922, Japan

J. Runstadler · F. Huettmann
Institute of Arctic Biology, Biology and Wildlife Department,
University of Alaska-Fairbanks (UAF), Fairbanks, AK 99775,
USA

Present Address:
J. Runstadler
Massachusetts Institute of Technology, Cambridge,
MA 02139, USA

reported for APMV-2 and APMV-3 [8, 16]. Xiao et al. [19] reported that APMV-6/duck/Italy/4524-2/07 (APMV-6/Dk/IT/4524-2/07), isolated from a duck in 2007 in Italy, was antigenically and genetically different from an APMV-6 prototype strain, APMV-6/duck/Hong Kong/18/199/77 (APMV-6/Dk/HK/77) [15]. This suggests the existence of a second antigenic group within the APMV-6 serotype. However, it remains unknown if such a variant virus is circulating in domestic and/or wild ducks because of a lack of reports on the isolation of viruses of this subgroup. It is also unclear whether it has emerged relatively recently.

While carrying out avian influenza (AI) surveillance in Hokkaido, Japan, in 2008, a hemagglutinating virus was isolated from a red-necked stint (*Calidris ruficollis*). However, since it was identified as neither AI virus (AIV) nor Newcastle disease virus (NDV) by RT-PCR [4, 13] at that time, we tried to identify the virus using electron microcopy (EM) and a rapid determination of viral nucleic acid sequence method (RDV) followed by serologic and genetic analyses.

In this study, we describe the genetic and antigenic characterization of an APMV-6 isolate from a red-necked stint that can be antigenically and genetically discriminated from the prototype APMV-6 (APMV-6/Dk/HK/77).

The virus with HA activity, which was isolated from a fecal homogenate sample using 10-day-old embryonated chicken eggs was tentatively designated as 8KS0813. Virus particles were purified from the 8KS0813-infected allantoic fluid by ultracentrifugation through 30 % and 60 % sucrose layers as described previously [7].

EM revealed that the morphology of purified 8KS0813 viral particles resembles that of members of the subfamily *Paramyxoviridae* [3, 7]. The majority of 8KS0813 particles were spherical in size, ranging from about 140 to 350 nm in diameter, and pleomorphic shapes were also observed (Supplementary Fig. S1). An envelope with spike-like projections surrounded the particles. The "herringbone-shaped" nucleocapsid, ranging from about 16 to 21 nm in diameter, was also seen.

The RDV method has previously been used to identify unknown or untypeable emerging viruses [11]. RDV was performed on the purified 8KS0813 virus with slight modifications. Briefly, viral RNA was extracted from the sample using a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany). Double-stranded cDNA was synthesized using a TransPlex Whole Transcriptome Amplification Kit (Sigma-Aldrich, Saint Louis, MO) in accordance with the manufacturer's instructions. However, only two forward primers, H1-1 (5′-AATTCGG CGGCCGCGGATCCCCGGGG-3′) and H1-7 (5′-ATTC GGCGGCCGCGGATCCCCGGCA-3′), were used to amplify the second cDNA library, together with a set of reverse primers for PCR described by Mizutani et al. [11]. Direct sequencing was performed using PCR products obtained from the second cDNA library with the forward primers. The sequences obtained using RDV were compared with other sequences available in GenBank using the BLAST program.

Sequence comparisons suggested that 8KS0813 was closely related to APMV-6/Dk/IT4524-2/07. During the cDNA library amplification, a total of 11 DNA fragments, ranging from 88 to 330 nucleotides (nt) in length, were obtained with 8KS0813. However, only three fragments (A, B and C), ranging from 136 to 185 nt, were mapped on the genome of APMV-6/Dk/IT4524-2/07 (accession no. GQ406232): fragment A (2381–2516 nt) from the phosphoprotein (P) gene, and fragments B (5283–5467 nt) and C (6159–6330 nt) from the F gene shared homology with the APMV-6/Dk/IT/4524-2/07 genome, with a range of 97.6 % to 100 % identity. In contrast, the nt sequence of the three DNA fragments was found to be approximately 80 % identical to that of APMV-6/Dk/HK/77 (accession no. EU622637). However, RT-PCR using the large gene primers for the prototype APMV-6 [14] was negative (data not shown). Therefore, additional analysis was required to fully identify the viral isolate.

Cross-hemagglutination inhibition (HI) tests were conducted using 8KS0813 and its chicken antiserum as well as reference APMV-1 to -9 serotype antigens (except APMV-5) and their chicken antisera according to the WHO manual [18] (Table 1). The titer was determined in twofold serial dilutions of the antiserum starting at a 1:8 dilution. Antiserum to APMV-6/Dk/HK/77 showed an HI antibody titer of 128 against the homologous strain, while the titer was 16 against 8KS0813 (8-fold lower). Conversely, antiserum to 8KS0813 showed an HI titer of 1,024 against the homologous strain, while the titer against APMV-6/Dk/HK/77 was 64 (16-fold lower). The antiserum against 8KS0813 did not react with the other serotypes tested.

Microneutralization tests using APMVs and 8KS0813 with antisera to 8KS0813 and APMV-6/Dk/HK/77 were performed using MDBK cells according to the WHO manual [18] to confirm the results of the HI tests. Neutralizing antibody titers were expressed as reciprocals of the highest dilution showing 50 % inhibition of infectivity. The neutralizing titer of the homologous 8KS0813-specific antiserum was 16-fold higher than that of the heterologous APMV-6/Dk/HK/77 and vice versa. No cross-reaction of 8KS0813-specific antiserum to the other APMV serotypes was observed (Supplementary Table S1).

The cross-HI and microneutralization tests demonstrated that 8KS0813 was antigenically related to APMV-6/Dk/HK/77 (prototype), although APMV-5, -10, -11 and -12 were not available for comparison. However, because of the antibody titer difference and the negative results in RT-PCR for APMV-6 as described above, 8KS0813 was

Table 1 Cross-hemagglutination inhibition (HI) test with 8KS0813 and APMVs[a]

Antigen	Antiserum to								
	APMV-1	APMV-2	APMV-3	APMV-4	APMV-6	APMV-7	APMV-8	APMV-9	8KS0813
APMV-1	512	N[b]	N	N	N	N	N	N	N
APMV-2	8	256	N	N	N	N	N	N	N
APMV-3	N	N	512	N	N	8	N	8	N
APMV-4	N	N	N	128	N	N	N	N	N
APMV-6	8	8	N	N	128	8	N	N	64
APMV-7	N	N	N	N	N	256	N	N	N
APMV-8	16	8	16	N	N	N	256	N	N
APMV-9	128	N	8	8	8	8	N	256	N
8KS0813	N	N	N	N	16	N	N	N	1024

[a] APMV-1/chicken/Japan/Ibaraki/85, 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

[b] HI titer <8

suspected to represent a new serotype of APMV or an antigenically and genetically unique APMV-6 strain. In addition, since APMV-6/Dk/IT/4524-2/07 and its antiserum were not available, we further tried to identify 8KS0813 using genetic analysis.

The full genome sequence of 8KS0813 was obtained by second-generation sequencing using the 454 Life Sciences GS FLX system (Roche Diagnostics Japan, Tokyo, Japan). The genome length of 8KS0813 was 16,230 nt, which was shorter (6 nt) than that of APMV-6/Dk/HK/77 (16,236 nt) and contained seven open reading frames (ORFs) with sequence identity to the N, P, M, F, SH, HN and L proteins of APMV-6 (Supplementary Table S2). The 3′ and 5′ ends of the 8KS0813 genome comprised leader and trailer regions in which the leader sequence was 55 nt in length. This length is typical for almost all members of the family *Paramyxoviridae*, including APMV-6 [12]. The 3′ terminus of the leader (UGGU) sequence was identical to those of viruses of the subfamily *Paramyxoviridae*. The trailer of the 8KS0813 genome was 45 nt in length, which was within the typical range (40–60 nt) of most of the viruses of the subfamily *Paramyxoviridae* [12]. The sequences of the gene start, gene end and intergenic regions of 8KS0813 were exactly the same as those of APMV-6/Dk/IT/4524-2/07 [19]. They were also similar to those of other known APMV-6 strains. The P gene of 8KS0813 contained the editing site UUUUUUCCC, which is exactly identical to that of the prototype APMV-6 strain.

Comparison of the full genome sequence of 8KS0813 with that of APMV-6 and other APMV serotype strains indicated that the nt sequence of 8KS0813 is 98.67 % identical to that of APMV-6/Dk/IT/4524-2/07. In contrast, a lower level of nt sequence identity (70.62–71.02 %) was found between 8KS0813 and three other APMV-6 strains isolated between 1977 and 2003. On the other hand, the sequence of 8KS0813 showed low identity (less than 49 %) to those of other APMV serotypes (data not shown). In addition, phylogenetic analysis of the full-length sequence of the HN gene revealed that 8KS0813 formed a cluster together with APMV-6/Dk/IT/4524-2/07 that was separate from other APMV-6 strains (Fig. 1). Xiao et al. [19] proposed that APMV-6/Dk/IT/4524-2/07 was antigenically and genetically different from the APMV-6 prototype strains and represented a subgroup within APMV-6.

Cleavage of the precursor fusion protein (F0) of NDV (APMV-1) is a key determinant of its infectivity and pathogenicity because NDV is not infectious if F0 is not cleaved by proteases [1]. The F protein cleavage site of virulent NDV contains a multiple basic aa sequence (^{113}**R**-X-**K/R**-**R***F^{117}), which is preferably recognized by ubiquitous cellular furin-like proteases. In contrast, avirulent NDV possessing dibasic residues at the cleavage site are cleaved by a secretory trypsin-like protease in particular tissues such as the respiratory and digestive tracts. The aa sequence at the F protein cleavage site of 8KS0813 (**R**-E-P-**R***L) was identical to that of APMV-6/Dk/IT/4524-2/07 [19]. In contrast, the prototype APMV-6 strain (APMV-6/Dk/HK/77) contains a single basic residue (P-E-P-**R***L). These data suggested that none of these viruses contain a preferred furin cleavage site similar to that of virulent NDV. From the F protein cleavage site aa sequences of 8KS0813 and APMV-6/Dk/HK/77, it can be predicted that both viruses should require a trypsin-like protease for replication in cultured cells or permissive hosts.

We investigated the growth characteristics of 8KS0813 and APMV-6/Dk/HK/77 in primary chicken embryo fibroblasts (CEF) and cell lines (MDCK, MDBK and Vero). Xiao et al. [19] reported that APMV-6/Dk/IT/4524-2/07 as well as APMV-6/Dk/HK/77 induced rounding of cells in six cell lines, including Vero, MDCK and MDBK

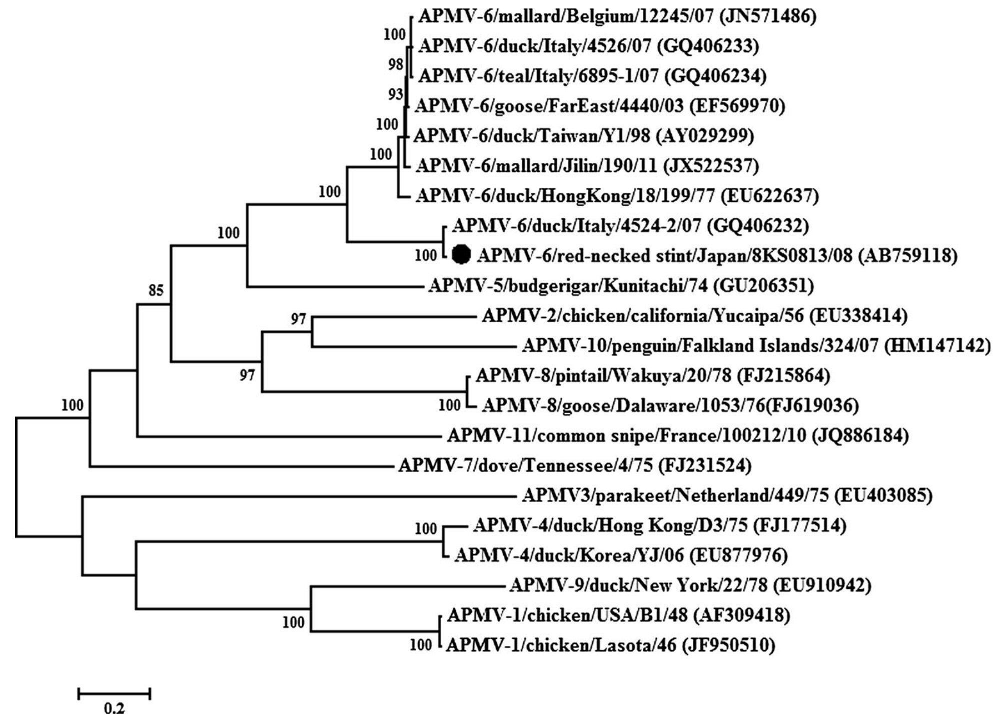

Fig. 1 Phylogenic analysis of the full-length HN genes of 8KS0813 and representative members of APMV serotypes 1-11. 8KS0813 (APMV-6/red-necked stint/Japan/8KS0813/08) is denoted by (●). The evolutionary history was inferred using the maximum-likelihood method. The evolutionary distances were calculated using the neighbor-joining model. The tree was constructed using bootstrap analysis (1,000 replicates) in the MEGA 5.0 program. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. Bootstrap values >50 % are shown at the nodes. The scale bar shows the number of base substitutions per site

Table 2 Growth of 8KS0813, APMV-6/Dk/HK/77 and avirulent NDV (clone 30) in cell cultures

Cell type	Virus titer ($TCID_{50}$/ml)					
	Dk/HK/77		8KS0813		NDV (Clone 30)	
	Tryp+[a]	Tryp−[b]	Tryp+	Tryp−	Tryp+	Tryp−
CEF	5.75	2.5	5.0	<1.5	7.25	<1.5
MDBK	7.25	3.5	6.5	2.5	7.25	2.5
MDCK	6.25	2.5	4.25	<1.5	2.5	<1.5
Vero	6.5	2. 5	6.25	<1. 5	7.5	<1.5

[a] The cell monolayer inoculated with the virus was maintained in the presence of trypsin

[b] The cell monolayer inoculated with the virus was maintained in the absence of trypsin

cells. The presence of trypsin had either a marginal effect or no effect on virus growth. In contrast to the observations reported by Xiao et al. [19], the growth of APMV-6/Dk/HK/77 was greatly enhanced by adding trypsin in the cultured cells, and 8KS0813 also required trypsin for virus growth to high titers (Table 2).

In the absence of trypsin, 8KS0813 could not grow in CEF, MDCK and Vero cells, and a low virus titer was obtained only in MDBK cells (Table 2). APMV-6/Dk/HK/77 could grow in all the cells tested in the absence of trypsin. However, when the supernatants of the inoculated cells with low virus titers in the absence of trypsin were transferred to newly cultured cells, virus growth in the absence of trypsin was not observed (data not shown). This result suggests that viruses with a nonfunctional, uncleaved F protein (F0) were released from the infected cells. The growth characteristics of 8KS0813 in the cultured cells were similar to those of avirulent NDV (Table 2). On the other hand, unexpectedly, 8KS0813 and APMV-6/Dk/HK/77 caused syncytia only in Vero cells, whereas cell rounding was observed in other cells.

Although the present study indicated that 8KS0813 and APMV-6/Dk/IT/4524-2/07 were genetically very close to each other, the growth characteristics of 8KS0813 in cultured cells seemed to be quite different from those of APMV-6/Dk/IT/4524-2/07 observed by Xiao et al. [19]. The reason for the discrepancy seen for viral growth in cultured cells between the two research groups is still unclear and awaits more study.

It is known that free-living birds, particularly migratory water birds, have the potential to carry and disseminate a number of pathogenic microorganisms, including viruses, bacteria, fungi, and parasites [6]. It has been estimated that members of at least nine virus families might utilize migratory birds for their dispersion in nature. Among these, AIV and APMV-1 (NDV) have frequently been detected in migratory birds [9]. However, reports on isolation of other APMV serotypes from these birds are limited in number.

Viral genomes accumulate mutations during virus replication in the host, and variant strains are accumulated more rapidly in the case of RNA viruses because the polymerase lacks a proofreading mechanism [5]. However, APMVs have historically been considered antigenically

and genetically stable, unlike influenza A viruses. For now, it remains unknown if the APMV-6 variant found here is widely circulating in domestic and/or wild birds, because we lack adequate surveillance information. It is also unclear how long ago it emerged. Recently, we isolated three APMV-6 viruses from wild ducks, and two of those appeared to be antigenically close to 8KS0813 (unpublished data). This finding suggests that APMV-6 has been diversifying and is maintained in wild water birds. Full characterization of such variants will help to understand the significance of changes occurring in APMV-6 in nature.

In conclusion, we isolated a variant of APMV-6 from a migratory red-necked stint. This viral isolate is genetically close to APMV-6/Dk/IT/4524-2/07, which has been proposed to represent a second subgroup of APMV-6 [19], and it differs from the prototype strain APMV-6/Dk/HK/77. It is interesting that two variant viruses isolated from different avian species and in different areas (Italy and Japan) quite far from each other showed similar genetic characteristics. However, viral growth properties in cultured cells seemed to be different between the two antigenic variants. We designated the new viral isolate as APMV-6/red-necked stint/Japan/8KS0813/2008 (8KS0813). To our knowledge, this is the first report on APMV-6 isolation from a member of the family *Scolopacidae*. The data contribute to the accumulated knowledge of genetic variants within the APMV-6 serotype.

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