РОЗДІЛ 1. ПРОБЛЕМИ БІОБЕЗПЕКИ ТА БІОЗАХИСТУ

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MOLECULAR GENETICS STUDIES OF NEW UNKNOWN AVIAN PARAMYXOVIRUS ISOLATED FROM WILD BIRD IN UKRAINE

Bolotin V.I.
National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine", Kharkiv, Ukraine, e-mail: vbolotin@hotmail.de

The current study covers phylogenetic analysis of Ukrainian APMV isolate from White-fronted goose. The aim of this work was to characterize new isolate and estimate its relationships to the other APMVs. Investigations were provided using bioifomatics and molecular genetics methods. As the results of PCR with the random primers and cloning into pCRII-TOPO100 transformed colonies of E. coli were analyzed and inserts were sequenced. It was found 3 regions, which could identify by BLAST. The first region with the length of 1387 nucleotides (nt) showed 98 % (1355 nt) degree of nucleotide similarity with the published in 2014 F gene sequence of APMV/Goose/Shimane/67/2000 isolate from Japan. From this region 344 nt had 66 % nucleotide sequence identity to the APMV 12 isolate Wigeon/Italy/3920_1/2005 at genome position 4760-5103. Next region (367 nt) had 70 % identity to the same APMV 12 isolate at position2419-2784. The last region (365 nt) showed 71 % identity to Newcastle disease virus strain M4 at position 12569-12928. This high divergence from the currently known APMV allows making the assumption that Ukrainian and Japanese isolate might be a new serotype of APMV. According to the obtained results we have confirmed circulation of novel APMV in wild birds on the territory of Ukraine. This requires providing further investigation to obtain full-genome sequence and study of biological properties of new APMV.

Keywords: avian paramyxovirus, serotype,sequencing, wild goose, phylogenetic analysis.

Avian paramyxoviruses (APMV) are members of the genus *Avulavirus* (subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales*) and have been isolated from different species of domestic and wild birds [1]. This genus includes Newcastle disease (ND) virus or APMV-1virus, and other serotypes of avian paramyxoviruses.

Currently there are 12 serologically different types of APMV based on antigenic differences revealed by serological tests. NDV causes economic loses all over the world through outbreaks in poultry. Other APMVs are partially characterized due to its economic importance [2]. Full genome sequences of almost all of APMVs have been published in GenBank. However, it is very little known about their biological properties. APMV-2, -3 and -7 cause mild infections in turkeys and chickens [3, 4]. APMV-5 has been isolated from budgerigars with acute fatal enteritis [5]. Serotype 6 is responsible for pneumonia in turkeys and avirulent for chickens [6]. APMV-4, -8 and -9 circulate mostly among wild birds [7]. Finally, the last three serotypes were described during study of isolates from penguins, snipe and Eurasian wigeon from Falkland Islands, France and Italy, respectively [8].

Wild birds as a natural source of new viruses in Europe migrate from the East with further spreading of infections. The previous study indicated the circulation of different APMVs in Ukraine, including APMV-4 and -6, which were genetically connected with isolates from Europe and Africa [9]. In this study molecular-genetics examinations of new unknown APMV isolate from White-fronted goose were carried out.

Materials and Methods. Isolate APMV/White-fronted goose/Ukraine/2011 was obtained from Department for Studying Avian Diseases of NSC "IECVM". Total RNA from the isolate was extracted using TRIzol SL Reagent (Invitrogen, USA). RT-PCR, cloning into TOPO and plasmid purification, sequencing and phylogenic analysis were done as described by Diel D.G. et al. [10]. In brief, PCR was performed with the one-step RT-PCR kit (Qiagen, USA) and the following set of degenerated random primers: beg2 (5'-CGCGTCGACTACTACGGGTAGA-3'), end-r (5'-GTACCCGGGGATCCTTTTTTCTAA-3'), FR26RV-N (5'-GCCGGAGCTCTGCA GATATC-3') and FR20RV (5'-GCCGGAGCTCTGCAGATATC-3'). After gel electrophoresis DNA bands with lengths of 0.4 to 1.5 kb were excised from the gels and purified by using the QuickClean DNA gel extraction kit (Qiagen, USA). The purified PCR products were cloned into the TOPO TA vector (Invitrogen, USA). Selected E. coli colonies were cultivated in 96 plates with the following plasmids extraction by using Wizard® MagneSil® Plasmid Purification System (Promega, USA) according to the manufacturer's instructions. Obtained samples subjected to DNA sequencing using M13 forward and M13 reverse primers.

Sequence editing and assembly of sequence contigs were carried out with the software package Laser Gene sequence analysis version 5.07 (DNAStar, Inc., USA). Nucleotide sequences were analyzed using the basic length alignment search tool (BLASTN 2.2.28) [11]. Heptad repeats in fusion protein were predicted by the Learn Coil-VMF program [12]. Potential N-linked glycosylation sites of F protein were predicted by the NetNGlyc 1.0 program of the Expasy proteomics server [13]. The phylogenetic tree was constructed using MEGA 5 by the maximum-likelihood method [14].The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [15].

Results. APMV/White-fronted goose/Ukraine/2011was isolated in 2011 in the South part of Ukraine from cloacal swabs of geese (*Anser albifrons*) that did not show any clinical signs. Virus was isolated using chicken embryos and serologically characterized as APMV-7 in hemagglutination-inhibition (HI) test using chicken antisera for representatives of APMV-1 to APMV-9 (Muzyka D.V., personal communication). However, study of the cDNA obtained from this isolate in PCR with specific for APMV-7 primers was not confirmed serological results.

As the next step the sequencing of the isolate was determined by using a shotgun PCR/sequencing approach. For this purpose PCR with the degenerated primers was provided and mass of amplicons were obtained (fig. 1). It was divided into two sectors ("A" and "B") in connections with length of the amplicons. Both sectors "A" and "B" included bands with length of approximately from 400 to 900 bp and 900 to1500 bp, respectively. After purification from the gel the amplicons were cloned into pCRII-TOPO with the following competent *E. coli* transformation.

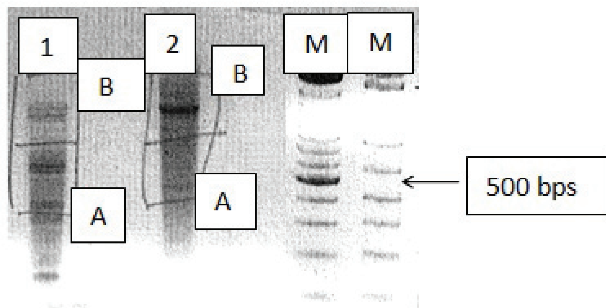

Figure 1. Agarose gel electrophoresis of PCR products from the tested APMV isolate using random primers beg2 and end-r (Lane 1), and FR26RV-N and FR20RV (Lane 2). Lane M, molecular weight markers (DNA molecular weight markers). Lane 1 and 2 were divided into 2 sectors (A and B) which were involved in shotgun PCR/sequencing approach.

Analysis of 100 transformed colonies of *E. coli* using traditional sequencing gave possibilities to find 3 regions, which could identify by BLAST. The first one was 1387 nucleotides (nt) and showed 98 % (1355 nucleotides) homology with the published in 2014 F gene sequence of APMV/Goose/Shimane/67/2000 isolate from Japan (Pubmed accession AB854168). There is no detail information concerning this strain, except MDT data (over 120 hours) [16]. However, in this study the deduced amino acid sequences for the F protein were as well compared. Among both viruses, the range of amino acid sequence identity was 99 % (4 substitutions: S5F, G10R, G105E, N413S). It was predicted that the F protein of APMV/White-fronted goose/Ukraine/2011 contains 2 heptad repeats (at positions 134-180 and 457-500), and 5 potential N-linked glycosylation sites located at the positions 78, 98,182, 357 and 438 amino acids. Obtained sequence is predicted to code a type I transmembrane protein and identified as F protein of Paramyxovirus. According to the published data, the F gene of Japanese APMV strain is 1638 nt long and encodes a protein of 545 amino acids, including the proteolytic cleavage site (^{102}QVRENR↓LVG^{110}). In comparison Ukrainian isolate has one substitution in cutting site (^{102}QVRGNR↓LVG^{110}).

Moreover, the region with the length of 367 bp had 70 % nucleotide sequence identity to the APMV-12 isolate Wigeon/Italy/3920_1/2005 at genome position 2419-2784 (table 1).

It was also identified the region with the length of 344 nt that showed 66 % similarity to the same APMV-12 isolate at position 4760-5103. The part of this region with the length of 146 nt had also level of nucleotide similarity of 73% with strain NDV08-046. The last region (365 nt) showed 71 % identity to Newcastle disease virus strain M4 at position 12569-12928. This strain was obtained in China from Muscovy duck.

A phylogenetic tree comparing F protein of Ukrainian isolate with the F proteins of 11 other APMVs available in the GenBank database is shown in Fig. 2. According to this data Ukrainian and Japanese APMVs are more closely related to the serotype 12 and cluster together with APMV 1 and 9.

Table 1 – The results of the shotgun PCR/sequencing of pCRII-TOPO plasmids with different inserts, which were obtained after RT-PCR with random primers and RNA of tested isolate

Sequencing data from contigs	Identities to other APMVs, number of identical nt/total number of nt, (%)	Position in genome
CATATGGAGACGGATCTCCGGGACCAGCAATCAGCACAGGCCTGAG TTGCGAAACCTTCCGCAAATCGTTAAGGGATGATATATTAGCATTCCC AGGATCCATTAGTTTCATCATACTTATATTCCCTTCCATTACTGCGACA GCAGTCTTCAATTGTTGTATGTCAGTCCTAATTAGTGGACAGCATGAC ACATGCTTTAAAATCAACTCTATTGAGTATTCAAGTTTACTCATCCTGT GATTCATGCTCTCCATCATCCCTAACATCGCGTGAGCAAAGTCTGCA AAGTTTGGGGCATTTGCCGCATCTACAGGCGAACTGCTGGGGTGTT GATGTGATGGGTGCACAGTCTGGGTTGCACCAGCTTGTGGTGATGA TCCCTTCTGTGCTCCATGATATTGGATGCTCTCTCCTGTGTCCCGGT CGCCACTT	Avian paramyxovirus 12 isolate Wigeon/ Italy/3920_1/2005, 256/367 (70%)	2419-2784
	Newcastle disease virus isolate QH4, 295/433 (68%)	2414-2836

<table>
<tr>
<td rowspan="3">ATTAGCTTGAATTAATGCTGTTGCAGCGGTAATCTGAGCAGCAGTTG
CAACACCTAAGGCAACACTCCCTATAATTGCCCCAACCAGTCTGTTT
CCTCTCACTTGAGTCGTTGAATTGCCTCTTATTGCAGACATTGCCGT
GGCAAGTGGTGTTAGAATGCGGGATAATGTTTCATTATAAGATTGCAA
TGATTGTTTGGCACAATCTTTCTTATTGTCAGGTAAATTTGGGATCAAT
TTGACAACTATAATTCCTGTTTGGGATGATGTATATATGCTAATGGGCC
GGTCACCAACAGGAACAATTCCTGCAGCAAGTGTTAATCTTCCGTCT
ATCGAGCCCTGACATGTCTGAGCAATCAATAGAATAGTGCTCAGGCG
GAAAATCTCCTGAAAGAAACGTGCCATCTCTGATTTGTGCATTTAGCT
GTGGCACATGTGACTTCTGCCCGTACTTTTTTCTATTGCAGAGATAAT
TTTAGCGCATATGATTTTAACAACATCCAGGGTGCATGGCATTGATAG
TTGATGCATGGGAATCTGCAGAGTTACTATCAGACAAAATAGCCGATT
CAGATACTGTTGATTAGAATTTTGTCTTTAATCATAAATGTTGATCTTG
CTTAAAGAAGTCAGTTGGATAGACAGATGATTTTTATCCGGTCTTTTTA
AATGGGTTGAATTTTCCATTTCTCCCAGATTTCTCAATTTTCGTGCTC
CCGACCACGAAGTCATCAGTTGTCGCAATAGCCCTATTGGTTCCCCC
TTGTATTATAATTTTGCATTCATGGAGAGAACCGGTCTGGCTCCATAG
TATCTTTGCAATTCCAGGGGCAGTCTGGCTTATAGGATAGCAGGCTG
TTCCCCTAGGTGAAAAGAAAGGACTCATCACTTTCGTTCGCTTTCCT
CTTGCTTTCAATGCAATTGAGGGACCGAAAATATCAAGCAGTCCTACA
CTGATCGACATCCTGCGAATTTTTAATTCAAGCTTGTCAACTGATATCT
TGTTCCCTTTGCTGTCAAGGCAGGAGATCATCCCGATGTGAATGAAA
AGGTCTGCGAAAAACTGATCGTTCCGCTTCATT</td>
<td>APMV/Goose/Shimane/67/2000
1355/1387 (98 %)</td>
<td>F gene from position 1 to 1355</td>
</tr>
<tr>
<td>Avian paramyxovirus 12 isolateWigeon/ Italy/3920_1/2005, 227/344 (65%)</td>
<td>4760-5982</td>
</tr>
<tr>
<td>Newcastle disease virus strain NDV08-046, 106/146 (73%)</td>
<td>4905-5050</td>
</tr>
<tr>
<td>ATGGACATGCTTGTGTTCAAGAATTATGAATTGAGCTTAGATCAATATA
CCACATTCGAATTAATGGGAATCTTGGCAATGACTACAGGGAAACTCA
TAGGTCAATCTATTATCcTCATATGATGACGAAACCTCAATTAAGAATG
ATGCTATTATCGTATATGACAACTCCCGGAATTGGATAAGTGAATTCCA
GAATTGTGATGCAATAAAATTGCTAGAGTATGYTGCAGTTGAAGTCCT
GCTGGACTGCGCCTATCAAATTTACTATCTGAGAGTAATASGTGTTGA
GAATATCATGCTATACATGAGTGACCTTTTGCAAAGTATGCCAGGGAT
CCTTGTTGGGCTAATTTAGCAGCAACAATACTTCYCATCCCGGAGTAT
TTTTGGAAGGCTGTAGCAGGGTCTGATATACTGCAGAG</td>
<td>Newcastle disease virus strain M4, 260/365 (71%)</td>
<td>12569-12928</td>
</tr>
</table>

Within the genus *Avulavirus*, different APMVs segregated into two clusters, one of them contained serotype 3, 4 and 11, and another combined NDV, APMV-2, -5, -6, -7, -8, -10, 12 and Ukrainian and Japanese unknown APMVs. In contrast, phylogenetic tree of *Avulavirus* based on genome sequence starting from N gene end to L gene start region showed that NDV clustered together with APMV-3, -4 and -9, whereas APMV-11 grouped together with APMV-2, -6, -7 and -8 [17]. This high divergence from the currently known APMV allows making the assumption that this isolate might be a new APMV.

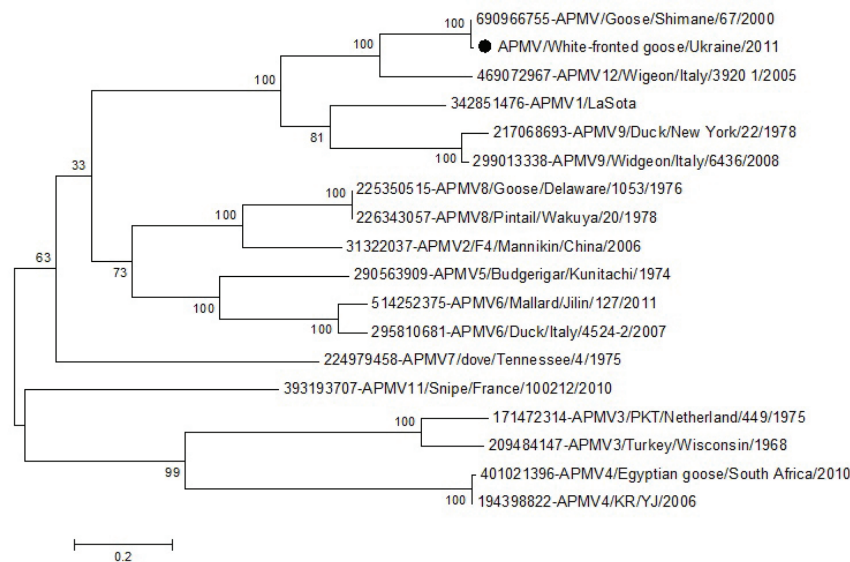

Figure 2. Molecular phylogenetic analysis of the fusion protein of Ukrainian and different APMVs by Maximum Likelihood method. Ukrainian isolate was marked with filled circle. The tree with the highest log likelihood (-10036.4776) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 425 positions in the final dataset. Evolutionary analysis was performed in MEGA5 [13].

Conclusion. According to the obtained results we have confirmed circulation of new unknown APMV in wild birds on the territory of Ukraine. Sequence alignment and phylogenic analysis showed similarity to uncharacterized APMV strain from Japan, APMV-1 and APMV-12. Further investigation will be carried out to obtain full genome-sequencing and detailed study of the biological properties of the virus.

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МОЛЕКУЛЯРНО-ГЕНЕТИЧНІ ДОСЛІДЖЕННЯ НОВОГО НЕВІДОМОГО ПАРАМІКСОВІРУСУ, ВИДІЛЕНОГО ВІД ДИКОЇ ПТИЦІ В УКРАЇНІ

Болотін В.І.
Національний науковий центр «Інститут експериментальної і клінічної ветеринарної медицини»,
м. Харків, Україна, e-mail: vbolotin@hotmail.de

У статті представлені результати дослідження нового параміксовірусу, виділеного від білолобої гуски на території півдня України. Метою даної роботи було охарактеризувати новий ізолят і вивчити філогенетичні зв'язки з параміксовірусами інших серотипів. Дослідження були проведені з використанням біоінформатичних і молекулярно-генетичних методів. На першому етапі досліджень проводили ПЛР з рандомними праймерами з наступним клонуванням продуктів ампліфікації у відкриту плазміду pCRII-TOPO 100 і трансформацією колоній E. coli, після чого аналізували отримані конструкції шляхом секвенування. За допомогою алгоритму BLAST визначені три послідовності, перша з яких довжиною 1387 нуклеотидів мала ступінь подібності 98 % (1355 ідентичних нуклеотидів) відносно опублікованої у 2014 р. послідовності F гена ізоляту APMV/Goose/Shimane/67/2000 з Японії. Крім цього, перша послідовність також мала у своєму складі ділянку довжиною 344 нуклеотидів зі ступенем подібності 66 % до параміксовірусу 12 серотипу (Wigeon/Italy/3920_1/2005) з розташуванням у межах геному від 4760 до 5103 позиції нуклеотидів. Наступна послідовність (367 нуклеотидів) показала 70 % ідентичність з APMV 12 ізолятом у позиції 2419-2784. Останній регіон (365 нуклеотидів) показав 71 % ідентичності до вірусу ньюкаслської хвороби штаму M4 в позиції 12569-12928. Такі значні відмінності від відомих у даний час параміксовірусів дозволяють зробити припущення про те, що український та японський ізоляти можуть належати до нового серотипу параміксовірусів. Згідно з отриманими результатами підтверджується циркуляція нового параміксовірусу серед диких птахів на території України. Необхідно провести подальші дослідження, які будуть спрямовані на вивчення послідовності повного геному і біологічних властивостей цього ізоляту.

Ключові слова: Білолоба гуска, пташиний параміксовірус, секвенування, серотип, філогенетичний аналіз