

## RESEARCH ARTICLE



### MOLECULAR AND PHYLOGENETIC CHARACTERIZATION OF THE HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 VIRUS DETECTED IN THE REPUBLIC OF MOLDOVA

Oxana GROZA<sup>id</sup>, Nicolae STARCIUC<sup>id</sup>

Technical University of Moldova, Chisinau, Republic of Moldova

Corresponding author: Oxana Groza, e-mail: [oxana.groza@doctorat.utm.md](mailto:oxana.groza@doctorat.utm.md)

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#### ABSTRACT

|                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
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| <b>Introduction</b>          | The H5N1 avian influenza virus is one of the most virulent antigenic combinations, including those with zoonotic pathogenic potential. As the number of avian influenza outbreaks has increased globally, a corresponding rise in outbreaks caused by this virus has also been recorded in the Republic of Moldova.                                                                                                                                                                                                                                                                                                                                                                                                       |
| <b>Materials and methods</b> | The descriptive study employed epidemiological, clinical and molecular biology (RT-PCR) research methods. The research material consisted of carcasses of domestic and wild birds, as well as blood serum samples collected from various regions of the republic. Identification of the influenza virus in the research material was carried out at the National Reference Laboratory Public Institution National Center for Animal, Plant Health and Food Safety (PI. NCAPHFS) and the EU Reference Laboratory (EURL, IZSve). The following diagnostic kits were used for virus identification: the "IndiSpin® Pathogen Kit" (QIAGEN® GmbH for INDICAL BIOSCIENCE) and the "Virotype® Influenza A RT-PCR Kit" (Germany). |
| <b>Results</b>               | The molecular structure of HPAI A(H5N1) viruses isolated from the Republic of Moldova demonstrated the presence of genetic mutations in the PB2 protein (E627K and/or D701N) associated with viral adaptation to mammalian hosts and isolates with European H5 viruses belonging to the EA-2021-I, EA-2022-CH, EA-2022-AB, and EA-2023-DB genotypes.                                                                                                                                                                                                                                                                                                                                                                      |
| <b>Conclusions</b>           | The molecular characterization of HPAI A(H5N1) isolated from birds in the Republic of Moldova demonstrated the presence of genetic mutations in the PB2 protein and in the hemagglutinin protein (S133A, S154N, and T156A), which are associated with adaptation to human-type receptors.                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| <b>Keywords</b>              | Virus, highly pathogenic, birds, molecular research, avian influenza.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |

#### CARACTERISTICA MOLECULARĂ ȘI FILOGENETICĂ A VIRUSULUI GRIPEI AVIARE ÎNALT PATOGEN H5N1 DETECTAT ÎN REPUBLICA MOLDOVA

|                            |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Introducere</b>         | Virusul gripei aviare H5N1 reprezintă una dintre cele mai virulente combinații antigenice, inclusiv cu potențial patogen zoonotic. Odată cu creșterea numărului de focare de gripă aviară la nivel global, s-a înregistrat o creștere a numărului de focare cu acest virus și pe teritoriul Republicii Moldova.                                                                                                                                                                                                                                                                                                                                                                                                                      |
| <b>Materiale și metode</b> | În prezentul studiu au fost utilizate cercetări epidemiologice, clinice și de biologie moleculară RT-PCR. În calitate de material de cercetare au constituit cadavrele păsărilor domestice și sălbatice și probele de ser sanguin colectate din diferite regiuni ale republicii, iar identificarea virusului gripal din materialul pentru cercetare s-a efectuat în cadrul Laboratorului Național de Referință Instituția Publică Centrul Național Sănătatea Animalelor, Plantelor și Siguranța Alimentară și Laboratorul de Referință UE (EURL, IZSve). În cercetare au fost folosite chiturile de diagnostic: "IndiSpin® Pathogen Kit" (QIAGEN® GmbH for INDICAL BIOSCIENCE și kitul "Virotype® Influenza A RT-PCR Kit", Germania. |
| <b>Rezultate</b>           | Structura moleculară a virusurilor HPAI A(H5N1) izolate din Republica Moldova a evidențiat prezența unor mutații genetice în proteina PB2 (E627K și/sau D701N) asociate cu adaptarea virală la gazde mamifere, precum și a unor izolate înrudite cu virusurile H5 europene aparținând genotipurilor EA-2021-I, EA-2022-CH, EA-2022-AB și EA-2023-DB.                                                                                                                                                                                                                                                                                                                                                                                 |
| <b>Concluzii</b>           | Caracterizarea moleculară a virusului HPAI A(H5N1) izolat de la păsări în Republica Moldova a demonstrat prezența unor mutații genetice în proteina PB2 și în proteina hemagglutinină (S133A, S154N și T156A), asociate cu adaptarea la receptorii de tip uman.                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| <b>Cuvinte-cheie</b>       | Virus, înalt patogen, păsări, cercetări moleculare, gripa aviară.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |

## INTRODUCTION

The avian influenza virus (AIV) is one of the best-known pathogens, and its persistent circulation has a significant impact on public health, the global economy, the poultry industry, international trade, and the health of wild birds. These are segmented, single-stranded RNA viruses belonging to the *Orthomyxoviridae* family and are highly variable (1). The genetic and antigenic variability of the surface glycoproteins HA and NA is particularly pronounced. To date, sixteen HA (H1–H16) and nine NA (N1–N9) subtypes circulate in wild waterfowl (2), and two new HA subtypes (H17 and H18) and two new NA subtypes (N10 and N11) have recently been identified in bats (3–6).

Compared with the classification based on the antigenic properties of HA and NA, avian influenza viruses are also classified according to their pathogenicity in avian species into two categories: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). Most avian influenza viruses are low pathogenic (LPAI) and cause either mild disease or asymptomatic infection, depending on the virus strain and the infected host species. However, certain subtypes, particularly H5 and H7, may evolve into highly pathogenic avian influenza (HPAI) viruses. The current and largest avian influenza epizootic in modern history began with the emergence of the highly pathogenic H5N1 avian influenza virus in Guangdong Province, China, in 1996 (7,8). In 2005, H5N1 viruses belonging to clade 2.2 caused the first major outbreak among wild birds at Qinghai Lake, China. Subsequently, through the migration of wild birds, the virus spread from Asia via Siberia to Europe, the Middle East, and Africa within a few months (9,10). The highly pathogenic avian influenza A(H5N1) virus became widely distributed, affecting both wild birds and domestic poultry across Asia, Europe, and Africa. By the end of 2021, it had also been detected in North America, indicating its further intercontinental spread (11). Outbreaks of highly pathogenic H5N1 avian influenza have caused substantial economic losses to the poultry industry, while sporadic transmission to humans in several countries has highlighted its potential threat to public health. Since 2003, a total of 889 sporadic human cases of avian influenza caused by different clades of HPAI A(H5N1) viruses have been reported across 23 countries (12). Although the exact number of farmed birds affected by these outbreaks is unknown, it is estimated to be in the hundreds of millions (10). Mortality among wild birds due to infection with highly pathogenic avian influenza (HPAI) viruses has historically been considered a rare event. HPAI A(H5N1) infection in wild birds was first reported in Hong Kong in 2002 (2). Wild birds constitute the natural reservoir of influenza A viruses, which typically cause asymptomatic infections.

Based on the above, interest in this disease has also increased in the Republic of Moldova, particularly in late 2023 and early 2024, following the occurrence of mass swan deaths in some bodies of water within the Republic of Moldova. The aim of this descriptive study was to identify and characterize the HPAI H5N1 virus isolated in the Republic of Moldova through whole-genome sequencing and phylogenetic analysis.

## MATERIALS AND METHODS

### Methodological Approach

A descriptive, quantitative epidemiological study was conducted, combining field surveillance with advanced laboratory diagnostic techniques. Primary diagnostic processing took place at the National Reference Laboratory (P.I. National Center for Animal and Plant Health, and Food Safety, Chisinau),

while confirmatory virological analyses, high-throughput sequencing, and phylogenetic tree reconstructions were conducted in collaboration with the EU Reference Laboratory for Avian Influenza and Newcastle Disease (EURL, IZSve, Legnaro, Italy). For the laboratory procedures, were followed these steps: Viral RNA Extraction: Performed using the IndiSpin® Pathogen Kit (QIAGEN / INDICAL BIOSCIENCE). Influenza A screening were executed via real-time RT-PCR targeting the Matrix (M) gene using the Virotype® Influenza A RT-PCR Kit (Germany). *Subtype identification* of H5, H7, and N1 genes was carried out using specific primers and probes (Generi Biotech; Primer Design Home of Genesing® H5N1 range), run on a Quant Studio 5 Real-Time PCR System (Thermo Fisher Scientific).

### Study Period and Study Area

The study period ran from December 2023 through 2024. Field sampling covered aquatic habitats – such as lakes and river basins – across northern and central districts like Sîngerei, Drochia, Rîșcani, and Florești, alongside poultry populations from backyard holdings. Whenever an H5N1 avian influenza outbreak was triggered a 3 km quarantine zone was established around the area and 59 samples were collected from it to closely monitor the epidemiological situation. Samples from suspected cases were gathered by inspectors from local NFSA (National Food Safety Agency) territorial subdivisions by filling out official minutes. They strictly followed the sampling, packaging, and transport rules laid out in the Regulation on the methodology for sampling, packaging, identification, and transport of samples intended for laboratory examinations in the field of animal health and welfare. All laboratory investigations were carried out at the National Reference Laboratory.

### Study Population

The study populations were focused on two main groups: wild aquatic birds living in natural habitats and domestic poultry kept in backyard holdings. Were collected and analyzed a total of 31,820 specimens during the surveillance period. The biological samples gathered included a tracheal and cloacal swabs collected from live birds, as well as parenchymal tissues (including lung, trachea, and brain) retrieved from carcasses and native blood serum used for antibody evaluation. The sampling strategy was divided into two main approaches: passive surveillance- investigations specifically targeting wild bird mortality within natural aquatic habitats and active surveillance – targeted monitoring carried out directly across backyard holdings.

### Statistical Analysis

The statistical analysis of data were gathered, organized, and evaluated using descriptive statistical techniques in Microsoft Excel. We determined positivity rates by dividing the number of positive real-time RT-PCR outcomes by the total number of samples tested. Microsoft Excel helped structure our database, compute absolute numbers and percentages, and track epidemiological variables across different districts and sample types. Furthermore, sequence alignments and phylogenetic tree building were carried out using standard bioinformatic tools along with reference sequences supplied by the EURL (IZSve). Following this, several high-mortality outbreaks were recorded along the Răut River – specifically in Moara de Piatră (Drochia district), Răcăria (Rîșcani district), and Putinești and Lunga (Florești district) – an escalation influenced in part by seasonal weather patterns.

## RESULTS

Based on epidemiological investigations, it was determined that in December 2023, cases of mass mortality among mute swans (*Cygnus olor*) were reported in the Republic of Moldova, in the area around the village of Cubolta in the Sîngerei district, from where samples were collected from 21 swans and sent to the P.I. National Center for Animal, Plant Health, and Food Safety (P.I. NCAPHFS). Laboratory results confirmed the presence of the highly pathogenic avian influenza virus (HPAI H5N1). Subsequently, cases of high mortality were recorded along the Răut River (Moara de Piatră village, Drochia District; Răcăria village, Rîșcani District; Putinești village; and Lunga village, Florești District). Subsequently, additional new cases of the disease were recorded in the northern districts of the republic, facilitated by climatic conditions (13). The number of positive avian influenza cases reached 245 out of 31,820 samples submitted to the laboratory from across the entire territory of the Republic of Moldova. The geographic distribution of confirmed cases of avian influenza is shown in Figure 1.

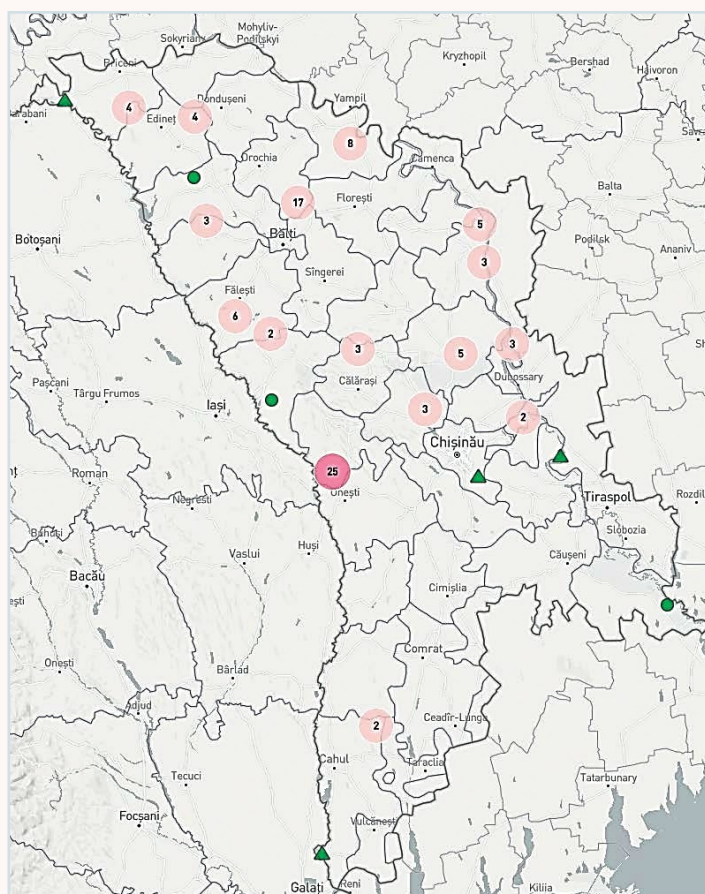


**Figure. 1.** Geographic distribution of avian influenza outbreaks in the Republic of Moldova during 2023–2024.

An analysis of the geographic locations of avian influenza cases reveals that most cases were recorded in the northern and central districts of the Republic of Moldova. In the southern region, only one case of avian influenza was recorded in the Cahul district; in the western districts, two cases were recorded, specifically in the Căușeni and Ștefan Vodă districts.

The laboratory investigations were aimed at detecting and typing the avian influenza virus, as well as detecting specific antibodies. The Animal Health and Veterinary Medicines Laboratory (LDSA) within the NCAPHFS Public Institu-

tion processed 31,820 samples between December 2023 and November 2024, conducting 57,502 laboratory tests, including blood serum samples using diagnostic methods recommended by the European Union Reference Laboratory for Avian Influenza and Newcastle Disease (Istituto Zooprofilattico Sperimentale delle Venezie (IZSve), Legnaro, Italy). The testing included the detection of antibodies against avian influenza viruses using the hemagglutination inhibition assay (HI), with the use of reference reagents: inactivated antigen, international reference strain: H5N8-A/turkey/Italy/7898/2014; H5N1-A/mallard/Italy/3401/05; H5N3-A/duck/Italy/775/04; H7N7A/macaw/England/626/80; H7N1-A/turkey/Italy/1067/99; – International reference positive serum – H5N1, H5N8, H7N1, H7N7; – International reference negative serum.

As a result of laboratory testing, the presence of the avian influenza virus was confirmed using the Real-Time RT-PCR method to identify the viral genome (matrix) in all samples submitted. Following the confirmation of avian influenza outbreaks in the Republic of Moldova, the National Agency for Food Safety (ANSA) implemented sanitary and veterinary measures to identify, localize, and eliminate the outbreaks, and during the quarantine periods, a varying number of biological samples (blood serum) were systematically collected and submitted, which included poultry from localities within the protection zones and surveillance zones.

Simultaneously, avian influenza virus identification was performed on 19 samples of pathological material (trachea and brain) at the P.I. NCAPHFS National Reference Laboratory, which confirmed the presence of the highly pathogenic H5N1 subtype in samples collected from wildlife. The results of this descriptive study are presented in Table 1.

**Table 1.** Identification and Pathogenicity of the Virus.

| Nr.                                                                                 | Analysis (Method)                                                                                                                            | Sub-analysis                                     | Results  |
|-------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|----------|
| <b>19 samples of pathological material (trachea and lungs) collected from swans</b> |                                                                                                                                              |                                                  |          |
| 1                                                                                   | Influenza A virus (Reverse Transcriptase Real-Time PCR qualitative / PDP VIR 018 2022 Rev. 7)                                                |                                                  | Positive |
| 2                                                                                   | Influenza A virus subtype H5 (Reverse Transcriptase Real-Time PCR qualitative / PDP VIR 143 2022 Rev. 3)                                     |                                                  | Positive |
| 3                                                                                   | Pathogenicity assessment and phylogenetic typing of the Eurasian H5 avian influenza virus by Reverse Transcriptase Real-Time PCR qualitative | H5 Clade 2.3.4.4b                                | Positive |
|                                                                                     |                                                                                                                                              | H5 CS-HPAI                                       | Positive |
| 4                                                                                   | Influenza A virus – neuraminidase subtype (qualitative PCR Real-Time)                                                                        | Avian influenza virus – neuraminidase subtype N1 | Positive |

An analysis of the phylogenetic results presented in Table 1 shows that the examination of the nine genetic segments demonstrates that the HPAI H5N1 viruses from clade 2.3.4.4b identified in the Republic of Moldova belong to two distinct genotypes. Two viruses belong to genotype DA, which began spreading in Europe in September 2023 and has been reported in Austria, Bulgaria, France, Germany, Italy, Romania, and Slovenia. At the same time, seven viruses belong to genotype DI, a new genotype detected in Poland between December 2023 and January 2024, indicating an event of genetic diversification and geographic spread that requires continuous monitoring. These results are presented in Table 2, which shows that in the confirmed avian influenza outbreaks in the Republic of Moldova, the avian influenza virus with the H5N1 antigenic combination was confirmed, with variable characteristics of the viral genome (Table 2) and a predominance of the DA and DI genotypes.

**Table 2.** Genomic Characterization of the HPAI H5N1 Virus by the EU Reference Laboratory.

| Virus                                  | Subtype | Date of Collection | Genotype |
|----------------------------------------|---------|--------------------|----------|
| A/swan/Moldova/8917-4 24VIR442-1/2023  | H5N1    | 15/12/2023         | DI       |
| A/swan/Moldova/8921-2 24VIR442-5/2023  | H5N1    | 16/12/2023         | DI       |
| A/swan/Moldova/8956 24VIR442-7/2023    | H5N1    | 18/12/2023         | DA       |
| A/swan/Moldova/9020 24VIR442-10/2023   | H5N1    | 20/12/2023         | DI       |
| A/swan/Moldova/9045 24VIR442-11/2023   | H5N1    | 21/12/2023         | DA       |
| A/swan/Moldova/9046 24VIR442-12/2023   | H5N1    | 20/12/2023         | DI       |
| A/swan/Moldova/9048 24VIR442-14/2023   | H5N1    | 20/12/2023         | DI       |
| A/swan/Moldova/9115-1 24VIR442-15/2023 | H5N1    | 28/12/2023         | DI       |
| A/swan/Moldova/9201-3 24VIR442-19/2023 | H5N1    | 27/12/2023         | DI       |

These results confirm that the molecular structure of viruses isolated in the Republic of Moldova shows that some virus isolates contain molecular markers with zoonotic potential, identified in viral proteins. These findings are summarized in Table 3.

**Table 3.** Molecular Markers Identified in the Viruses Analyzed by the EU Reference Laboratory.

| Protein | Mutation                    | Effect                                                                                     | Samples                                              |
|---------|-----------------------------|--------------------------------------------------------------------------------------------|------------------------------------------------------|
| HA      | E251K                       | Increased virus binding to a2-6                                                            | A/swan/Moldova/8956_24VIR442-7/2023_H5N1_2023-12-18  |
|         | S107R; T108I                | Increased virulence in mice; Increased pH of fusion                                        | All                                                  |
|         | S133A                       | Increased pseudovirus binding to a2-6                                                      | All                                                  |
|         | S154N                       | Increased virus binding to a2-6                                                            | All                                                  |
|         | T156A                       | Increased virus binding to a2-6; Increased transmission in guinea pigs                     | All                                                  |
|         | V182N                       | Increased virus binding to a2-6; Decreased virus binding to a2-3                           | All                                                  |
| M1      | I43M                        | Increased virulence in ducks; Increased virulence in mice                                  | All                                                  |
|         | N30D                        | Increased virulence in mice                                                                | All                                                  |
|         | T215A                       | Increased virulence in mice                                                                | All                                                  |
| NS      | C138F                       | Increased viral replication in mammalian cells; Decreased interferon response              | All                                                  |
|         | C138F; NS-1:K55E; NS-1:K66E | Enhanced replication in mammalian cells; Decreased interferon response                     | All                                                  |
|         | I106M                       | Increased viral replication in mammalian cells; Increased virulence in mice                | All                                                  |
|         | L103F; NS-1:I106M           | Increased virulence in mice                                                                | All                                                  |
|         | P42S                        | Increased virulence in mice; Decreased antiviral response in mice                          | All                                                  |
| PA      | I38V                        | PA inhibitor (PAI) baloxavir susceptibility                                                | A/swan/Moldova/9048_24VIR442-14/2023_H5N1_2023-12-20 |
|         | N383D                       | Increased polymerase activity in mammalian cells;                                          | All                                                  |
|         | N409S                       | Increased polymerase activity in mammalian cells; Increased replication in mammalian cells | All                                                  |
|         | S37A                        | Increased polymerase activity in mammalian cells                                           | All                                                  |

| Protein | Mutation                                          | Effect                                                                                                                          | Samples                                                                                                     |
|---------|---------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------|
| PB1     | D3V                                               | Increased polymerase activity in mammalian cells; Increased replication in mammalian cells                                      | All                                                                                                         |
|         | D622G                                             | Increased polymerase activity in mice; Increased virulence in mice                                                              | All                                                                                                         |
| PB2     | I292V                                             | Increased polymerase activity in mammalian cells, Increased virulence in mice; Increased polymerase activity in mammalian cells | Seven out of nine                                                                                           |
|         | K389R                                             | Increased polymerase activity in mammalian cells; Increased replication in mammalian cells                                      | All                                                                                                         |
|         | L89V; G309 D                                      | Increased polymerase activity in mammalian cells; Increased virulence in mice                                                   | All                                                                                                         |
|         | L89V; G309 D; T339K; R4 77G; I495V; K627E; A67 6T | Increased polymerase activity in mammalian cells; Increased virulence in mice                                                   | A/swan/Moldova/9045_24VIR44 2-11/2023_H5N1_2023-12-21, A/swan/Moldova/8956_24VIR44 2-7/2023_H5N1_2023-12-18 |
|         | V598T                                             | Increased virulence in mice; Increased polymerase activity in mammalian cells; Increased replication in mammalian cells         | All                                                                                                         |

Analyzing the data in Table 3, we observe that HA (hemagglutinin) alters receptor specificity ( $\alpha$ 2,3-avian/ $\alpha$ 2,6-human) and protein stability; in particular, the E251K mutation increases binding to the  $\alpha$ 2,6 (human) receptor, and the virus gains affinity for human respiratory cells, posing an increased zoonotic risk. In turn, the S107R and T108I mutations demonstrate increased virulence in mice and result in greater stability, as well as easier activation within the human respiratory tract. For this reason, the S133A mutation results in binding to the  $\alpha$ 2,6 receptor, which represents an adaptation to mammals, while the S154N mutation results in binding to the  $\alpha$ 2,6 receptor, which promotes infection in humans. Although the V182N mutation increases binding to  $\alpha$ 2,6 and decreases binding to  $\alpha$ 2,3, this shift is indicative of a mutation associated with increased human tropism. Analyzing the data regarding the PB2 protein (Polymerase Basic 2), we note that it plays a critical role in viral replication and adaptation to mammals, and the I292V mutation is associated with increased virulence in mammals and reflects enhanced adaptation to mammalian hosts. At the same time, the mutations L89V, G309D, T339K, R477G, I495V, K627E, and A676T are associated with increased viral replication and virulence in mammals; in contrast, the K627E mutation is one of the best-known mutations associated with adaptation to humans.

## DISCUSSION

The results of the descriptive study confirmed the presence and circulation of the avian influenza virus, which, depending on certain circumstances (contact with various bird species, transmission via various vectors such as domestic and wild animals, and humans), is capable of altering certain genes in the viral genome and acquiring new pathogenic properties for other species of birds and animals, as well as posing a risk to public health. These findings are consistent with those reported by other authors (5, 8, 12). Based on data presented by the European Union Reference Laboratory for Avian Influenza & Newcastle Disease, the Istituto Zooprofilattico Sperimentale delle Venezie in Legnaro, Italy, in collaboration with the National Reference Laboratory P.I. NCAPHFS, we can note that the molecular and phenotypic analysis of the H5N1 HPAI virus identified in the Republic of Moldova reveals two distinct genotypes with increased virulence and pathogenicity. Specific genetic changes

in the HA and PB2 proteins resulting from mutations isolated from wildlife (swans) may pose an increased zoonotic risk to humans, and the data obtained through molecular sequencing allow for a rapid and effective assessment of the avian influenza risk to the agri-food sector.

It is also important to note that PB2 contains the most obvious zoonotic mutations that would allow the virus to replicate efficiently in a human host (15, 16). Nevertheless, the avian influenza virus has the potential to increase the number of incursions, given the high viral loads carried by migratory birds (16). It is also encouraging that, to date, the avian influenza virus does not have the ability to cause sustained human-to-human transmission; however, this phenomenon poses a major risk for the future and requires constant and thorough monitoring.

## CONCLUSIONS

The molecular characterization of HPAI A(H5N1) viruses isolated from wild birds and domestic poultry in the Republic of Moldova during 2023–2024 demonstrated the presence of genetic mutations that distinguish these viruses from the genetic profiles of H5N1 viruses identified in several European countries. Genome analysis further revealed mutations in the PB2 protein (E627K and/or D701N) associated with viral adaptation to mammalian hosts, while also confirming the genetic relationship of the Moldovan isolates with European H5 viruses belonging to the EA-2021-I, EA-2022-CH, EA-2022-AB, and EA-2023-DB genotypes.

Analysis of the hemagglutinin (HA) protein identified the receptor-binding mutations S133A, S154N, and T156A, which are associated with adaptation to human-type receptors, in the majority of H5N1 viruses circulating in the Republic of Moldova. These mutations were likewise detected in viruses circulating in several European countries during the same period, highlighting the continued evolution of H5N1 viruses and emphasizing the importance of ongoing molecular surveillance to assess their zoonotic potential.

In this context, continuous surveillance and epidemiological monitoring of avian influenza remain national priorities for both the poultry sector and public health. Effective prevention and control of HPAI depend on robust laboratory diagnostic capacity, accurate molecular characterization, and the timely implementation of veterinary public health measures aimed at mitigating the spread of avian influenza viruses.

**CONFLICT OF INTEREST** The authors declare that there is no conflict of interest.

**ETHICAL APPROVAL** In conducting the research and reporting the results, the authors complied with all requirements of professional ethics.

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