UDC 578.832.1:578.4

NOVEL AVIAN PARAMYXOVIRUSES AMONG WILD BIRDS IN KAZAKHSTAN

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ABSTRACTS

This article presents the results of paramyxovirus monitoring in the avifauna of Kazakhstan from 2002 - 2015. New data were obtained on the epidemiology, evolutionary variability and phylogenesis of paramyxoviruses. Seventy paramyxovirus isolates belonging to serotypes PMV-1, PMV-4, PMV-6, PMV-8, PMV-13, PMV-16 and PMV-20 were isolated from feral birds in Kazakhstan. Viruses were isolated from 23 avian species belonging to six families in five orders: Anseriformes (swans, geese, and ducks), Charadriiformes (snipes and gulls), Falconiformes (eagles), Columbiformes (doves and pigeons), and Passeriformes (crows). Novel results showed that avian paramyxoviruses PMV-13 and PMV-20 were phylogenetically distant from each other and from the other serotypes. They formed separate branches showing different evolutionary origins from other circulating viruses in the territory of the Republic. These findings form an essential part of the study of avian viral biology and provide insight into methods for control and prevention of viral infections in avifauna. Viral monitoring should be carried out on an ongoing basis.

Key words: paramyxovirus, avulavirus, bird, order, family, species, subtype, genome, sequencing, phylogeny.

INTRODUCTION

Avian paramyxoviruses are RNA-containing viruses belonging to the genus *Avulavirus* of the family *Paramyxoviridae*, capable of causing diseases with various clinical manifestations in the majority of wild and domestic birds. Up to 2010, nine antigenically different serotypes (PMV-1-PMV-9) were known, which were then supplemented with three new serotypes of PMV-10, 11 and 12 [1, 2, 3, 4].

Currently, research on APMV is widely carried out in various regions of the world, so a large program is implemented out within the framework of the European Network of Excellence (EPIZONE) with the participation of many countries of the Old World. The isolation and description of new serotypes in Kazakhstan will make a significant contribution to the global research process.

APMV serotype 13 found in Japan, Kazakhstan and Ukraine was described in 2013 - 2015 [5, 6, 7]. In 2017 six novel APMV serotypes were announced: from ducks in Japan and Korea [8, 9], from shorebird in Brazil [10], and three novel serotypes were simultaneously isolated from Antarctic penguins [11]. These data suggest that APMVs circulate widely in wild populations and that there is a high likelihood of novel genetically distinct variants emerging.

Among avuloviruses, the most prevalent is Newcastle disease virus (NDV), belonging to PMV-1, capable of causing epizootics with high mortality, up to the death of all infected livestock of both domestic and wild birds [1]. Representatives of other serotypes cause diseases of respiratory and reproductive organs of lower severity in birds [12].

The APMV-1 genome is a single-stranded negative polarity RNA encoding eight proteins: surface proteins are haemagglutinin-neuraminidase (HN), fusion protein (F) and internal are RNA-dependent RNA polymerase (L), matrix protein (M), phosphoprotein (P) and nucleoprotein (NP), as well as proteins V and W. The role of each of these proteins in NDV replication has been well studied [13, 14, 15], but the most important amino acid sequence in the manifestation of virulence is the amino acid sequence of the F protein cleavage site [16].

Wild birds, mostly waterfowl, are known as the main reservoirs of the APMV-1, 4, 6, 8 and 9 in nature; they play a key role in their maintenance in the biosphere and are a potential natural source of new dangerous variants of viruses [1].

The molecular characterization of the entire genome of the APMV by the *Sanger* sequencing remains technically complicated since these viruses are poorly represented in the Genebank database, which makes it difficult to find conservative regions and design of overlapping primers. Numerous studies on the sorting out of genomes of of APMV-2 [17], APMV-3 [18], APMV-4 [19], APMV-5 [20], APMV-6 [21], APMV-7 [22], APMV-8 [23], APMV-9 [24] reference strains made a significant contribution to the understanding of genome organization of the representatives of genus *Avulavirus* and showed their considerable diversity within serotypes.

Seventy isolates of APMV of the serotypes APMV-1, APMV-4, APMV-6, APMV-8, APMV-13, APMV-16, APMV-20 were isolated in Kazakhstan upon long-term ecological-virological studies. The viruses are isolated from birds of 20 species of six families of orders *Anseriformes* (Swans, Gees, and Ducks), *Charadriiformes* (Snipe, Gulls), *Falconiformes* (Eagles), *Columbiformes* (Doves, Pigeons), *Passeriformes* (Crows).

The most common in the wild avifauna of Kazakhstan, as well as throughout the world, were APMV-1 (41 isolates), then APMV-4 (13), APMV-8 (8), APMV-13 (4), APMV-6 (3), APMV-16, APMV-20.

In the autumn of 2015, during an outbreak of acute infectious disease among the vaccinated domestic chickens in the Almaty region, 12 hemagglutinating agents were isolated, which were attributed to APMV-1 according to the results of the haemagglutination inhibition (HI) test. At the same time, in the same region, three velogenic isolates of NDV were isolated from synanthropic birds (crows, pigeons) [25].

In autumn 2014, ten isolates APMV-1 were isolated from wild birds in South Kazakhstan (Chokpak Pass) and identified using the recommended multiplexes RT-PCR and HI test. The results suggest a possible relationship of epizootic outbreaks of Newcastle disease in populations of wild, synanthropic and domestic birds.

During of ecological and virological studies APMV-4, APMV-6, APMV-8 were isolated from the representatives of *Anseriformes* and *Charadriiformes* (*Anatidae* and *Scolopacidae* families, respectively) in 2002-2013 for the first time on the territory of the Republic of Kazakhstan. According to the results of genetic studies, the Kazakhstan's APMV-1 strains showed a close relationship with the European variants. The isolates of APMV-4 of 2003, as well as APMV-6 and APMV-8, isolated in 2013, were 99% identical with viruses of these serotypes from the Far East [26].

The circulation of APMV-4, APMV-6 was detected on the coastal area of Caspian Sea; the prevalence of APMV-1, APMV-4, APMV-6, APMV-8, APMV-13 among wild birds in the

south-eastern regions of the republic is shown. The avifauna of Northern and Central Kazakhstan is harboring of APMV-1, APMV-4, APMV-8, and APMV-13[6, 26, 27] .

It was shown that the genome of the Kazakh isolate APMV-6/red-crested pochard/Balkhash/5842/2013 is similar to that of the reference variant and contains 16236 nucleotides. Like other representatives of this serotype, it consists of seven genes arranged in the following order: *3'-NP-P-M-F-SH-HN-L-5 '*, and differs from other serotypes by the content of the additional gene encoding the small hydrophobic (*SH*) protein [28].

Analysis of the nucleotide sequences of the Kazakhstan isolate genes showed its 99% similarity by three internal (*M, HN* and *L*) genes with the APMV-6/mallard/Jilin/127/2011 virus. For surface *HN* and *F* genes, the closest relatedness is also noted with this virus.

Molecular analysis of the nucleotide sequence of the F-gene of the Kazakhstan strain APMV-6/red-crested pochard/Balkhash/5842/2013 showed the presence of unique substitutions in the positions C651T, A726G, T1155C, A1377G and T1431C. The revealed differences indicate that the Kazakhstan strain APMV-6/red-crested pochard/Balkhash/5842/2013 differs from the reference strain (APMV-6/duck/HongKong/18/199/1977) and other APMV-6 viruses and is a novel natural variant [28].

Subsequently, based on the nucleotide sequences of the *F* gene, the amino acid compositions of functionally active cleavage sites of the fusion proteins of strains from GenBank and Kazakhstan isolate were identified. It is shown that all of them are similar to each other and have in this area the PEPR↓L sequences characteristic of low pathogenic viruses.

Analysis of the nucleotide sequences of the Kazakhstani isolate APMV-8/white-fronted goose/Northern Kazakhstan/5765/2013 with those of APMV-8/goose/Delaware/1053/76 from GenBank showed a high degree of their relationship (99%) in *M, L, HN* and *F* genes, the similarity in the *NP* gene reached 98%.

The sequence length of the five newly generated APMVs was identical to the two previously reported strains, goose/Delaware/1053/76 and pintail/Wakuya/20/78 (GenBank accession numbers FJ215863 and FJ215864, respectively): each of which consists of 15,342 nucleotides. In general, the genomes of APMV-8 strains (and APMV-1, -2, -3 and -4) consist of six tandemly linked genes in the order of *3′-NP-P-M-F-HN-L-5′* yet lack the *SH* protein gene present in APMV-6 [27].

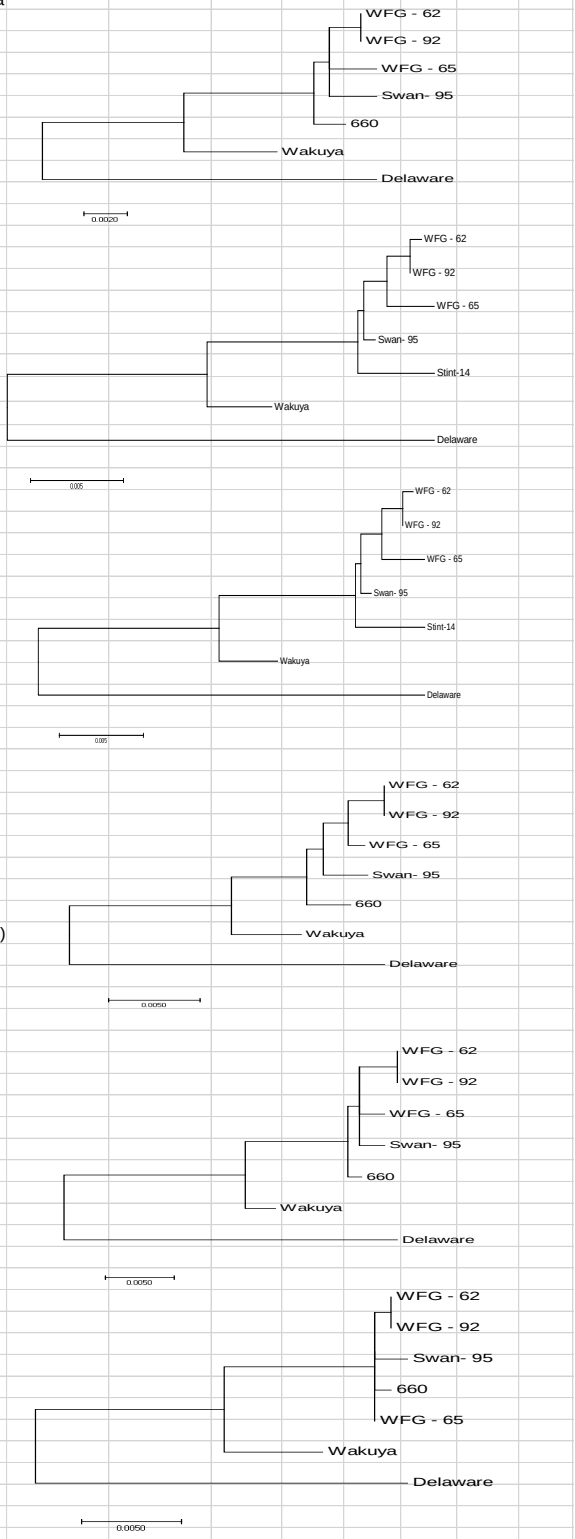
The five new isolates revealed several genetic differences amongst themselves. Two isolates, WFG-62 and WFG-92, showed the highest similarity, with only 3 nucleotide differences in the whole genome, but the other three viruses differed from 51 to 99 nucleotides.

The extragenic 3`-leader and 5`-trailer regions contain the conserved promoter sequences. Therefore, it is not surprising that the 3′-leader region (comprising 55 nucleotides) of the five isolates from Kazakhstan were identical to that of the Wakuya strain. However, the Delaware strain differed by a single nucleotide at position 52. Furthermore, the 5′-trailer region of the Kazakhstan strains showed no variability among the three White-fronted goose isolates but 1 and 3 nucleotide differences were observed when compared to the sequences of the Whooper Swan and the Little Stint strains, respectively [27].

On the basis of comparative phylogenetic analysis, the presence of a separate cluster of APMV-8 was established. It was shown that APMV-8/white-fronted goose/Northern Kazakhstan/5765/2013 differs in evolution from the other viruses of this serotype and formed a separate branch within this cluster.

The phylogenetic tree generated from the complete genome sequences perfectly reflects the trees for the single genes (figure 1).

		WFG-62	WFG-92	WFG-65	Swan-95	Stint-14	Wakuya
NP	WFG-92	0 (0)					
	WFG-65	5 (0)	5 (0)				
	Swan-95	5 (1)	5 (1)	5 (1)			
	Stint-14	5 (1)	5 (1)	6 (1)	6 (2)		
	Wakuya	17 (1)	17 (1)	18 (1)	18 (2)	16 (2)	
	Delaware	40 (3)	40 (3)	41 (3)	40 (4)	39 (4)	35 (3)
	17.57 (1.67)						
	0.049						
F	WFG-92	1 (0)					
	WFG-65	7 (1)	6 (1)				
	Swan-95	6 (1)	5 (1)	7 (2)			
	Stint-14	12 (2)	11 (2)	13 (3)	8 (3)		
	Wakuya	23 (2)	22 (2)	24 (3)	19 (3)	23 (4)	
	Delaware	56 (5)	55 (5)	51 (4)	54 (6)	57 (7)	47 (3)
	24.14 (2.86)						
	0.055						
HN	WFG-92	2 (2)					
	WFG-65	10 (2)	8 (0)				
	Swan-95	10 (5)	8 (3)	12 (3)			
	Stint-14	16 (7)	14 (5)	18 (5)	13 (5)		
	Wakuya	32 (7)	30 (5)	34 (5)	30 (6)	30 (6)	
	Delaware	64 (9)	62 (7)	66 (7)	62 (8)	64 (8)	56 (8)
	30.52 (5.38)						
	0.066						
L	WFG-92	0 (0)					
	WFG-65	19 (4)	19 (4)				
	Swan-95	37 (9)	37 (9)	31 (7)			
	Stint-14	44 (8)	44 (8)	37 (6)	38 (3)		
	Wakuya	79 (16)	79 (16)	72 (14)	73 (11)	67 (10)	
	Delaware	200 (26)	200 (26)	194 (24)	191 (21)	183 (20)	176 (20)
	86.67 (12.48)						
	0.051						
M	WFG-92	0 (0)					
	WFG-65	5 (0)	5 (0)				
	Swan-95	5 (2)	5 (2)	4 (2)			
	Stint-14	5 (0)	5 (0)	4 (0)	4 (2)		
	Wakuya	14 (1)	14 (1)	13 (1)	13 (3)	11 (1)	
	Delaware	41 (4)	41 (4)	40 (4)	40 (6)	40 (4)	36 (5)
	16.43 (2)						
	0.06						
P	WFG-92	0 (0)					
	WFG-65	1 (0)	1 (0)				
	Swan-95	3 (0)	3 (0)	2 (0)			
	Stint-14	2 (1)	2 (1)	1 (1)	3 (1)		
	Wakuya	16 (8)	16 (8)	15 (8)	17 (8)	16 (9)	
	Delaware	43 (18)	43 (18)	42 (18)	44 (18)	43 (19)	39 (14)
	16.76 (7.14)						
	0.036						



The tables of the left side represent the number of nucleotide and amino acid (in bracket) variability among isolates, including the overall mean distance. The phylogenetic trees for each gene were represented at the right side.

Fig. 1. Genetic and phylogenetic analyses of six genes of five Kazakhstan APMV-8 and two previously reported isolates.

The tree clearly indicates the very close genetic relationship between the different APMV-8 strains isolated from wild birds in Kazakhstan and show very few genetic variations. The

APMVs isolated from the White-fronted Geese show a closer relationship among themselves when compared to the strains isolated from the Whooper Swan and the Little Stint. In addition, the APMV-8 isolated from the Whooper Swan is more closely related to the strains from the White-fronted geese than to that of the Little Stint.

During the monitoring of pathogens of viral infections in wild bird populations in 2013, the staff of the laboratory of virus ecology of the Institute of Microbiology and Virology managed to isolate hitherto not identified APMV's novel serotype from the White-fronted Goose in Northern Kazakhstan [6]. The authors obtained the complete nucleotide sequence of the genome of isolate designated as APMV-13/White-fronted Goose/Northern Kazakhstan/5751/2013, which is available in the GenBank database under No. KU646513.

CONCLUSION

As a result of molecular analysis of the Kazakhstan isolate APMV-13, the nucleotide sequences of all genes were identified in the following order: *3'-NP-P/V/W-M-F-HN-L-5 '*, which encode eight proteins: NP (493 amino acid residues [aa]), P (397 aa), V (241 aa), W (150 aa), M (366 aa), F (545 aa), HN (549 aa), and L (2,199 aa).

Phylogenetic studies showed that APMV-13 forms a monophyletic clade with serotypes APMV-1, -9, and -12. Inside this cluster, APMV-13 forms a pair with APMV-12.

Three isolates APMV/gull/Kazakhstan/5976/2014, APMV/gull/Kazakhstan/ 5977/2014 and APMV/gull/Kazakhstan/5979/2014, were obtained from independent samples during annual surveillance for avian influenza and paramyxoviruses in wild birds from the Caspian Sea coast in Western Kazakhstan, and were initially identified as putative paramyxoviruses on the basis of electron microscopy. HI test with antisera to nine known APMV serotypes (APMV1-9) indicated no relation to any of them. Next generation sequencing of whole genome sequences indicated the three isolates were genetically identical, and had a nucleotide structure typical for all APMVs, consisting of six genes 3'-NP-P-M-F-HN-L-5'. Phylogenetic analyses, and assessment of amino acid identities, suggested the most closely related lineages to be APMV-2, 8, 10 and 15, but the novel isolate had less than 64% identity to them and all other known avian paramyxoviruses. This value was above levels considered to generally define other APMV serotypes. Estimates of the evolutionary divergence of the nucleotide sequences of the genomes of APMVs have shown that novel Kazakhstan APMV strain was closest to APMV-2, APMV-8, APMV-10 and APMV-15, with calculated distance values of 2.057, 2.058, 2.026 and 2.286 respectively, which is above values considered to differentiate other serotypes (observed minimum was 1.108 between APMV-1 and recently isolated APMV/UPO216/Korea) [29]. Together, the data suggest that isolate APMV/gull/Kazakhstan/5976/2014 and other two should be considered as the first representative of a novel APMV-20 group, and is the first time that avian paramyxoviruses have been found infecting members of the gull family, extending the known taxonomic host range [29, 30].

As a result of molecular genetic studies, data on the circulation in Kazakhstan of novel for scientific community APMV serotypes 13 and 20 were confirmed (according to the new taxonomic classification from 2017) [31].

Acknowledgements

This work was supported by the Ministry of Education and Science of the Republic of Kazakhstan (grant No. AP05133370).

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