

ANNOTATION

to the doctoral thesis for Doctor of Philosophy (PhD) degree on the specialty
«8D05105-Biotechnology»

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«Genotyping of avian influenza strains wide spread in the territory of the Republic
of Kazakhstan»

General characterization of the dissertation research. The work is devoted to the identification and isolation of strains of avian influenza virus circulating in the territory of the Republic of Kazakhstan in 2020-2022, the study of their virological and molecular genetic properties, as well as the identification of signs of phylogenetic relationship in order to recognize their distribution and evolution.

Research relevance. Influenza outbreaks in poultry farms cause significant economic damage worldwide, leading to the complete destruction of poultry. Migratory birds, especially waterfowl, are a natural reservoir of avian influenza viruses (AIV). Thus, the infection gets into water bodies and can be transmitted to other poultry through domestic waterfowl. In order to somehow predict the possibility of introducing the virus and prevent its widespread distribution, forecasting is important. The introduction of forecasting of varying duration into practice allows us to significantly improve the strategy and tactics of epizootological monitoring of avian influenza, and minimize the negative consequences of the growth of epizootic activity of foci.

Influenza is caused by viruses belonging to the genus *Influenza virus* of the *Orthomyxoviridae* family. There are four types of influenza virus - A, B, C and D. The genome of avian influenza type A is a single-stranded RNA-containing virus with a segmented genome. They are classified into subtypes based on the antigenicity of the glycoproteins hemagglutinin (HA) and neuraminidase (NA). Currently, 19 hemagglutinin and 11 neuraminidase subtypes of viruses are known, which occur in many different combinations. Influenza viruses affect not only birds but also humans, birds, and various mammals. Cases of human infection with AIV subtypes H1-H3, H5, H7, and H9 have been reported. Molecular changes in the RNA genome occur due to two main mechanisms: point mutation (antigenic drift) and reassortment of RNA segments. Point mutations cause minor changes in the antigenic nature of the virus and are the main reason for annual vaccination against influenza A. Recombination occurs when a host cell is infected with two or more types of avian influenza virus, resulting in the emergence of a new type.

In recent years, the number of outbreaks of highly pathogenic avian influenza among domestic and wild birds has increased sharply. Several subtypes of AIV, namely H5, H7 and H9, overcome the interspecies barrier, infecting humans and other mammals.

In Kazakhstan, in the summer of 2005, the first outbreak of highly pathogenic avian virus H5N1 was registered at the Nan poultry farm, located in the village of Golubovka in the Pavlodar region. The last major spread of AIV, which caused mass deaths of poultry at poultry farms, was registered in seven regions of Kazakhstan in 2020. This year, outbreaks of the disease have been registered in dozens of countries around the

world, causing enormous damage to the poultry industry. As a result of genetic analysis of the pathogen, it was found that the virus is a reassortant closely related to the influenza virus, registered in Iraq in May of the same year.

The epizootic situation among poultry in many countries of the world, including the Republic of Kazakhstan, remains tense, and outbreaks of infectious viral diseases occur from time to time. Monitoring the epizootic situation is the main element of anti-epizootic measures to prevent the emergence and spread of bird flu among agricultural birds. Some types of bird flu can be zoonotic and therefore pose a serious threat to poultry farming in Kazakhstan. Since the bird flu virus is constantly evolving through mutations and recombination, it has a significant impact on the health and productivity of animals.

The spread of the virus is facilitated by the wide nesting areas of many birds encountered during seasonal migrations. In the republic, 130 species of birds have been registered during the nesting, molting, seasonal migrations and wintering periods. Every year, the number of nesting bird species reaches 10 million, 2-3 million birds arrive for molting, and about 50 million migratory birds stop at water bodies during spring and autumn migrations. At the same time, migratory birds fly over and nest almost throughout the entire territory of the republic. Therefore, closely related strains of the influenza A virus can be detected in birds from different remote areas and, conversely, different variants of the virus can be isolated in limited areas. This is confirmed by the chronology and nature of outbreaks, phylogenetic analysis data in published studies.

Therefore, an outbreak of avian influenza, which is recorded on a large scale, draws special attention to the need to combat the spread of AIV and requires timely and effective preventive measures.

Systematic molecular biological screening of biological samples collected from wild birds during spring-summer migration and from poultry kept in private farms near the migration route of birds in various regions of Kazakhstan significantly expands knowledge in the field of identifying patterns of distribution in the ongoing evolution of AIV, and can also contribute to the selection of relevant vaccine strains and minimize the role of birds as a source of new, potentially dangerous variants of avian influenza.

Research objects: avian influenza virus type A (*Influenzavirus*) isolated from nasopharyngeal and cloacal swabs, as well as pathological material of sick birds.

Research aim. Investigation of molecular biological characteristics and phylogenetic analysis of avian influenza strains circulating among wild and domestic birds in the Republic of Kazakhstan in 2020-2022.

Research objectives:

1. Collection of samples from poultry kept in private farms near the bird migration route in various regions of Kazakhstan for the purpose of monitoring AIV. Conduct serological and molecular biological screening of the collected samples.
2. Conducting sampling of birds during an outbreak of AIV and determining the virus subtype using PCR screening.
3. Isolation of virus strains in chicken embryos and sequencing of their complete genome using the Sanger method.
4. Phylogenetic analysis of all eight genes of the studied AIV strains and identification of molecular markers in order to determine their pathogenicity.
5. Comparative study of biological properties and antigenic relationships of AIV

strains.

6. Development of a PCR test system for the diagnosis of AIV.

Research Methods. The work was carried out using virological, serological (enzyme-linked immunosorbent assay, GGA, HAI, etc.), molecular biological methods (polymerase chain reaction, sequencing) studies and bioinformatics analysis methods were used (Clustal W algorithm, "neighbor joining" method, Tamura-Nei model, BLASTn analysis).

Scientific novelty

- In the period 2020-2022, AIV monitoring was carried out in six regions located on the route of migratory birds and biological samples were collected from poultry in private farms located near the bird migration route;

- Four AIV strains were isolated - A/Chicken/North Kazakhstan/184/2020 (H5N8), A/Mute swan/Mangystau/9421/2022 (H5N1), A/Caspian tern/Atyrau/9184/2022 (H5N1) and A/Chicken/Almaty/220/2020 (H9N2) from wild and domestic birds and their genomes were fully sequenced;

- A phylogenetic analysis and molecular genetic characterization of the studied strains were carried out;

- For the first time in Kazakhstan, the H9N2 strain was isolated from poultry;

- New unique point mutations that were not previously registered in the AIV genome were identified; a real-time PCR test system for the detection and differentiation of avian influenza virus based on the A/Chicken/North Kazakhstan/184/2020 (H5N8) strain isolated from the North Kazakhstan region was developed and a patent was obtained.

Theoretical and practical significance

1. The complete genome sequencing of A/Chicken/North Kazakhstan/184/2020 (H5N8), A/Mute swan/Mangystau/9421/2022 (H5N1), A/Caspian tern/Atyrau/9184/2022 (H5N1) and A/Chicken/Almaty/220/2020 (H9N2) was carried out. A comparative analysis of the studied strains with strains registered in Kazakhstan and neighboring countries showed their close relationship with the viruses of China, Russia and the Middle East.

2. For the first time in Kazakhstan, the H9N2 influenza strain - A/Chicken/Almaty/220/2020 was isolated from poultry and molecular markers associated with the adaptation of AIV to mammals were determined.

3. The international GenBank and GISAID databases freely contain nucleotide sequences of all genes of AIV A/Chicken/North Kazakhstan/184/2020 and A/Caspian tern/Atyrau/9184/2022 under numbers OP740951.1 – OP740959.1 and OQ804408.1 – OQ804415.1, which makes it possible to use them for local and global comparative phylogenetic analysis with AIV strains currently circulating.

4. 2 patents for utility models were received: No. 7497 "Method for developing a real-time PCR test system for detecting and differentiating avian influenza virus" and No. 8586 "Avian influenza virus strain clade 2.3.4.4b A/ Caspian tern/Atyrau/9184/2022 (H5N1), used for the preparation of diagnostic drugs and vaccines".

5. A methodological guide "Zoning and regionalization of the territory of the Republic of Kazakhstan taking into account the analysis of the risks of introduction and the possibility of spreading the highly pathogenic avian influenza virus" has been

published.

Provisions, submitted for the defense:

1. In various regions of Kazakhstan in 2020-2022, 3383 biological samples were collected from wild and domestic birds. Screening of samples in ELISA, PCR and RTGA revealed circulation of AIV. Of the samples obtained from the Northern, Western and South-Eastern regions of Kazakhstan, four AIV strains were isolated from chicken embryos.

2. According to phylogenetic analysis, three genotypes of the avian influenza virus were identified in the territory of the Republic of Kazakhstan, belonging to the subtypes H5N1, H5N8 and H9N2.

3. Molecular biological and phylogenetic analysis of the HA gene in the studied Kazakhstani isolates showed that the three H5 isolates are closely related strains, and the H9N2 strain is not related to the previously isolated Kazakhstani H9N2 viruses isolated from wild birds.

4. In the structure of the NA protein of the A/Chicken/Almaty/220/2020 (H9N2) virus, a deletion of the TEI amino acids (encoding threonine (T), glutamic acid (E) and isoleucine (I)) was found at position 63-65, which is a highly pathogenic marker of the A/Duck/HongKong/Y280/97 strain isolated during the epidemic of poultry in China.

5. Molecular markers associated with the adaptation of AIV to mammals and previously unreported point mutations were identified in the genome of the studied isolates.

Research results and conclusions:

1. As a result of serological screening of 3 286 blood serum samples collected from poultry in private households in Almaty, East Kazakhstan, Akmola, West Kazakhstan, Kostanay and North Kazakhstan regions, 100 samples were found to be positive for H5 virus antibodies. And molecular biological screening of these samples by the PCR with reverse transcription method revealed a weakly positive result for the M-gene in only 5 samples collected in the Akmola region.

2. As a result of screening of 109 samples collected in the Almaty, North Kazakhstan, Mangystau and Atyrau regions in 2020 and 2022, during the outbreak of AIV, the circulation of subtypes H5N8, H9N2 and H5N1 was detected.

3. Of the 10 PCR-positive samples on chicken embryos, four strains were isolated: A/Chicken/Almaty/220/2020, A/Chicken/North Kazakhstan/184/2020, A/Mute swan/Mangystau/9421/2022 and A/Caspian tern/Atyrau/9184/2022.

4. As a result of phylogenetic analysis, the cleavage site of the HA gene of the H5N8 isolate - A/Chicken/North Kazakhstan/184/2020 and H5N1 - A/Mute swan/Mangystau/9421/2022 and A/Caspian tern/Atyrau/9184/2022 - consists of the sequence PLREKRKR↓GLF, which is possibly identical to the motif of highly pathogenic AIV variants detected in European countries, including the UK in 2016-2017. And the motif of the A/Chicken/Almaty/220/2020 (H9N2) cleavage site - PSRSSR↓GLF was the same as the low-pathogenic H9N2 isolates of the Y280 lineage registered in China in 2018.

5. Despite the low pathogenicity of the A/Chicken/Almaty/220/2020 isolate, several molecular markers were found in the H9N2 genome that increase its adaptability to mammals. The nucleotide sequence of the complete genome of AIV A/Chicken/North

Kazakhstan/184/2020 (OP740951.1 - OP740959.1) and A/Caspian tern/Atyrau/9184/2022 (OQ804408.1 - OQ804415.1) has been published in the international GenBank and GISAID databases.

6. The A/Chicken/North Kazakhstan/184/2020(H5N8) isolate, isolated from the North Kazakhstan region, served as the basis for the development of a real-time PCR test system for the detection and differentiation of avian influenza viruses.

Relation to the main scientific works' plan. The dissertation work was carried out in the virology laboratory of the Kazakh Veterinary Research Institute within the framework of the Ministry of Agriculture of the Republic of Kazakhstan BR10764899-OT-22 "Study the epizootic characteristics of the country's territory for especially dangerous diseases and develop veterinary and sanitary measures to improve their effectiveness" and BR10764975-OT-22 "Develop and propose for production means and methods for diagnostics, disease prevention, therapy of infected animals", as well as in the virus ecology laboratory of the Scientific and Production Center "Microbiology and Virology" within the framework of the project of the Ministry of Education and Science of the Republic of Kazakhstan AP19678255 "Study of the epidemic potential of natural variants of influenza A viruses circulating among birds and mammals in Kazakhstan".

Work approbation. The results of the dissertation work were published and presented at the international conference of students and young scientists "Farabi Alemi" (Almaty, 2021) and the scientific conference Achievements of young scientists and specialists of Kazakhstan in the field of veterinary science, biotechnology and food safety (Almaty, 2020). Also partially included in the research report for projects BR10764899-OT-22, BR10764975-OT-22 and AP19678255.

Publications. According to the requirements of the Regulations on the Dissertation Council of Al-Farabi KazNU the materials of the dissertation are reflected in 12 publications, including 2 articles in journals included in the Web of Science database, 5 articles from the list of the Committee for Control in the Sphere of Education and Science and 1 dissertation at an international conference. Also, 2 patents for utility models were received - No. 7497 (2022) and No. 8586 (2024).

The content and structure of the dissertation. The dissertation is set out on 114 pages and consists of the following sections: list of abbreviations, introduction, literature review, materials and methods, results, conclusions, 186 references. The thesis contains 18 tables, 31 figures and five appendices.