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APPLICATION OF MASSIVE PARALLEL SEQUENCING FOR THE INVESTIGATION OF WILD BIRDS VIRUSES

Abstract. Identification of viral pathogens is of great importance for the diagnostics of infectious diseases in humans and animals. Almost all outbreaks of dangerous infections in the last two decades have been caused by new viruses, most of which originated from a natural reservoir.

Experimental studies on avian paramyxovirus (APMV) of serotype 1 have shown that wild birds can spread and introduce mild or non-pathogenic virus variants into the poultry population, which, after several passages in the organism of susceptible birds, often acquire highly pathogenic properties.

Using the new technology of massive parallel sequencing, information on the genetic structure of wild bird viruses belonging to the *Paramyxoviridae* family was obtained. The high efficiency of the method is shown, which allows simultaneous sequencing of the complete genomes of viruses without prior knowledge of their belonging to any family. The data obtained will allow us to expand our knowledge of the course of the natural evolution of migratory bird viruses.

Keywords: Virus, Massive Parallel Sequencing, Wild Birds, Complete Genome of the Virus, RNA, DNA, Bioinformatic Analysis.

Introduction. Recent studies confirm the priority role of wild birds as a natural reservoir and the source of genetic material for the emergence of new epizootic variants of viruses.

Ecological and epizootological assessment of the state of viral populations in birds is important for practical veterinary medicine when defining the cause of the outbreaks and controlling emergent epidemic situations. Since Kazakhstan is located in the center of the Eurasian continent and important wild birds' migration flyways cross its territory, this can serve as a factor of introduction of new pathogenic variants of viruses, the study of the genetic diversity of viruses circulating in the organism of wild birds is an urgent issue.

To date, genetic studies of viruses with the sequencing of their genes have been conducted using the widely used and well-proven Sanger method. With the development of technology, new methods have emerged into the arena, which are gradually becoming routine in the world's scientific laboratories. One of these new methods is massive parallel sequencing, also called Next Generation Sequencing (NGS), which provides a high-performance analysis of huge amounts of data on the nucleotide sequences contained in the sample.

In order to study the capabilities of this technology in obtaining the complete genomes of wild bird viruses, unidentified hemagglutinating agents without preliminary knowledge of their belonging to a certain family of viruses were sequenced.

Materials and methods. Field samples were collected from wild and domestic birds according to the Office International des Epizooties (OIE) [1] and before researches were stored in liquid nitrogen (-196ºC).

Isolation of virus and recovery passages were conducted by the inoculation of each sample into the allantoic cavity of 9-10 days old Embryonated Chicken Eggs (ECE) and subsequently incubated at + 36ºC for 48 hours according to certified methods recommended by the OIE [2].

Viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden) according to the manufacturer's recommendations.

NGS libraries were prepared using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA). Complementary DNA from RNA was synthesized using random hexamer primers by reverse transcription. Sequencing was performed on Illumina MiSeq Sequencer (USA).

Bioinformatic analysis was performed using UGENE 1.20 (Russia) [3] and Tablet (UK) [4] software.

Alignment of gene sequences and phylogenetic analyses by Maximum Parsimony were carried out using MEGA 6.0 [5].

Results. A virological screening of samples from archival materials collected in Western, Southeast and Central Kazakhstan in different periods from wild aquatic birds belonging to the families of *Anatidae*, *Laridae*, *Scalopacidae* and *Charadriidae* of *Anseriformes* and *Charadriiformes* orders. As a result of inoculastion of samples into10-days-old ECE, haemagglutinating agents were isolated, of which RNA was isolated and their concentration was measured (table 1).

Table 1 – Initial RNA concentrations of virus isolates for sequencing

Hemagglutinating agent	Conc., ng/ul
chicken/Almaty/36/2015	100,0
pygmy cormorant/Kyzylkol/7074/2016	>8,0
barn swallow/ Kyzylkol /7079/2016	>8,0
mallard/ Korgaljyn /6769/2015	>8,0
great black headed gull/Atyrau/6452/2015	18,0
white fronted goose/Northern Kazakhstan /5751/2013	>8,0
white fronted goose /Northern Kazakhstan/5759/2013	26,3
white fronted goose /Korgaljyn/1791/2006	9,2
black headed gull/Balkhash/5844/2013	>8,0
great black headed gull /Atyrau/5541/2013	>8,0
gull/Aktau/5976/2014	>8,0
aquatic bird/Alakol/6952/2016	23,2

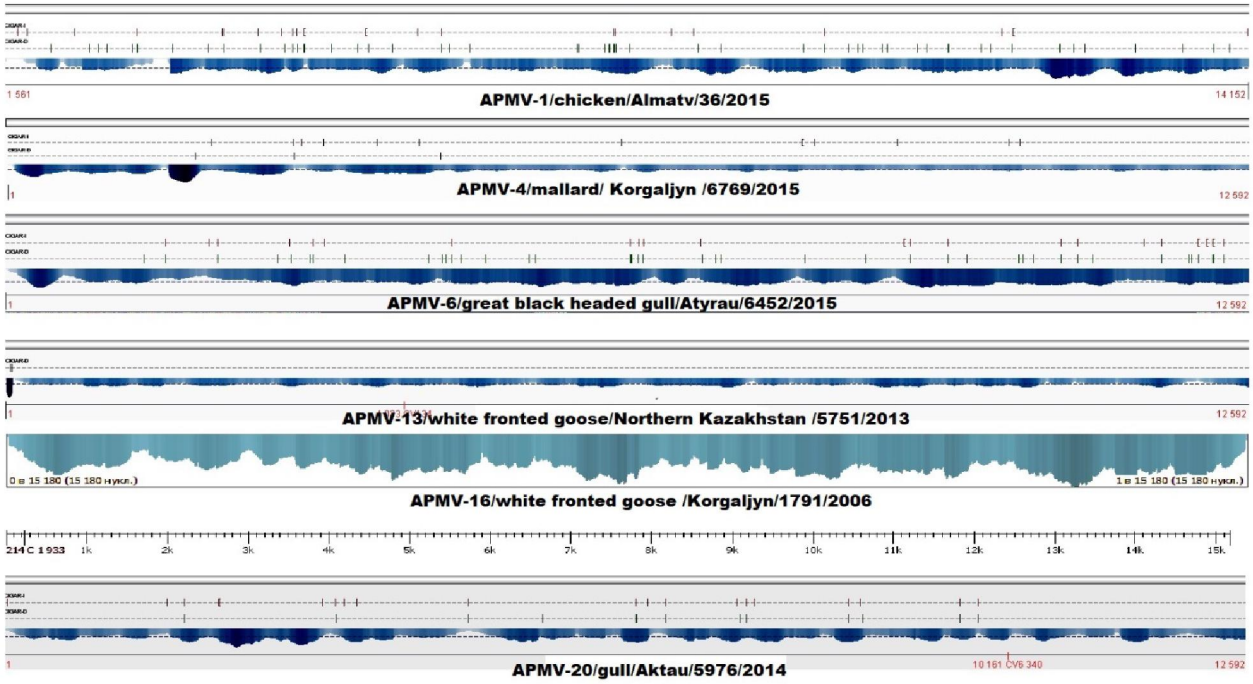
As can be seen from table 1, RNA concentrations ranged from 8.0 to 26.3 ng/ul, which, according to the recommendation of the sequencing kit manufacturer, is sufficient for the production of libraries.

Cytoplasmic and mitochondrial ribosomal RNAs (rRNAs) were removed for further sequencing of the complete viral nucleotide sequences using specific oligonucleotides, in addition to the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA) kit used.

RNA fragmentation was performed to a size of about 300-400 bp. using an enzymatic method at different temperatures, using bivalent cations included in the kit. From the RNA fragments, the first cDNA chain was synthesized using reverse transcriptase and random primers followed by a second strand synthesis using DNA polymerase I and RNase H.

The adenine molecule was then attached to the obtained cDNA fragments and subsequently the adapters were ligated. Illumina adapters were used for preparation of the library of fragmented cDNA. The products were purified and amplified in PCR. The quality of the prepared libraries was checked on the Bioanalyzer 2100 (Agilent Technologies, USA). Sequencing was performed on a next generation sequencer Illumina MiSeq (USA), using a reagent kit v.3.

For bioinformatic analysis, the resulting sequences were collected and processed in the UGENE 1.20 software (Russia). As a result, the complete genomic sequences of the viruses were obtained (figure).



View of the complete genomes of sequenced viruses in Tablet and UGENE 1.20 software

Figure show that the coverage of the virus genome was even and varied from 4,521 to 5,500 rids in different regions. Complete genome sequences of viruses belonging to *Paramyxoviridae* family of APMV-1, APMV-4, APMV-6, APMV -13, APMV -16 and APMV -20 serotypes were obtained. The full names of isolates with identified serotypes are presented in table 2.

Table 2 – Identified serotypes of paramyxoviruses and their genomes

Вирусы	Genome Length, nt
APMV-1/chicken/Almaty/36/2015	15097
APMV-4/pygmy cormorant/Kyzylkol/7074/2016	15054
APMV-4/barn swallow/ Kyzylkol /7079/2016	15054
APMV-4/mallard/ Korgaljyn /6769/2015	15054
APMV-6/great black headed gull/Atyrau/6452/2015	16236
APMV-13/white fronted goose/Northern Kazakhstan /5751/2013	15996
APMV-13/white fronted goose /Northern Kazakhstan/5759/2013	15996
APMV-16/white fronted goose /Korgaljyn/1791/2006	15180
APMV-20/black headed gull/Balkhash/5844/2013	15786
APMV-20/great black headed gull /Atyrau/5541/2013	15786
APMV-20/gull/Aktau/5976/2014	15786
APMV-20/aquatic bird/Alakol/6952/2016	15786

Discussion. Identification of new pathogens is of great importance for the diagnosis of infectious diseases in humans and animals. Almost all outbreaks of dangerous infections in the last two decades have been caused by new pathogens, such as the severe acute respiratory syndrome virus (SARS) [6], hanta virus Sin Nombre [7], the 2009 pandemic influenza virus H1N1 [8], and the newly described EMC coronavirus [9], most of which originate from a natural reservoir.

Modern technologies make it possible to identify viruses using a wide range of methods. Traditional methods include electron microscopy, cell culture and infection of live organisms, as well as serological studies [10], but all they have their limitations. For example, many viruses are not able to be cultivated in

laboratory and can be characterized only by molecular methods [11], such as the use of hybridization microchips [12] and PCR [13].

Amplified products as the result of hybridization and PCR require final identification by sequencing. The limitation of these methods is the need to know the sequence of nucleotides before the study, which is not always possible.

Massive parallel sequencing or next generation sequencing (NGS), provides a high-performance analysis of huge amounts of data on the nucleotide sequences contained in a particular sample. So, it makes possible to identify all the nucleic acids of different organisms present in a sample and this hugely increases the possibilities of genetic researches.

Perhaps the most obvious application of these technologies is the sequencing of the genome. Although viral genomes are relatively small, but their scientific value is often extremely important, and this technology can be a highly effective way of obtaining the complete sequence of the viral genome.

This study made it possible to simultaneously obtain the complete genomes of paramyxoviruses of various serotypes using the method of massive parallel sequencing. It is known that wild ornithofauna plays a key role in maintaining APMV in the biosphere and is a potential natural source of the emergence of new dangerous variants of viruses.

Experimental researches on APMV-1 showed that wild birds can spread and introduce low- or non-pathogenic variants into poultry, which after a few passages *in vivo* become highly pathogenic [14]. For this reason, continued monitoring of the APMV in the wild is one of the most important tasks for ensuring the safety of poultry.

The obtained data on the complete genomes of paramyxoviruses using new technologies will allow us to expand our knowledge about the course of the natural evolution of viruses of migratory birds.

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ТҮЗ ҚҰСТАРЫНЫҢ ВИРУСТАРЫН ЗЕРТТЕУДЕ ЖАППАЙ БІР МЕЗГІЛДЕ СЕКВЕНДЕУ ӘДІСІН ҚОЛДАНУ

Аннотация. Вирустық патогендерді идентификациялаудың адам мен жануарлардың инфекциялық ауыруларын балаудағы маңызы зор. Соңғы екі онжылдықтағы қауіпті инфекциялардың барлығын дерлік жаңа вирустар шақырды. Олардың басым бөлігі табиғи резервуарларда туындады.

Серотүрі 1 парамиксовирусы (ПМВ) негізінде сынақтық зерттеулер, түз құстарының үй құстары арасына вирустардың әлсіз немесе зардапсыз нұсқаларын енгізуге және таратуға қабілетті екенін, олардың жиі жағдайда бірнеше пассаждан кейін бейім құстардың ағзасында зардаптылығы жоғары қасиетке ие болатынын көрсетті.

Жаңа, жаппай бір мезгілде секвендеу технологиясын қолдану нәтижесінде парамиксовирустар туыстатығына жататын түз құстары вирустарының генетикалық құрылымдары жайында мәліметтер алынды. Бір уақытта вирустардың қай туыстастық өкілі екенін алдын-ала білмей ақ, олардың толық геномын секвендеуге мүмкіндік беретін аса тиімді әдіс екені анықталды. Алынған мәліметтер жыл құстары вирустарының табиғи эволюциясы барысы жайында біздің біліміміздіі нығайтады.

Түйін сөздер: вирус, жаппай бір мезгілде секвендеу, жабайы құс, вирустың толық геномы, РНҚ, ДНҚ, биоинформатикалық талдау.

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ПРИМЕНЕНИЕ МЕТОДА МАССОВОГО ПАРАЛЛЕЛЬНОГО СЕКВЕНИРОВАНИЯ ПРИ ИССЛЕДОВАНИИ ВИРУСОВ ДИКИХ ПТИЦ

Аннотация. Идентификация вирусных патогенов имеет огромное значение для диагностики инфекционных заболеваний человека и животных. Почти все вспышки опасных инфекций последних двух десятилетий были вызваны новыми вирусами, большинство из которых происходили из природного резервуара.

Экспериментальные исследования на примере парамиксовируса (ПМВ) серотипа 1 показали, что дикие птицы способны распространять и заносить слабо- или непатогенные варианты в популяцию домашних птиц, которые через несколько пассажей в организме восприимчивых птиц зачастую приобретают высокопатогенные свойства.

С использованием новой технологии массового параллельного секвенирования получены сведения о генетической структуре вирусов диких птиц, принадлежащим семейству парамиксовирусов. Показана высокая эффективность метода, который позволяет одновременно секвенировать полные геномы вирусов без предварительного знания об их принадлежности к какому-либо семейству. Полученные данные позволят расширить наши знания о ходе естественной эволюции вирусов перелетных птиц.

Ключевые слова: вирус, массовое параллельное секвенирование, дикие птицы, полный геном вируса, РНК, ДНК, биоинформационный анализ.

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