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Comparative Study of Highly Pathogenic Avian Influenza Strains Isolated in Ukraine in 2005 and 2008

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Objective. To research the molecular characteristics of two HPAI strains – A/Ch/Syvash/02/05/H5N1 and A/Ch/Krasnogvardeysk/58/08/H5N1, which were identified as representatives of the highly pathogenic H5N1 viruses. **Methods.** RNA extraction, real-time polymerase chain reaction (PCR). **Results.** The phylogenetic studies revealed that the above mentioned strains belong to two various genetic lineages originated from the Eastern European strains isolated in 2005, but differ from the viruses introduced to the Central and Western Europe in 2005/2006, and also the lineages consisting of H5N1 viruses isolated in the Europe and Middle East in late 2007. **Conclusions.** Relying on experimental studies, it can be concluded that the strains of A/Ch/Syvash/02/05/H5N1 and A/Ch/Krasnogvardeysk/58/08/H5N1 are highly pathogenic.

Keywords: avian influenza virus, real-time PCR, sequencing, phylogenetic analysis.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) is a widespread avian viral disease. The form of the disease caused by H5N1 subtype has affected many Asian, European and African countries in last five years. The system of effective control and prevention of HPAI is based on the monitoring and specific preventive measures [1, 2].

The diagnosis of avian influenza should be a complex of virological, serological and molecular techniques. The last group is represented by detection and identification methods for virus type and subtype recognizing using classical and real-time reverse transcription polymerase chain reaction (RT-PCR, rRT-PCR), followed by assessment of virulence by the establishment of amino acid sequence at the cleavage site of hemagglutinin (HA) [3]. Phylogenetic studies aiming to establish relationship between virus isolates are also becoming a part of routine diagnostic procedures.

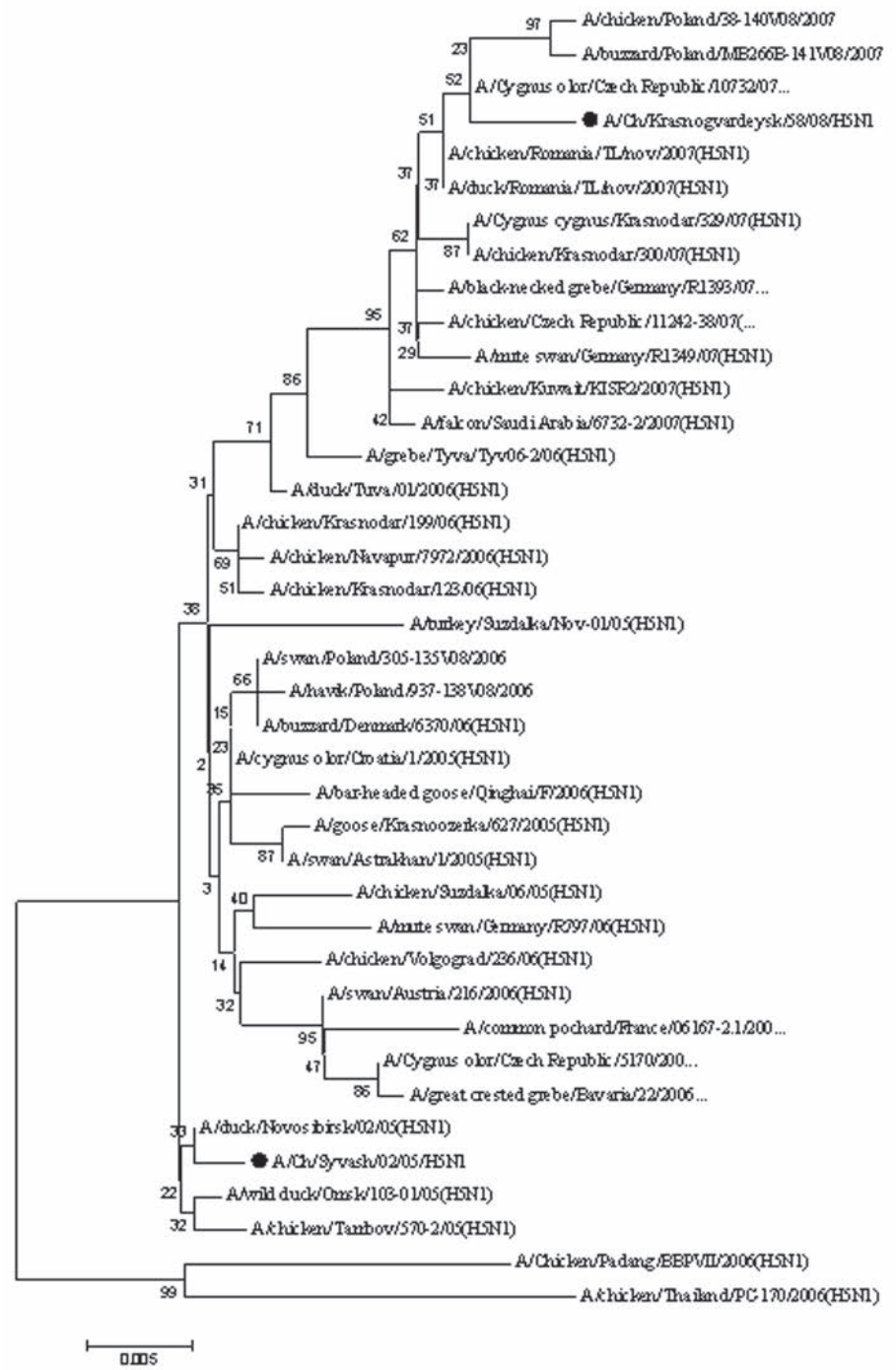
The aim of this study was to confirm the HPAI nature of Ukrainian strains isolated during the Crimea outbreaks, and to compare them to other HPAI strains from various countries isolated in 2005−2007.

MATERIALS AND METHODS

This study was performed in the Molecular Diagnostics Department of the National Scientific Center – Institute of Experimental and Clinical Veterinary Medicine (Kharkiv, Ukraine) and Department of Poultry Diseases of the National Veterinary Research Institute (Pulawy, Poland).

Viruses. A formalin-inactivated seed of two HPAI isolates − A/Ch/Syvash/02/05 and A/Ch/Krasnogvardeysk/58/08 – was used as a material for RNA extraction.

RNA-extraction and (r)RT-PCR. RNA extraction was performed with the use of RNA-extraction kit (QIAGEN, USA) based on the affine sorbtion method. The real time RT-PCR for *M* and *H5*, *N1* genes was carried out based on the previously described protocols [4−6] with own modifications. The amplification of approximately 300 bp region encompassing the cleavage site *HA* gene was carried out in the classical RT-PCR [7]. For phylogenetic studies, two additional RT-PCRs



The phylogenetic relationship of Ukrainian strains − A/Ch/Syvash/02/05/H5N1 and A/Ch/Krasnogvardeysk/58/08/H5N1 (highlighted with «•» sign) and other European and Asian HPAI viruses (constructed by NJ, BS value = 1000)

were performed using primers designed in the NVRI, Pulawy. All classical RT-PCRs produced products consisting of three overlapping regions of the total length of approx. 1200 bp. Both real-time and classical RT-PCR were performed using the Qiagen reagents.

Sequencing. Sequencing of amplified products after purification (Qiagen DNA Purification kit) was performed in the Genomed Sequencing Center (Warszawa) by the ABI-technology. The corrected sequences were aligned with HA-gene sequences of H5N1 viruses isolated in the Asian and European countries between 2005−2008 (n = 37) using Clustal W. The phylogenetic tree was constructed by the Neighbour Joining algorithm (BS value = 1000) in Mega 3.1 software [8]. The translation of nucleotides into amino acids was done (Mega 3.1) and the sequence of amino acids at the cleavage site of *HA* was established.

RESULTS AND DISCUSSION

The first step of our research showed that viruses A/Ch/Syvash/02/05 and A/Ch/Krasnogvardeysk/58/08 are the representatives of Influenza A virus. More accurate typing gave us the possibility to determine the subtype of isolates as H5N1 according to the results of *HA* and *NA* genes amplification by rRT-PCR.

The results were confirmed in the conventional RT-PCR(s)/H5. The PCR products of A/Ch/Syvash/02/05/H5N1 and A/Ch/Krasnogvardeysk/58/08/H5N1, obtained in 40-cycles and 45-cycles amplification runs, were clearly visible on electrophoregramms (the size of the bands was 545, 300, and 651 bp in three applied PCR) and did not contain any additional unspecific bands. Purified PCR-products were determined in the concentrations of 19.7 and 24.9 ng/µl, respectively.

The amino acid sequences of the HA cleavage site were typical of HPAI (the motif PQGERRRKKR*GLF found in both isolates contained multiple basic amino acids).

At the nucleotide level, both Ukrainian isolates shared approx. 97 of similarities. The phylogenetic analysis (Figure) revealed that they located on different branches of phylogenetic tree. The strain A/Ch/Syvash/02/05/H5N1 located closely to some H5N1 strains isolated in Russia but it was clearly different from other strains isolated in Europe in 2005 and 2006. That would suggest that it does not belong to the lineage, which was introduced to the Central and Western Europe in 2005/2006. The strain A/Ch/Krasnogvardeysk/58/08/H5N1 grouped together with H5N1 strains isolated in Europe (Poland, Romania, Russia, Germany, Czech Republic) and the Middle East (Kuwait, Saudi Arabia) in the second half of 2007.

CONCLUSIONS

In accordance with experimental data, the strains A/Ch/Syvash/02/05/H5N1 and A/Ch/Krasnogvardeysk/58/08/H5N1 were determined as highly pathogenic. They belong to two separate genetic lineages, what means that there were at least two independent introductions of H5N1 to the Ukrainian poultry flocks. These findings, based on the analysis of 864 bp region of HA gene (approx. half of the *HA* gene), are statistically reliable, but need to be confirmed by the sequencing of the whole genome.

PROSPECTS FOR FURTHER STUDIES

Experimental data will be used for GenBank submission, in future it is planned to make full-genome sequencing of HPAI strains A/Ch/Syvash/02/05/H5N1 and A/Ch/Krasnogvardeysk/58/08/H5N1.

Порівняльний аналіз ізолятів вірусу високопатогенного грипу птиці, виділених в Україні у 2005 та 2008 рр.

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Мета. Вивчити молекулярні характеристикі двох штамів вірусу грипу птиці − A/Ch/Syvash/02/05/H5N1 і A/Ch/Krasnogvardeysk/58/08/H5N1, ідентифіковані як представники високопатогенного вірусу H5N1. **Методи**. РНК-екстракція, полімеразно-ланцюгова реакція у реальному часі. **Результати**. Філогенетичні дослідження показали, що зазначені штами належать до двох різних генетичних ліній та походять від штамів зі Східної Європи, виділених у 2005 році, але відрізняються від вірусів, поширених у Центральній і Західній Європі у 2005/2006, а також лінії, що поєднує віруси H5N1, ізольовані в Європі і на Близькому Сході наприкінці 2007 року. **Висновки**. Грунтуючись на експериментальних дослідженнях, можна зробити висновок стосовно того, що штами A/Ch/Syvash/02/05/H5N1 і A/Ch/Krasnogvardeysk/58/08/H5N1 є високопатогенними.

Ключові слова: вірус грипу птиці, ПЛР у режимі реального часу, секвенування, філогенетичний аналіз.

Сравнительный анализ изолятов вируса высокопатогенного гриппа птицы, выделенных в Украине в 2005 и 2008 гг.

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Цель. Изучить молекулярные характеристики двух штаммов вируса гриппа птицы − A/Ch/Syvash/02/05/H5N1 и A/Ch/Krasnogvardeysk/58/08/H5N1, идентифицированных как представители высокопатогенного вируса H5N1. **Методы.** РНК-экстракция, полимеразная цепная реакция в реальном времени. **Результаты.** Филогенетические исследования показали, что указанные штаммы относятся к двум разным генетическим линиям и происходят от штаммов из Восточной Евро-

пы, выделенных в 2005 году, но отличаются от вирусов, распространенных в Центральной и Западной Европе в 2005/2006, а также линии, объединяющей вирусы H5N1, изолированные в Европе и на Ближнем Востоке в конце 2007 года. **Выводы.** Основываясь на экспериментальных исследованиях, можно сделать вывод о том, что штаммы A/Ch/Syvash/02/05/H5N1 и A/Ch/Krasnogvardeysk/58/08/H5N1 являются высокопатогенными.

Ключевые слова: вирус гриппа птицы, ПЦР в реальном времени, секвенирование, филогенетический анализ.

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