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ISOLATION AND MOLEKULAR-GENETIC CHARACTERISTICS OF THE NOVEL AVIAN PARAMYXOVIRUS APMV-13

Abstract. The article presents the data on the isolation, identification and phylogenetic analysis of the novel avianparamyxovirus(APMV) serotype. Eighteen positive samples of APMVwere obtained during reverse transcription-polymerase chain reaction screening of 204 samples collected in five regions of Kazakhstan. The sequencing results of the L-gene fragment and BLAST analysis indicated on circulation of previously unknown avian paramyxovirus novel serotype in the populations of wild birds of Kazakhstan. Full genome sequencing of the isolate APMV-13/white-fronted goose/North Kazakhstan/5751/2013 was performed on the next generation sequencing platformHiSeq 3000 (Illumina). The sequence of genes was determined as 3'-NP-P/V/W-M-F-HN-L-5′, encoding eight proteins characteristic to the avian paramyxoviruses. Phylogenetic studies have shown that the avian paramyxovirus serotype 13is a novel natural variant, significantly different from other serotypes.

Key words: paramyxovirus, APMV-13, polymerase chain reaction, gene, sequencing, phylogenetic analysis.

Introduction. Avian paramyxoviruses (APMV) are RNA-containing viruses that form the *Avulavirus* subfamily belonging to the *Paramyxoviridae* family and can cause diseases with different clinical manifestations in most species of wild birds. According to the new classification, *Avulaviruses* on the basis of phylogenetic differences are divided into three genders –*Metaavulavirus, Orthoavulavirus, Paraavulavirus*. Until 2015, twelve serotypes of APMV (APMV-1-12) were known [1-4].

In 2015-2017 the reports were published about the discovery of seven novel serotypes of the APMV: from wild geese in Japan [5], Kazakhstan [6] and Ukraine[7], threefromducks in Japan [8], Korea [9] and from sandpiper in Brazil [10]; three more viruses were simultaneously isolated from antarctic penguins [11]. These data suggest that APMV are actively circulating in the wild avifauna and there is a high probability of the occurrence of other pathogenic variants.

To date, study of APMVs is widely conducted in various regions of the world, so a large program is carried out within the framework of the European network of excellence (EPIZONE) with the participation of many Old World countries.

Isolation and description of novel serotypes in the territory of Kazakhstan will make a significant contribution to this research.

The aim of the paper is to describe APMVs of novel serotypes circulating in Kazakhstan avian populations, to study their virological and molecular genetic features.

Materials and methods. For virological studies, samples were collected in the form of cloacal, tracheal washings from birds of water and near-water complexes. The washes were collected with a sterile cotton swab, placed in vials of medium 199 containing a complex of antibiotics (penicillin 2000 U/ml, streptomycin 2 mg/ml, gentamicin 50 μg/ml, nystatin 50 U/ml) and bovine serum albumin (0.5%/ml). For the droppings and cloacal swabs, the concentration of antibiotics was fivefold increased. Samples before virological studies were stored in liquid nitrogen (-196 °C).

Isolation and recovering passages were carried out by inoculation of each sample of the test material into the allantoic cavity of three 10-11 day embryonated chicken eggs (ECE) and then incubating at 35°C for 48-72 hours. Allantoic fluids for the presence of the virus were checked in hemagglutination(HA) test using a 0.75% suspension of chicken red blood cells. The infectious titer was calculated by the Reed-Muench method [11] and expressed in lg of $EID_{50}/_{0,2ml}$.

For removing of non-specific inhibitors of agglutination, the sera were pretreated with a receptor-destroying enzyme (RDE) from V. Cholerae filtrate (Denka Seiken Co., Ltd. Tokyo, Japan). To 1 part of undiluted serum 3 volumes of RDE were added at a working dilution of 1:50. The mixture was left at 37°C for 18 hours, then 6 parts of physiological saline was added to obtain the final dilution of the serum (1:10), and then heated at 56° C for 30 minutes.

The serotypes of APMV isolates were established in the hemagglutination inhibition (HI) test [12] with a panel of polyclonal diagnostic sera directed to: APMV-1/chicken/La Sota/46; APMV-2/Chicken/Yucaipa/56; APMV-3/Turkey/Wisconsin/68; APMV-4/duck/Hong Kong/D3/75; APMV-5/Budgerigar/Japan/Kunitachi/1975; APMV-6/duck/Hong Kong/199/77; APMV-7/dove/Tennessee/4/75; APMV-8/goose/Delaware/1053/76; APMV-9/duck/New York/22/78 provided by prof. M. Lipkind (Kimron Veterinary Institute, Beit-Dagan, Israel), additionally were updated from the National Reference Laboratory for the NDV, Friedrich-Loffler Institute, InselRiems, Germany.

RNA isolation was performed using a QIAamp Viral RNA Mini kit (Qiagen GmbH, Hilden) in accordance with the manufacturer's recommendations. RNA was extracted from 140 µl of clinical samples and eluted in a final volume of 50 µl.

The cDNA was prepared by reverse transcription reaction using the universal random hexamer primer.

Analyzes of reverse transcription PCR (RT-PCR) were performed on the basis of a one-step protocol using the appropriate RT-PCR kit (AccessQuick One-Step RT-PCR Kit, Promega) according to the manufacturer's instructions using a Pan-paramyxovirus primer to L-gene [14].

The reaction was carried out in an Eppendorf Gradient thermocycler with the following parameters: reverse transcription at 48 °C for 45 min, initial 2 min denaturation at 95 °C and amplification in 30 cycles, including denaturation (94 °C, 30 sec), primer annealing (55 °C, 30 sec) and chain extension (72 °C, 30 sec) followed by final elongation at 72 °C, 10 min.

DNA sequencing was performed using termination dideoxynucleotides on an automatic 8-capillary sequencer ABI 3500 DNA Analyzer (Applied Biosystems, USA).

For the sequencing of viral RNA on a HiSeq device (Illumina, USA), a double-stranded cDNA, which was synthesized using the RiboClone (Promega, USA) kit, was used as the template. For fragmentation of the cDNA to a size of about 250 b.p. the enzymatic method using transposase from the Nextera XT Library Preparation Kit (Illumina, USA) was used. In preparing the library of fragmented DNA, Illumina adapters were used. The quality of the prepared libraries was checked on the Bioanalyzer 2100 (Agilent Technologies, Germany). Sequencing was performed using the MiSeq Reagent v.2 kit (Illumina, USA). The resulting sequences were collected and analyzed using UGENE 1.20 software (Russia).

A TruSeq Stranded Total RNA kit with Ribo-Zero (Illumina, USA) was used to sequence viral RNA on a high-performance HiSeq 3000 device (Illumina, USA), according to the manufacturer's recommendations.

Alignment and phylogenetic analysis of sequenced genes with nucleotide sequences from Genebank was carried out using the computer program MEGA 6.0 by the method of attaching neighbors based on 1000 samples, model Tamura-Nei.

Results. Virological screening of 204 biological samples (cloacal and tracheal swabs) collected from165 bird individuals of *Anatidae, Laridae, Scolopacidae* and *Charadriidae* families of the orders *Anseriformes* and *Charadriiformes*in West, South and Central Kazakhstan in 2013was carried out to identify APMVserotypes.

APMV isolates were cloned by inoculation of 10-11 day-oldECEwith virusdiluted from 10^{-1} to 10^{-7}. The titer of virus-containing allantoic fluid in HA test at a dilution of 10^{-6} was 1:128 t - 1:512. For further molecular studies RNA was isolated from the virus suspension purified through a sucrose density gradient.

As a result of primary inoculation of 10-11 day-oldECE with samples, 20 hemagglutinating agents were isolated. PCR identification with primers to the conserved fragment of the L-geneallowed 15 agents to be assigned asAPMV.

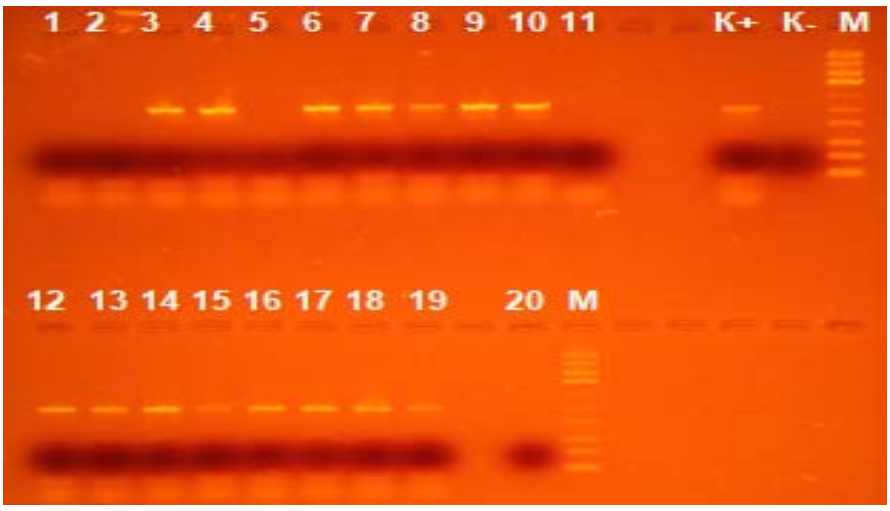
Table represents the results of HI test of APMV isolates with homologous and reference diagnostic sera.

Hemagglutinationassay results of APMV isolates from wild birds with hyperimmunerabbit and reference sera

Isolate	Immuneserumtostrain:									
	APMV-1	APMV-2	APMV-3	APMV-4	APMV-5	APMV-6	APMV-7	APMV-8	APMV-9	APMV-**13**/ WFG /North KZ/5751/2013
APMV-13/WFG*/ North Kazakhstan /5750/2013	80	0	0	0	0	0	0	0	40	**320**
APMV-13/WFG/North Kazakhstan /5751/2014	80	0	0	0	0	0	0	0	40	**320**
APMV-13/WFG/North Kazakhstan/5753/2014	80	0	0	0	0	0	0	0	40	**320**
APMV-13/pintail/North Kazakhstan/5759/2014	80	0	0	0	0	0	0	0	40	**320**
*White fronted goose.										

As can be seen from Table, the hemagglutinating activity of the Kazakhstan APMV isolates, including APMV-13/white-fronted goose/North Kazakhstan/5751/2013, were inhibited by homologous immune serum (1: 320),and they did not react or reacted in low titers with reference sera against to viruses of serotypes 1-9.

As a result of PCR specific 700 b.p.products of paramyxovirus L-gene were amplified in 15 samples.



Note: "M" is the DNA marker; "K +" - positive control; K- »- negative control; No. 1-20 of the sample number.

Figure 1 – Results of PCR with RNA from materials from wild birds of Western Kazakhstan

Sequencing of L-gene amplification products and subsequent BLAST analysis in GenBank indicated the belonging of four of them to APMV-1, six to APMV-8 and one to APMV-6. Sequence analysis of the four remaining unidentified APMV isolates of 2013 showed their significant genetic divergence by conservative fragment of the L gene with the known serotypes of the APMV (figure 2), suggesting that novelhither to unidentified APMV circulate in waterfowls of Kazakhstan.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Max score	Total score	Query cover	E value	Ident	Accession
Avian paramyxovirus 12 isolate Wigeon/Italy/3920_1/2005, complete genome	289	289	91%	3e-74	73%	KC333050.1
Newcastle disease virus isolate chicken/BYP/Pakistan/2010, complete genome	143	143	91%	2e-30	67%	JN682210.1
Newcastle disease virus isolate NDV2K35/CH/TN/2003, complete genome	140	140	91%	3e-29	67%	KF740478.1
Newcastle disease virus strain cormorant/US(WI)/18719-03(USGS)/2003, partial genome	140	140	91%	3e-29	67%	GQ288385.2
Pigeon paramyxovirus 1 strain PPMV-1/Belgium/03-05843/2003, partial genome	138	138	66%	1e-28	70%	JX901118.1
Newcastle disease virus isolate chicken/CP/Pakistan/2010, complete genome	134	134	91%	1e-27	67%	JN682211.1
Newcastle disease virus isolate 2009_Mali_ML008, complete genome	132	132	83%	4e-27	67%	JF966387.1
Newcastle disease virus strain chicken/Sukorejo/019/10, complete genome	131	131	91%	2e-26	66%	HQ697255.1
Newcastle disease virus strain cormorant/US(CA)/92-23071/1997, partial genome	131	131	91%	2e-26	67%	GQ288388.2
Newcastle disease virus strain cormorant/Canada/95DC2345/1995, partial genome	131	131	91%	2e-26	67%	GQ288384.2

Figure 2 – BLAST-analysis of nucleotide sequences of unidentified Kazakhstan isolate APMV/White-fronted goose/North Kazakhstan/5751/2013

Analyzing of the L-gene of unidentified isolate APMV/White-fronted goose/North Kazakhstan/5751/2013) demonstratetheir most similarity (73%) to the referencestrain of APMV serotype 12 [13], with the remaining viruses from Genbank, the divergence index was more than 33%, which presumably attributed this strain to thenovel serotype.

In bioinformatic analysis, the obtained sequences were preliminarily assembled using the CLC Assembly Cell software (Qiagen, USA), (figure 3).

Figure 3demonstrates that the nucleotide sequences of all six APMV-13 genes were obtained in the following order: 3'-NP-P/V/W-M-F-HN-L-5 ', which encode eight proteins: NP (493 amino acids (aa), P (397 aa), V (241 aa), W (150 aa), M (366 aa), F (545 aa), HN (549 aa), and L (2199 aa).

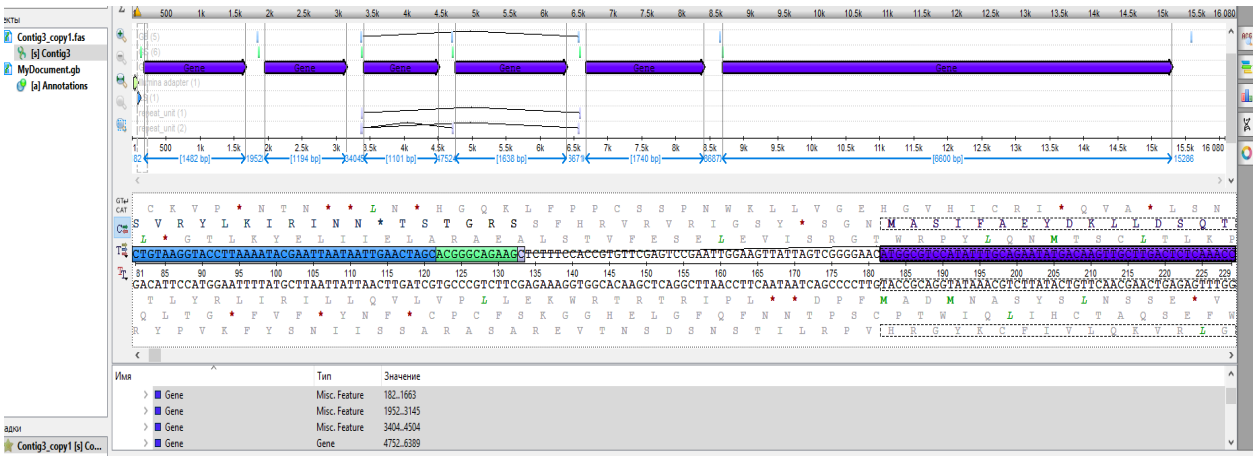


Figure 3 – View of full sequenced genome of APMV-13/ white-fronted goose/North Kazakhstan /5751/2013 in UGENE program

Next Generation sequencingof full genome of isolates and subsequent BLAST analysis identified as novel APMV serotype13.

The results of phylogenetic analysis of novel Kazakhstan APMV with representatives of serotypes 1-12 from GenBankare shown in figure 4.

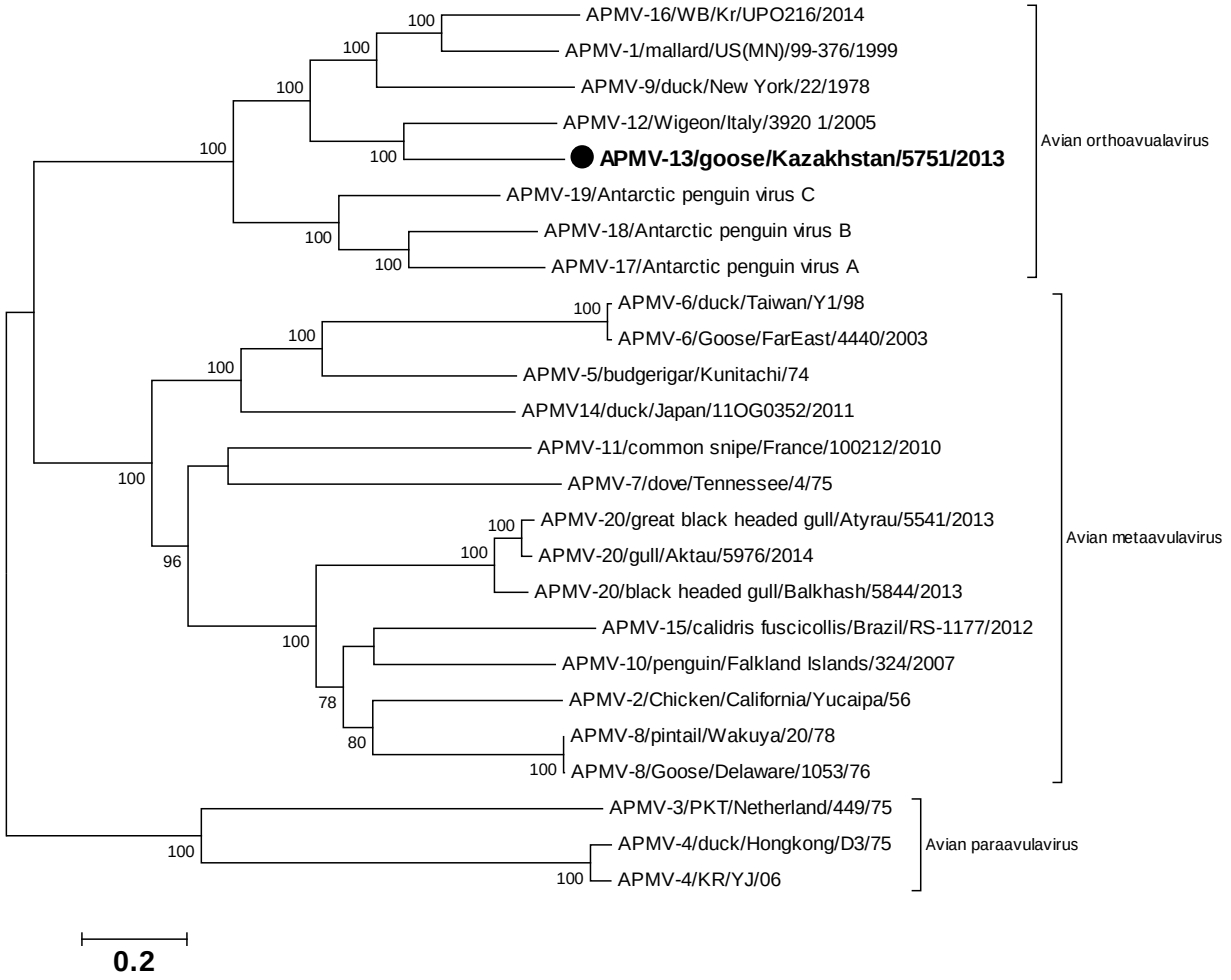


Figure 4 – Phylogenetic relationship of the novelavian paramyxovirus
APMV-13/White-fronted Goose/Northern Kazakhstan/5751/2013 with other avian paramyxoviruses serotypes

As it can be seen in Figure 4, the Kazakhstan isolate APMV-13, together with the APMVserotypes 1,9,12 and 16, formed a separate monophyletic group, within which the most phylogenetically similar was APMV-12, isolated in 2012 in Italy.

Thus, as a result of molecular genetic studies, data on the circulation of novel avian paramyxovirus serotype 13 were confirmed in Kazakhstan (according to the new taxonomic classification from 2017).

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ЖШС «Микробиология және вирусология ҒӨО», Алматы, Қазақстан

ҚҰС ПАРАМИКСОВИРУСТАРЫНЫҢ ҒЫЛЫМҒА ЖАҢА ПМВ-13 ТҮРІН БӨЛУ ЖӘНЕ МОЛЕКУЛАЛЫ-ГЕНЕТИКАЛЫҚ СИПАТТАУ

Аннотация. Мақалада құстардың жаңа серотүрін бөлу, ажыратып балау мен филогенетикалық талдау нәтижелері сипатталады. Қазақстанның бес облысынан жиналған 204 сынаманы кері транскрипция - полимеразды тізбекті реакция скринингтеу нәтижесінде 15 нұсқасы парамиксовирустарға оң нәтиже берді. L-генінің бөлігін секвендеу әдісімен және келесілік BLAST-талдау нәтижесінде Қазақстандағы түз құстары популяциясында ПМВ белгісіз түрінің айналымда жүргенін айғақтайтын мәліметтер алынды. Соңғы үлгідегі HiSeq 3000 (Illumina) секвенаторында қазақстандық APMV-13/white-fronted goose/North

Kazakhstan/5751/2013 бөліндісі геномын толық секвендеу жүргізілді. Құс ПМВ тән, сегіз ақуыз кодтайтын 3'-NP-P/V/W-M-F-HN-L-5' гендерінің тізбегі анықталды. Филогенетикалық зерттеу нәтижесі қазақстандық ПМВ жаңа 13-серотүрі табиғи жаңа нұсқа болып саналады және өзге серотүрлерден едәуір айырмашылығы бар.

Түйін сөздер: парамиксовирус, APMV-13, полимераздытізбекті реакция, ген, секвендеу, филогенетикалық талдау.

М. Х. Саятов, А. Б. Сейдалина, К. О. Карамендин, А. И. Кыдырманов, Е. Т. Касымбеков, К. Д. Даулбаева, Е. Я. Хан, С. А. Сулейменова, К. Х. Жуматов

ТОО «НПЦ микробиологии и вирусологии», Алматы, Казахстан

ИЗОЛЯЦИЯ И МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКАЯ ХАРАКТЕРИСТИКА НОВОГО ДЛЯ НАУКИ ПАРАМИКСОВИРУСА ПТИЦ APMV-13

Аннотация. В статье приведены результаты изоляции, идентификации и филогенетического анализа парамиксовируса (ПМВ) птичнового серотипа. При скрининге 204 образцов, собранных в пяти областях Казахстана в обратной транскрипции-полимеразной цепной реакции, обнаружены 18 положительных на ПМВ проб. Методом секвенирования фрагмента L-гена и последующего BLAST-анализа показана циркуляция в популяциях диких птиц Казахстана ПМВ птичнового ранее неизвестного серотипа. На секвенаторе нового поколения HiSeq 3000 (Illumina) проведено полногеномное секвенирование казахстанского изолятаAPMV-13/white-frontedgoose/NorthKazakhstan/5751/2013. Определена последовательность генов 3'-NP-P/V/W-M-F-HN-L-5', кодирующих восемь белков, характерных для ПМВ птиц. Филогенетические исследования показали, что казахстанский изолят ПМВ серотипа-13 является новым природным вариантом, значительно отличающимся от других серотипов.

Ключевые слова: парамиксовирус, APMV-13, полимеразная цепная реакция, ген, секвенирование, филогенетический анализ.

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