

Characterization of avian paramyxovirus type 6 isolated from a Eurasian teal in the intersection of migratory flyways in Russia

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Abstract The complete genome sequence was determined for avian paramyxovirus (APMV-6) serotype 6 strain teal/Chany/455/2009, isolated from a teal (*Anas crecca*) in Siberia. Siberia is crossed by four major migration flyways and represents the major breeding area for many wild bird species in the Palearctic. Strain teal/Chany/455/2009 is genetically closely related to Kazakh and Chinese strains and belongs to the genetic group of duck/Hong Kong/18/199/77-like APMV-6 viruses. We show that the virus has low pathogenic potential according to genetic markers and animal model experiments.

Keywords Avian paramyxovirus type 6 · Teal · Siberia · Russia

Avian paramyxoviruses are isolated regularly from birds throughout the world. Ducks are usual natural hosts of avian paramyxovirus serotype 6 (APMV-6). Other hosts include geese, rails, and turkeys [1]. APMV-6 causes mild respiratory disease, slightly elevated mortality and decreased of egg production in turkeys [1]. APMV-6 virus was isolated for the first time in Hong Kong (1977,

domestic duck). This strain was named duck/Hong Kong/18/199/77, and it is considered the prototype strain of APMV-6 [2].

Genetically, APMV-6 is divided into two subgroups. The first subgroup consists of strains similar to the prototype strain duck/Hong Kong/18/199/77. The completely sequenced strains duck/Taiwan/Y1/1998, goose/FarEast/4440/2003, mallard/Belgium/12245/07, mallard/Jilin/127/2011, mallard/Jilin/190/2011, and red-crested pochard/Balkhash/5842/2013 belong to this subgroup. The second subgroup is represented by strains genetically related to the strains red-necked stint/Japan/8KS0813/2008 and duck/Italy/IT4524-2/2007 [3, 4].

Strains of the first genetic subgroup (duck/Hong Kong/18/199/77-like viruses) are characterized by their 16,236-nt genome length and strains of the second genetic subgroup (red-necked stint/Japan/8KS0813/2008 and duck/Italy/IT4524-2/2007) are characterized by their 16,230-nt genome length. Strains red-necked stint/Japan/8KS0813/2008 and duck/Italy/IT4524-2/2007 have a 6-nt deletion in the downstream untranslated region of the F gene. The nucleotide sequence (nt) similarity between the genetic subgroups is only 70 %, while nucleotide sequences identity between strains of the duck/Hong Kong/18/199/77-like subgroup is 94–98 %. All APMV-6 genomes consist of seven genes in the pattern 3'-N-P/V/W-M-F-SH-HN-L-5', which differs from that of other APMVs by the presence of an additional gene encoding the small hydrophobic protein (SH). Strains of the duck/Hong Kong/18/199/77-like genetic subgroup are characterized by a monobasic F protein cleavage site (P-E-P-R↓L). The F protein cleavage site of red-necked stint/Japan/8KS0813/2008 and duck/Italy/IT4524-2/2007 is R-E-P-R↓L and has two basic amino acids (R).

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There are only nine complete genome sequences and three partial sequences of APMV-6 isolates in GenBank (20.06.16). According to the GenBank database, the earlier APMV-6 strain Goose/Goose/FarEast/4440/2003 was isolated in Russia (from domestic goose). In the present study, we report the complete genome sequence of the teal/Chany/455/2009 (APMV6-455) strain, isolated from a clinically healthy wild teal (*Anas crecca*) in the Asian portion (Siberia) of Russia in 2009.

As a result of an influenza virus surveillance program in Siberia (Russia) in 2009, a virus causing hemagglutination was isolated from wild migratory duck – teal (*Anas crecca*). The individual bird from which APMV6-455 was isolated appeared to be healthy. The isolated hemagglutinating viral agent showed 128 hemagglutination units (HAU) per 50 μ l. This virus was shown by sequencing to be paramyxovirus type 6. APMV6-455 virus-containing allantoic fluid showed high infectivity in the allantoic cavities of 10-day-old embryonated eggs, with the titer of 8.5 log₁₀ EID₅₀/ml and demonstrated a maximal hemagglutinin production of 128 hemagglutination units (HAU) per 50 μ l in the allantoic fluids of embryonated eggs inoculated with the virus. However, the APMV6-455 virus did not cause the death of chicken embryos. The mean death times (MDT) of the virus in 10-day-old embryonated chicken eggs were more than 120 h, indicating that APMV6-455 is avirulent for chickens. The absence of the pathological alterations in chorioallantoic membranes indicated a low pathogenic potential of the virus for chicken embryos.

We first examined the virion of allantoic-grown APMV-6 virus, using electron microscopy. The morphology of

virions was found to be typical for paramyxoviruses: enveloped, sphere-shaped and pleomorphic [5] (Fig. 1). The virions showed low variability in size, ranging approximately from 150 to 220 nm in diameter. Envelope glycoproteins were densely packed on the virion surface.

To determine pathogenic potential of this virus, 6-week-old chickens were infected intravenously with APMV6-455, and results showed that the virus was a non- or low-pathogenic virus (IVPI = 0). Pathogenicity in day-old chickens by intracerebral inoculation was also examined. None of the chicks showed clinical symptoms or died, and the ICPI of APMV6-455 in chickens was 0.0, a value that is typical for non-pathogenic isolates. Thus, both the ICPI and the IVPI of APMV6-455 in SPF chickens were found to be zero. These results suggest that APMV6-455 is apathogenic.

We evaluated the pathogenicity of APMV6-455 for mice as a small animal model for infection with APMV. We inoculated BALB/c mice intranasally and monitored clinical signs and viral replication in the direct target organ, lungs. The mice did not lose weight or show signs of disease at 14 days after intranasal infection. We also did not observe active replication in lungs by infection of Vero cells. These results suggest that APMV6-455 is non-pathogenic for mice and does not replicate actively in the lungs of mice.

Efficient genome replication of members of the family *Paramyxovirinae* depends on the genome nucleotide length. It should be an even multiple of six, and this principle is known as the ‘rule of six’ [6]. The complete genome of teal/Chany/455/2009 has been sequenced and found to be 16,236 nt in length and thus follows the “rule of six”. According to multiple alignment of nucleotide sequences, all duck/Hong Kong/18/199/77-like viruses are characterised by a 16,236-nt genome length. Viruses of another subgroup are characterized by 16,230-nt genome length. This genome consists of seven genes in the order 3’N-P-M-F-SH-HN-L5’. The F protein cleavage site is the monobasic site P-E-P-R↓L, which is detected in the other strains of duck/Duck/Hong Kong/18/199/77 subgroup.

In our study, we have identified the first APMV-6 isolate from teal (*Anas crecca*) in Siberia. We conducted whole-genome sequencing and detailed analysis. APMV-6-455 falls into the phylogenetic group of duck/Hong Kong/18/199/77-like viruses (Fig. 2) but forms a subgroup separated from duck/Hong Kong/18/199/77, duck/Taiwan/Y1/1998 and goose/FarEast/4440/2003 (an APMV-6 strain isolated earlier in Russia). APMV-6-455 is closely related to the Kazakh and Chinese strains red-crested pochard/Balkhash/5842/2013, mallard/Jilin/127/2011 and mallard/Jilin/190/2011 according to phylogenetic analysis of full-genome and single gene sequences. The phylogenetically investigated strain isolated in 2009 is more closely related to red-

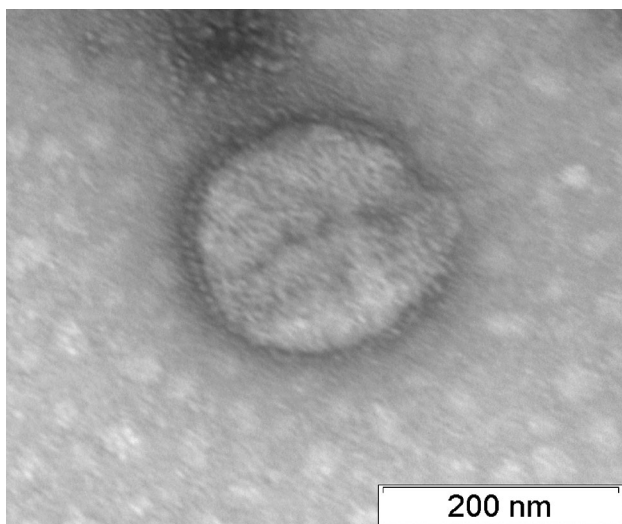


Fig. 1 Electron microscopy image of a virus particle of avian paramyxovirus serotype 6 strain teal/Chany/455/2009. The particle is ~220 nm in diameter

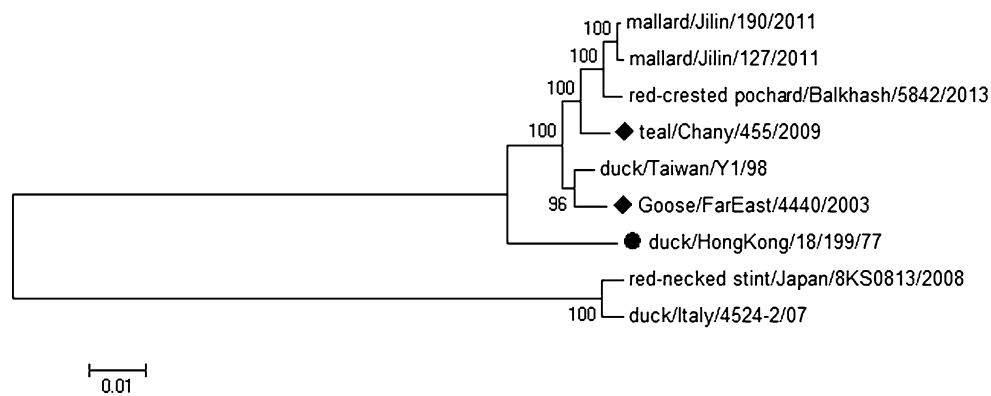


Fig. 2 Phylogenetic analysis of full-length genome sequences of APMV-6 isolates. Trees were constructed by bootstrap analysis (1,000 replicates) using the MEGA 5 program. The neighbor-joining statistical method (model: maximum composite likelihood) was used.

crested pochard/Balkhash/5842/2013 than to Chinese strains from 2011. This is probably because the Chany lake system and Balkhash Lake are geographically close and connected by migratory flyways.

We calculated p-distances between all APMV-6 strains of the duck/Hong Kong/18/199/77-like group and found that some strains differed at the nucleotide level but were closely related or identical at the amino acid level. In particular APMV-6-455 was identical to duck/Taiwan/Y1/1998 in the aa sequences of the NP, M and F proteins, and aa sequences of other strains, excluding duck/Hong Kong/18/199/77, were related to each other. At the next step of amino acid sequence analysis, the number of substitutions per 100 aa between strains was calculated for different proteins and for full-genome sequences. We found that the proteins accumulated substitutions in different amounts. The proteins ranked in the order NP-F-M-L-HN-P-SH in increasing number of substitutions per 100 aa. Major changes in the amino acid sequences have occurred for 21 years between duck/Hong Kong/18/199/77 and duck/Taiwan/Y1/1998, as indicated by analysis of the p-distance and number of substitutions per 100 aa.

For the surface proteins F, SH and HN, an analysis of the pattern of amino acid substitutions was performed. Different types of substitution positions were found. The SH protein is characterized by a high frequency (7.04 per 100 aa) of mutations common to all strains in the HK-like genetic group, distinguishing them from the prototype strain duck/Hong Kong/18/199/77. Furthermore, the SH protein contains more (2.82 per 100 aa) variable positions of amino acid substitutions than other surface proteins of APMV-6. The HN protein is characterized by a high frequency of sporadic/unique substitutions (5.4 per 100 aa) and substitutions detected in different groups of strains (1.5 per 100 aa), and F protein characterized by low frequency of amino acid substitutions.

(a), phylogenetic tree for the full-length genome sequence; (b), phylogenetic tree for the NP gene; (c), phylogenetic tree for the P gene; (d), phylogenetic tree for the M gene

Because of the spread of many infections with increasing pathogenic potential that can become dangerous (SARS, MERS, influenza), we evaluated the pathogenic potential. We found the F protein cleavage site to be the monobasic site P-E-P-R↓L, which is marker of avirulent APMV. Our results of pathogenic tests suggest that APMV6-455 is apathogenic. Similar observations have been reported for other APMV-6 serotypes isolated from wild ducks [2, 7]. The MDT and study of infected embryonated chicken eggs confirmed that APMV6-455 is avirulent for chickens.

We used BALB/c mice as an animal model that supports replication of all of the APMVs in the respiratory tract except for APMV-5, which failed to cause detectable infection [8]. Viral replication occurred mostly in the upper and lower respiratory tracts. We found no significant replication in organs and no clinical signs of disease or death. Together with of ICPI and IVPI tests on chickens, the results suggest that APMV6-455 has low pathogenic potential. Such results obtained using this animal model should be useful for risk assessment of APMV-6.

The isolation of avian paramyxovirus from migratory waterfowl is sporadic and is often done in the context of other monitoring programs, such as those for avian influenza viruses (AIVs). AIV and APMV-1 (NDV) have frequently been detected in migratory birds, which is recommended for disease control in domestic birds [9–11]. However, reports on isolation of other APMV serotypes from these birds are limited in number [12].

We isolated the first APMV-6 strain from one of the most numerous species – common teal (*Anas crecca*) – in a hotspot for Avian Diseases Surveillance in Asia – the Chany lake system [13–15]. Siberia, with the Chany lakes system, is one of the most important breeding and staging areas for migratory waterfowl in North Eurasia [16]. Hundreds of thousands of waterfowl of numerous species

from multiple flyways converge in and disperse from this region annually; therefore, this region may be a key area for potential intra- and interspecific spread of infectious pathogens among migratory waterfowl in Eurasia. A similar picture can be observed on the North American continent [17]. The common teal is one of the most numerous species in Siberia. Teals breeding in southwestern Siberia form very large flocks on Chany Lake for moulting before they depart to wintering areas, mostly in a westward direction. The main wintering areas appear to be situated in Western Europe, the Mediterranean and Caspian Sea areas, and India and Pakistan [16].

There are a limited number of available APMV-6 sequences, and it is therefore not possible to analyse the phylogeography of the virus. However, it is most likely that the virus is associated with populations of wild aquatic birds, mostly ducks of the genus *Anas*. This is confirmed by the known isolates isolated from ducks in Europe and our phylogenetic analysis. As in this study, APMV-6 is isolated more rarely from ducks of the genus *Anas* [17–19] than from migrating birds gulls and shorebirds, for which isolation of APMV-1, -4, and -6 has been reported [20]. Isolation of APMV-6 from shorebirds has been reported in Germany with a prevalence of 2.4 % [21]. Because ducks are highly migratory, crossing Eurasia in different directions twice a year during the long migrations [18, 19], they present a possible mechanism for the global movement of these viruses. For teal, the most important route is the Black Sea/Mediterranean Flyway, which suggests probable circulation of the virus in populations associated with it. On the other hand, we have identified the virus at the point of the largest crossing of migration routes in Eurasia in the breeding season, with a high level of contact of molting birds from different populations and ways to contact and transmit the virus. Our phylogenetic analysis confirmed this.

Nucleotide sequence accession number. The complete genome sequence of APMV-6/teal/Chany/455/2009 has been deposited in GenBank under the accession no. KT962980.

Compliance with ethical standards

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Conflict of interest The authors have no conflict of interest to declare.

Ethical approval All applicable international guidelines for the care and use of animals were followed. All procedures performed in studies involving animal models were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration. Animal experiments were approved by the Committee on Biomedical Ethics, Research Institute of Experimental

and Clinical Medicine, Russian Academy of Sciences, Novosibirsk (Protocol no. 27 from 19.11.2015).

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