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

Rapeewan Thampaisarn[a,e], Vuong N. Bui[c], Dai Q. Trinh[c], Makoto Nagai[b], Tetsuya Mizutani[b], Tsutomu Omatsu[b], Yukie Katayama[b], Dulyatad Gronsang[a,e], Duong H.T. Le[d], Haruko Ogawa[a], Kunitoshi Imai[a,*]

[a] Diagnostic Center for Animal Health and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
[b] Research and Education Center for Prevention of Global Infectious Diseases of Animals, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan
[c] National Institute of Veterinary Research, 86 Truong Chinh, Dong Da, Hanoi, Viet Nam
[d] Pasteur Institute of Ho Chi Minh City, 167 Pasteur, District 3, Ho Chi Minh City, Viet Nam
[e] Faculty of Veterinary Science, Mahidol University, Nakhon Pathom 73170, Thailand

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ABSTRACT

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 avian paramyxovirus (APMV). Electron micrographs showed that the morphology of the virus particle was similar to that of APMV. The complete genome of this virus comprised 15,444 nucleotides complying with the paramyxovirus "rule of six" and contains six open reading frames (3′-N-P-M-F-HN-L-5′). 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 lentogenic strain of APMV-1, the K residue at position -1 of the cleavage site was first discovered in APMV members. The phosphoprotein gene of isolate 11OG0352 contains a putative RNA editing site, 3′-AUUUUCCC-5′ (negative sense) which sequence differs from that of other APMVs. The intracerebral pathogenicity index test did not detect virulence in infected chicks. In hemagglutination inhibition (HI) tests, an antiserum against this virus did not detectably react with other APMVs (serotypes 1–4, 6–9) except for low reciprocal cross-reactivity with APMV-6. We designated this isolate, as APMV-14/duck/Japan/11OG0352/2011 and propose that it is a novel APMV serotype. 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.



## 1. Introduction

The family *Paramyxoviridae* which includes numerous viruses that infect humans and animals, comprises the *Paramyxovirinae* and *Pneumovirinae* subfamilies. Virions consist of a lipid bilayer envelope surrounding a nucleocapsid and contain a single molecule of linear, negative-sense, single-stranded RNA. The morphology of virions is generally spherical, ≥150 nm in diameter, although pleomorphic as well as filamentous forms are also observed (Lamb and Parks, 2013).

The subfamily *Paramyxovirinae* comprises five genera. The genus *Avulavirus* includes different serotypes of avian paramyxoviruses (APMVs). Nine serotypes of APMVs, APMV-1 to APMV-9 have been defined using hemagglutination inhibition (HI) tests (Wang et al., 2012). However, different serotypes cross-react (Alexander et al., 1983; Kessler et al., 1979; Lipkind and Shihmanter, 1986;

* Corresponding author at: Diagnostic Center for Animal Health and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan.
*E-mail address:* imaiku@obihiro.ac.jp (K. Imai).

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Shortridge et al., 1980; Tumova et al., 1979). Further, antisera against APMV serotypes are not always available in many laboratories. The presence of new serotypes in wild bird populations had not been long reported since the 1970s; however, four novel APMVs were recently isolated: 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). These four viruses were differentiated from the nine known serotypes and were proposed as a new serotype according to the results of HI tests using limited numbers of serotype antisera, genetic analyses, or both.

Most research has focused on APMV-1, also termed Newcastle disease virus, because its velogenic strain can cause severe disease in poultry. However, little is known about other APMVs. For example, APMV-2, -3, -6, and 7 are associated with mild respiratory disease and decreased egg production in turkeys (Alexander, 1997; Awang and Russell, 1990; Bankowski et al., 1981; Saif et al., 1997). APMV-5 isolated from an outbreak in budgerigar does not affect chickens and ducks (Kim et al., 2012; Nerome et al., 1978), while APMV-12 and APMV-13 are avirulent for chickens (Terregino et al., 2013; Yamamoto et al., 2015).

The surveillance of avian influenza (AI) virus in migratory wild birds in Hokkaido Prefecture in northern Japan has been conducted in our laboratory since 2009 (Abao et al., 2013; Bui et al., 2011). In 2011, a hemagglutinating viral agent was isolated from duck feces, which was not be genetically and/or antigenically identified as an AI virus or as an APMV (serotypes 1–13). Therefore, we concluded that this isolate is a novel APMV serotype. Here, we described the first isolation of APMV serotype 14, designated APMV-14/duck/Japan/11OG0352/2011 (isolate 11OG0352) and report the virological, serological, and genetic characterization of this virus.

## 2. Materials and methods

### 2.1. 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) of northern Japan were collected during AI virus 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 10-day-old embryonated chicken eggs. After incubating at 37 °C for three days, allantoic fluids were tested for hemagglutination (HA) activity using 0.5% chicken red blood cells according to Manual for the Laboratory Diagnosis and Virological Surveillance of influenza (WHO, 2011).

### 2.2. 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%).

### 2.3. 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).

### 2.4. Nucleotide sequencing

The nucleotide sequence of the full 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.

### 2.5. Phylogenetic analysis

Nucleotide sequences of the whole genome and individual genes were aligned using the MUSCLE command. Pairwise comparison and evolutionary distance estimation between sequences were conducted in MEGA6 using the p-distance model and the Kimura 2-parameter model, respectively (Kimura, 1980; Tamura et al., 2013). Phylogenetic trees were generated by MEGA6 using the maximum-likelihood method with 1000 bootstraps.

### 2.6. Cross-HI test

HI tests were conducted according to the guidelines of the OIE (World Organization for Animal Health, 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 by 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.

### 2.7. 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 (CEF) in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum