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Phylogenetic analysis of influenza A viruses (H3N2) circulating in Zhytomyr region during 2013–2014 epidemic season

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Aim. To perform phylogenetic analysis of the hemagglutinin (HA) and neuraminidase (NA) genes of influenza A(H3N2) viruses circulating in the Zhytomyr region during 2013–2014 epidemic season. To make comparison of the HA and NA genes sequences of the Zhytomyr region isolates with the HA and NA genes sequences of influenza viruses circulating in the world. **Methods.** Laboratory diagnosis was conducted by real-time polymerase chain reaction (RT-PCR). In this study the sequencing and phylogenetic analysis were carried out. **Results.** For the first time the genes of influenza A(H3N2) viruses isolated in the Zhytomyr region during 2013–2014 epidemic season, coding hemagglutinin and neuraminidase were compared with their orthologs. According to the results of this comparison the phylogenetic tree was constructed. Additionally, the amino acid substitutions of the influenza viruses circulating in Ukraine and worldwide were analyzed. **Conclusions.** The nucleotide sequences of the influenza A(H3N2) viruses genes HA and NA isolated in the Zhytomyr region were identified. Based on the nucleotide sequences of HA and NA we constructed the influenza virus phylogenetic tree demonstrating that the virus isolated in the Zhytomyr region was closely related to the Ukrainian isolate from Kharkov and in the world to the isolates from Germany, Romania, Italy.

Keywords: A(H3N2) influenza viruses, phylogenetic analysis, epidemic season, mutations.

Introduction

Influenza viruses are the most prevalent pathogens of human respiratory infections and the most significant because they cause high morbidity and mortality [1]. Influenza affects all age groups of population, but the highest incidence is recorded among children and adolescents [2]. Influenza viruses have the ability to cause annual epidemy, and sometimes – global pandemic. This is applied to the majority of influenza A viruses. These pathogens are more variable than the influenza B and influenza C viruses due to unique antigenic properties of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) [3].

Influenza viruses change the composition of their surface antigens with high evolutionary rate that allows them to evade the action of the immune system, and thus to keep themselves in the human population [4]. There are two main evolutionary mechanisms,

which allow influenza viruses to constantly evolve and re-infect their hosts, namely, an antigenic drift and antigenic shift [3, 5–7]. The antigenic drift is a result of the gradual accumulation of mutations that are fixed in the viral genome. Such mutations can lead to minor changes in the viral proteins resulting in better survival of these viruses, including the capacity to escape immune system recognition. During the antigenic shift, influenza A virus can get segment HA, and possibly NA segment or another segment of the genome of different influenza virus subtype, resulting in a radically new influenza virus with new properties [6]. A high variability of influenza viruses makes it necessary to conduct a comparative study of antigenic and biological properties of influenza virus during epidemics [5]. Also, surveillance and control of antigenic properties of circulating influenza viruses in the population are annually required for the defining of a new variant strain for vaccine [2, 8–10].

Thus, the aim of our research was to perform phylogenetic analysis of HA and NA genes of influenza A(H3N2) viruses, which circulated in the Zhytomyr region during the 2013–2014 epidemic season and their comparison with those which circulated in the world.

Materials and Methods

The material for the study were the clinical samples of nasopharyngeal swabs, pharyngeal swab, nasopharynx and nose, autopsy material selected in the first three days and no later than the 5th day of illness from the patients with suspected influenza and SARI (severe acute respiratory infections) [11]. The patients were hospitalized in the Zhytomyr region children and adult infectious departments of hospitals. The autopsy material from dead patients was obtained from the regional department of morbid anatomy and office forensics.

For carrying out the work there were used virological and molecular genetic methods of research.

Laboratory diagnosis was conducted by real-time PCR (polymerase chain reaction). To detect influenza virus types A and B there were used extraction kits (Total RNA Mini Kit Spin Format, Bio-Rad, USA) and reagent for reverse transcription (iScript cDNA Synthesis Kit, Bio-Rad, USA), primers and probes respective markers – Univ inf A, sw A, sw H1, H1, H3, Univ inf B, RNase P (Biosearch, USA).

The researches were carried out by the protocol (it was given by WHO) of polymerase chain reaction with reverse transcription real-time to detect and study influenza A (H1N1) pdm from the Center of Disease Control and Prevention (CDC) United States (version 2009) [12].

Virological studies were carried out by the method of isolation and cultivation of influenza viruses in MDCK cell (epithelial cells kidneys female Cocker-Spaniel) [10]. The isolates were later used for the strain identification and sequencing.

The sequencing of influenza viruses isolates was carried out in the WHO Collaborating Centre in London. The sequences of influenza viruses isolated in other countries were found using web-site GISAID (the Global Initiative on Sharing All Influenza Data – http://platfom.gisaid.org). The package of software MEGA 6.0 for carrying out the phylogenetic analysis was used [13].

Results and Discussion

Influenza A (H3N2) virus in the Zhytomyr region was detected during two epidemic seasons: 2011–2012 and 2013–2014. In 22 samples (30.5 %) of the 72 samples tested during the epidemic season 2011–2012 influenza A (H3N2) viruses were detected by PCR in real time **[12]**.

In this study, we have characterized influenza A(H3N2) viruses circulating during 2013–2014 epidemic season in the Zhytomyr region.

There were examined 67 nasopharyngeal swabs from patients with severe course of SARI and samples of autopsy material. 298 tests by PCR in real time were carried out. This was done for the monitoring of influenza viruses circulation among different groups of the Zhytomyr region population during 2013–2014 epidemic season. Influenza A (H3N2) viruses were detected in 18 samples (26.9 %). Other influenza viruses in the epidemic season were not found. The obtained results indicate widespread influenza among the different age groups of the Zhytomyr region, but mostly children and young adults were involved in the epidemic process.

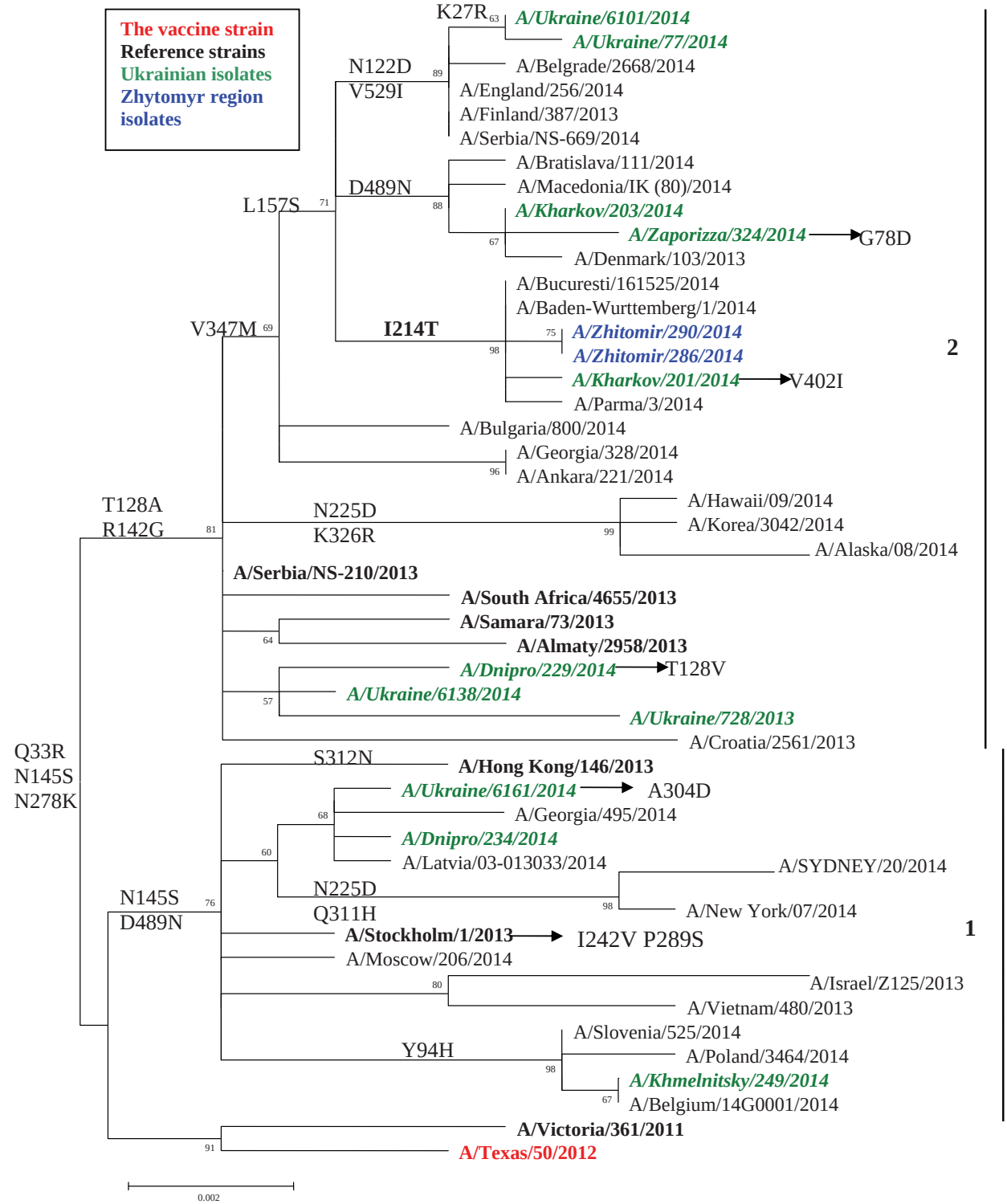

Fig. 1. Phylogenetic analysis of the HA gene of influenza A (H3N2) viruses isolated in the Zhytomyr region during 2013–2014 epidemic season

For isolation of influenza viruses in MDCK cell culture we used five samples, in which influenza A(H3N2) viruses were detected [10].

For comparison, in our research we used the segments that encode the surface proteins HA and NA. These proteins are mostly exposed to antigenic drift and shift to avoid the effects of human immune system. Hemagglutinin is a receptor-binding protein that is the main target of the immune response. Therefore it is the most variable protein in influenza viruses as amino acid substitutions in the antigenic sites (A, B, C, D, E) of HA molecules affect the antigenic properties of influenza virus. These events influence the change of vaccine strains. He The neuraminidase protein is less variable, but amino acid substitutions in it affect the resistance of influenza viruses to the antiviral drugs (neuraminidase inhibitors) [14].

The isolates were sent to the World Influenza Centre (London) for sequencing. In the epidemic season 2013–2014 for the first time there were made the sequences of genes HA and NA of influenza A(H3N2) viruses isolated from the clinical material from patients of the Zhytomyr region and they were subsequently included in the GISAID database of influenza viruses worldwide.

We found a very high similarity (99 %) of the HA and NA genes of the Zhytomyr region viruses with the HA and NA genes of influenza viruses in the group with T214I substitution on the phylogenetic tree using BLAST system. Comparison of the nucleotide sequences of the Zhytomyr region virus HA and NA with those of the T214I group viruses revealed several synonymous nucleotide substitutions and no substitutions that alter amino acid sequences of the proteins.

The HA and NA phylogenetic tree of the Zhytomyr region strains and strains from all over the world was generated using MEGA 6.0. Evolutionary distance was calculated using the maximum composite likelihood method. Statistical significance of the tree topology was tested by bootstrap analysis of 100 pseudoreplicate datasets.

The 2728 HA and NA protein sequences of influenza A(H3N2) viruses of different strains circulated during 2013–2014 epidemic season in the world were downloaded from the GISAID database.

By random sampling we selected 48 isolates from around the world during 2013–2014 epidemic season and constructed the phylogenetic tree of HA genes (Fig. 1).

All the HA genes of the Zhytomyr region isolates, as all Ukrainian and worldwide isolates studied with vaccine strain A/Texas/50/2012 and reference strains: A/Victoria/361/2011, A/Hong Kong/146/2013, A/Stockholm/1/2013, A/Serbia/NS-210/2013, A/South Africa/4655/2013, A/Samara/73/2013, A/Almaty/2958/2013 fell within the A/Victoria/208 genetic and into the of genetic subgroup 3C of group 3 [15]. All submitted isolates acquired in the evolution new joint (Q33R), group (L157S, I214T, N122D) and individual (V402I) amino acid substitution in a part of hemagglutinin compared with vaccine strain and reference strains. Therefore, they can be divided into two groups. The amino acid substitutions that define these groups are:

The first genetic group included the isolates that had substitution N145S (asparagine to serine) and D489N (aspartic acid to asparagine). The group combined some viruses from Ukraine and the world and reference strains A/Hong Kong/146/2013 and A/Stockholm/1/2013.

In turn, the reference strain A/Stockholm/1/2013 has substitution I242V and P289S, and virus A/Hong Kong/146/2013 acquired a new glycosylation site S312N.

The second genetic group comprised a large quantity of Ukrainian viruses including the Zhytomyr region isolates and reference strains that had T128A (resulting in the loss of a potential glycosylation site), R142G substitutions, along with the isolates from all over the world. The group combined some viruses from Ukraine, the world and reference strains A/Serbia/NS-210/2013, A/So uth Africa/4655/2013, A/Samara/73/2013, A/Almaty/2958/2013.

The Zhytomyr region isolates were found to be most closely related to the Ukrainian isolate from Kharkov as well as to the isolates from Germany, Romania, Italy. They have gained substitution I214T (isoleucine to threonine).

Thus, analyzing the HA gene sequences of the Zhytomyr isolates we can conclude that the strains recorded in the Zhytomyr region, are similar to those

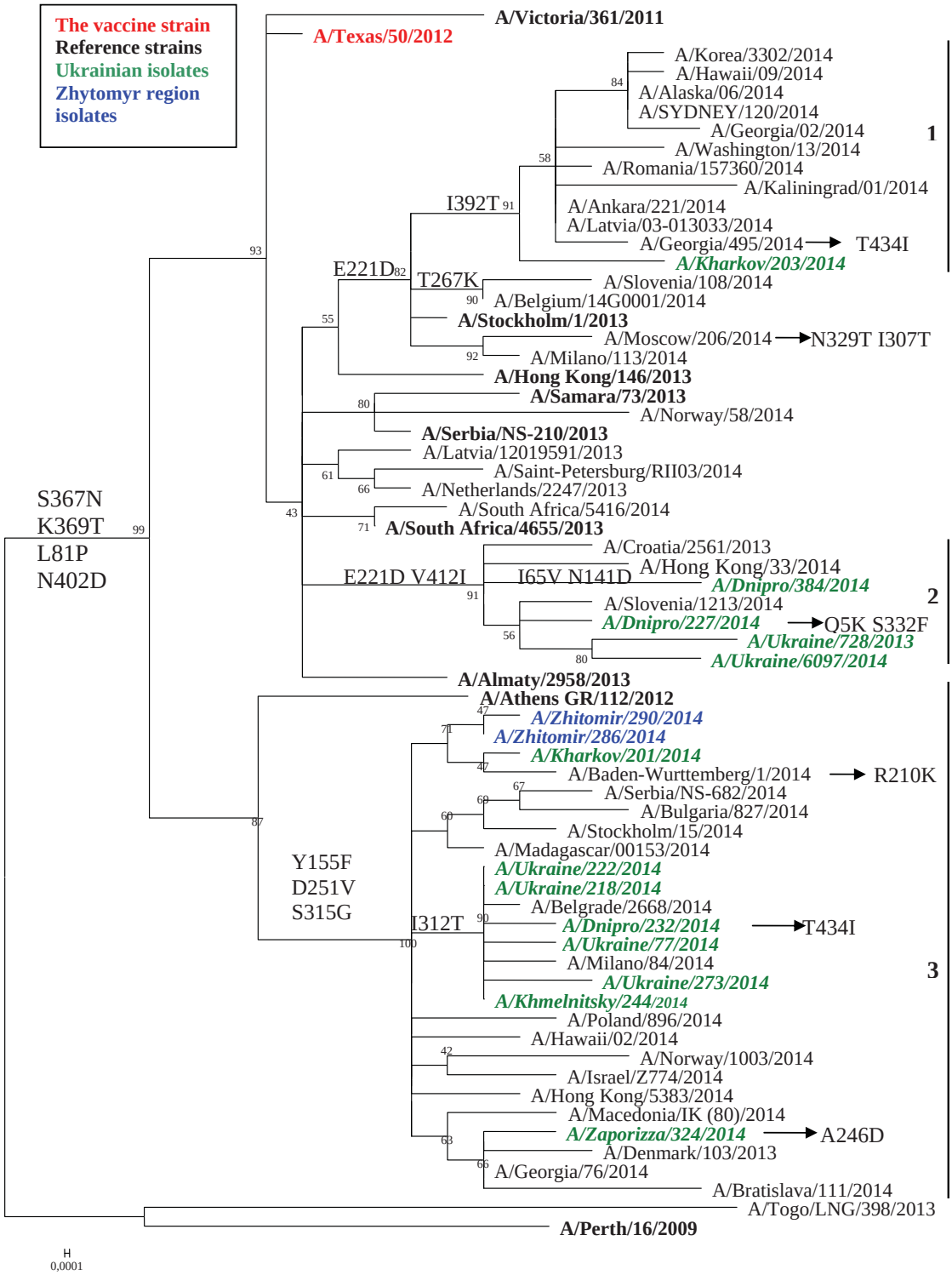

Fig. 2. Phylogenetic analysis of the NA gene of influenza A (H3N2) viruses isolated in the Zhytomyr region during 2013–2014 epidemic season

from other regions of Ukraine (the Kharkiv region) or from abroad (Germany, Romania, Italy). This is due to a high level of the migration both within the country and abroad.

In the phylogenetic tree of the NA genes 65 isolates from around the world were presented (Fig. 2).

The analysis of the NA genes of the Zhytomyr region influenza viruses showed similar results with the analysis of the HA gene sequences. All investigated isolates are within the genetic subgroup 3C of A/Victoria/208 genetic clade, keeping mutation S367N and N369T. These mutations are typical for the group 3. The mutations L81P and N402D (loss glycosylation site) are typical for the subgroup 3C.

All studied isolates of the 2013–2014 epidemic season were genetically similar to the vaccine strain A/Texas/50/2012, but had some genetic differences, so they were divided into three genetic groups.

The first group includes the viruses from different countries including Ukraine – A/Kharkov/203/2014 (V396I) with substitution I392T (isoleucine to threonine).

The second group is based on the Ukrainian isolates and the isolates from different countries of the world with replacement E221D (glutamic acid to aspartic acid substitution) and V412I (valine to isoleucine substitution).

Our isolates were in the group which included the Ukrainian isolates and the isolates from around the world. In this group there were found new substitutions Y155F, D251V, S315G. Our isolates are located along with the viruses from Kharkiv and Baden-Württemberg. This group is very heterogeneous and is similar to the reference virus A/Athens GR / 112/2012 more than to new reference viruses.

Conclusions

The Zhytomyr region isolates were found to be most closely related to the Ukrainian isolate from Kharkov and in the world to the isolates from Germany, Romania, Italy. All these viruses are specified by the substitution I214T. Our influenza viruses of 2013–2014 epidemic season have a high genetic relationship (99 %) to the viruses carrying the I214T substitutions. All nucleotide substitutions in the genomes of the Zhytomyr region isolates are synonymous. The Zhytomir region isolates were placed in a dominant subgroup world 3C of A/Victoria/208 genetic clade. They, like many Ukrainian and world isolates, were similar to the reference strain A/AthensGR/112/2012, more than to new reference viruses.

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Філогенетичний аналіз вірусів грипу A(H3N2), що циркулювали в Житомирській області протягом епідемічного сезону 2013–2014рр.

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Мета. Зробити філогенетичний аналіз генів гемаглютиніну (НА) і нейрамінідази (NA) вірусів грипу A(H3N2), що були поширені в Житомирській області під час епідемічного сезону 2013–2014рр. і порівняти їх з тими, що циркулювали в світі. **Методи.** Лабораторну діагностику здійснювали за допомогою ПЛР у реальному часі (real-time RT-PCR). Було проведено секвенування та філогенетичний аналіз. **Результати.** В епідемічному сезоні 2013–2014 рр. вперше було зроблено секвенування генів НА та NA вірусів грипу A(H3N2), виділених з клінічного матеріалу хворих з Житомирської області та в подальшому увійшли до бази даних вірусів грипу з усього світу (GISAID). **Висновки.** Житомирські ізоляти розташувались поряд з українським ізолятом з Харкова, а серед світових поряд з ізолятами з Німеччини, Румунії, Італії. В послідовностях генів гемаглютиніну було виявлено заміщення I214T. Виділені нами ізоляти в епідемічному сезоні 2013–2014рр. мали високу генетичну спорідненість (99 %) до вірусів, що знаходяться в групі на філогенетичному дереві, тому що мають синонімічне заміщення. Житомирські ізоляти розташувались у домінуючій групі в світі 3С групи 3 генетичного кластеру A/Victoria/208 і були подібними до вакцинного штаму A/Texas/50/2012. В послідовностях генів нейрамінідази суттєвих заміщень виявлено не було. Житомирські ізоляти, як і багато українських та світових ізолятів виявились подібними до референс-штаму A/AthensGR/112/2012, ніж до більш нових референс-вірусів.

Ключові слова: віруси грипу A(H3N2), філогенетичний аналіз, епідемічний сезон, мутації.

Филогенетический анализ вирусов гриппа A(H3N2) которые циркулировали в Житомирской области в период эпидемического сезона 2013–2014гг.

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Цель. Провести филогенетический анализ генов гемагглютинина (НА) и нейраминидазы (NA) вирусов гриппа A(H3N2), которые циркулировали в Житомирской области во время эпидемического сезона 2013–2014гг. и сравнить их с таковыми со всего мира. **Методы.** Лабораторную диагностику проводили с помощью ПЦР в реальном времени (real-time RT-PCR). Было проведено секвенирование и филогенетический анализ. **Результаты.** В эпидемическом сезоне 2013–2014гг. впервые было сделано секвинирование генов НА та NA вирусов гриппа A(H3N2), виделенных из клинического материала больных в Житомирській области и в дальнейшем вошли в базу данных вирусов гриппа со всего мира (GISAID). **Выводы.** Житомирские изоляты разместились рядом с украинским изолятом из Харькова, а среди мировых рядом с изолятами из Германии, Румынии, Италии. В последовательностях генов гемагглютинина было выявлено замещение I214T. Выделенные нами изоляты в эпидемическом сезоне 2013–2014гг. имели высокое генетическое сходство (99 %) к вирусам, которые находяться в группе на филогенетическом дереве, так как имеют синонимическое замещение. Житомирские изоляты разместились в доминирующей субгруппе в мире 3С группы 3 генетического кластера A/Victoria/208. В последовательностях генов нейраминидазы существенных мутаций не выявлено. Житомирские изоляты, как много украинских и мировых изолятов оказались подобными к референс-штамму A/AthensGR/112/2012, чем к более новым референс-вирусам.

Ключевые слова: вирусы гриппа A(H3N2), филогенетический анализ, эпидемический сезон, мутации

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