



Research article

Isolation of avian influenza viruses in Moscow region in 2022-2023

Anastasia Treshchalina¹ , Daria Gordeeva¹ , Elizaveta Boravleva¹ , Alexandra Gambaryan^{1*} , Alla Belyakova¹ , Alexei Prilipov² , Galina Sadykova² , Tatiana Timofeeva² , and Natalia Lomakina²

¹ Chumakov Federal Scientific Center for the Research and Development of Immune and Biological Products. Village of Institute of Poliomyelitis, Settlement "Moskovsky", 108819, Moscow, Russian Federation

² The Gamaleya National Center of Epidemiology and Microbiology of the Russian Ministry of Health, Moscow, Gamaleya St., 18, 123098, Russian Federation



Article History:

Received: 18-Feb-2026

Accepted: 03-April-2026

*Corresponding author:

Alexandra Gambaryan

al.gambaryan@gmail.com

Abstract

Wild waterfowl serve as the primary hosts of influenza A viruses, from which sporadically emerging lineages adapt to chickens, horses, pigs, and humans. This highlights the importance of controlling influenza viruses in wild populations. During the autumn migration of wild ducks through Moscow, monitoring for avian influenza was conducted. Analysis of wild duck influenza viruses from 2006 to 2021 revealed a shift in prevalence from European to Asian lineages. The present study investigated viruses isolated in 2022–2023. Twelve virus strains were isolated and subjected to whole-genome sequencing. Phylogenetic analysis indicated relationships with viruses previously isolated in Kazakhstan, Egypt, India, Bangladesh, China, and Korea. In contrast, viruses from 2008 to 2010 were primarily isolated in the Netherlands and Sweden, with fewer cases in Mongolia and Ukraine. These findings confirm earlier observations that the number of viruses of European origin in Moscow has declined over time. Genomic positions associated with host adaptation and pathogenicity were analyzed. All isolates possessed amino acids characteristic of avian influenza viruses at the proteolytic site, the receptor-binding region, positions 627 and 701 of PB2, position 66 of PB1-F2, and the PDZ domain in the NS protein. Infection of mice elicited a strong immune response. While the viruses did not cause mortality in adult mice, infection of young mice (weighing 8–10 g) with a high dose of H3N8 viruses resulted in death. These results emphasize the necessity for continued monitoring of influenza viruses in wild populations.

Keywords: Avian influenza viruses, circulation, monitoring, pathogenicity

Citation: Treshchalina, A. A., Gordeeva, D. R., Boravleva, E. Yu., Gambaryan, A. S., Belyakova, A. V., Prilipov, A. G., Sadykova, G. K., Timofeeva, T. A., and Lomakina, N. F. 2026. Isolation of Avian influenza viruses in Moscow region in 2022-2023. *Ger. J. Microbiol.* 6 (1): 36-45. <https://doi.org/10.51585/gjm.2026.1.0060>

Copyright: © 2026 Authors. Published by GMPC as an open-access article under the terms and conditions of the [Creative Commons Attribution 4.0 International License](https://creativecommons.org/licenses/by-nc/4.0/) (CC BY-NC), which allows unrestricted use and distribution in any forums, provided that the original author(s) and the copyright owner(s) are credited and the original publication in this journal is cited.

Introduction

Influenza A viruses are prevalent in nature and display significant genetic diversity due to the high variability of their genomes. These viruses infect a range of avian and mammalian species, including humans. Wild waterfowl of the orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, and waders) serve as the primary natural hosts of influenza A viruses and are typically asymptotically infected (Webster, 1992; Yoon et al., 2014;

Subbarao et al, 2006).

Influenza A viruses are classified into 18 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes, with a potential H19 subtype recently identified (Capua and Alexander, 2004; Fereidouni et al., 2023). Although H17 HA and H18 HA have been detected in bats, the 17 HA and 9 NA subtypes are responsible for the observed diversity in avian populations. Wild birds are essential for maintaining and

replenishing the viral gene pool. During autumn migration, substantial viral exchange and genome reassortment occur within bird aggregations (Steel and Lowen, 2014). Avian influenza viruses (AIV) have contributed to the emergence of pandemic variants of human influenza viruses. Pandemic strains have resulted from the transmission of entire viruses, as in the 1918 Spanish flu and the 2009 pandemic, as well as from the reassortment of individual genomic segments, as observed in 1957 and 1968 (Horimoto and Kawaoka, 2001). Recognition of these mechanisms has increased interest in synanthropic avian influenza viruses.

Between 2006 and 2021, waterfowl influenza viruses were monitored in autumn (Treshchalina et al., 2022). Samples for virus isolation, specifically duck feces, were collected from the banks of 20 water bodies in Moscow and the Moscow region, resulting in the isolation of more than 56 AIV strains. Findings obtained through 2022 have been published in several studies (Lomakina et al., 2009; Postnikova et al., 2021; Treshchalina et al., 2022; Boravleva et al., 2022; Postnikova et al., 2023; Treshchalina et al., 2024). In 2022–2023, 12 virus strains representing the H3N8, H4N6, H6N2, and H11N9 subtypes were isolated. This study focuses on the characterization of these viruses. The complete primary structure of all newly isolated viruses was determined, and their relationships with previously isolated viruses from Moscow, as well as with currently circulating Eurasian avian influenza viruses, were analyzed. The pathogenicity of the isolates in mammals was assessed using a mouse model.

Material and methods

Virus isolation

Viruses were isolated from duck feces collected along pond banks. Fecal samples were suspended in twice their volume of phosphate-buffered saline (PBS) supplemented with antibiotics: 0.1 mg/ml kanamycin, 0.4 mg/ml gentamicin, 0.01 mg/ml nystatin, and 2% MycoKill AB solution (PAA Laboratories GmbH, Pasching, Austria). Following centrifugation at 4000 rpm for 10 minutes, the supernatant was used to infect 10-day-old specific pathogen-free (SPF) chicken embryonated eggs (CE), obtained from the Ptichnoye poultry farm in Moscow, Russia. After 48 hours, virus-containing allantoic fluid (VAF) was collected. Samples

positive in the hemagglutination assay were cloned by three passages at limiting dilutions. Virus content in VAF was quantified as hemagglutinating units. All isolated strains were deposited in the virology repository of the M.P. Chumakov Federal Scientific Center for Research and Development of Immunobiological Products, Moscow, Russia. Strain names and GenBank accession numbers are provided in Table 1.

Ethics Statement

Studies involving animals were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 18 March 1986). The study design was approved by the Ethics Committee of the Chumakov Federal Scientific Center for Research on Animal Health, Russian Academy of Sciences (Approval from 2 December 2014 and from 25 March 2025). All measures were taken to alleviate animal suffering. All tests were conducted in accordance with the standard for the care and maintenance of laboratory animals, GOST 33215-2014.

Sequencing

Viral RNA was extracted from 140 µl of allantoic fluid from infected chicken embryos using the QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany). Full-length viral genome fragments were generated sequentially: cDNA synthesis was performed by reverse transcription with the universal terminal primer u12 and the MMLV enzyme (Alfa-Ferment LLC, Moscow, Russia), followed by PCR amplification with specific terminal primers (Stech et al., 2008) and Pfu polymerase (Alfa-Ferment LLC, Moscow, Russia). PCR was conducted on a Mastercycler nexus gradient (flexlid) (Eppendorf, Hamburg, Germany) with the following program: 94°C for 2 minutes, then 30 cycles of 94°C for 15 seconds, 52°C for 15 seconds, and 72°C for 1 minute. Amplified fragments were separated by electrophoresis in 1–1.3% agarose gel and extracted using the Diatom DNA Elution kit (Isogene Laboratory LLC, Moscow, Russia, No. D1031). Sequencing was performed using terminal or internal primers, the BrightDye™ Terminator Cycle Sequencing Kit v3.1 (Nimagen, the Netherlands), and an ABI PRISM 3100-Avant automated DNA sequencer (Applied Biosystems 3100-Avant Genetic Analyzer, Foster City, USA). Nucleotide sequence assembly and analysis were

performed using the Lasergene software package (DNASTAR Inc., Madison, WI, USA).

Phylogenetic analysis

For phylogenetic tree construction, all nucleotide sequences of European and Asian AIVs were downloaded from the GISAID database for each influenza genome segment. A BioPython script was used to randomly select 100 sequences. The initial dataset was supplemented with sequences of Moscow isolates and their 10 most homologous relatives for each, followed by removal of duplicate entries. Sequence alignment was performed using BioEdit v.7.7.1 (<https://bioedit.software.informer.com>) with the ClustalW algorithm (<http://www.clustal.org/>). Phylogenetic trees were constructed in MEGA XII (<https://www.megasoftware.net/>), with the optimal model selected using MEGA XII tools. Bootstrap analysis was performed with 1000 replicates to evaluate statistical significance. Tree visualization and annotation were conducted using iTOL v7.2 (<https://itol.embl.de/>) (accessed March 12, 2025). Molecular sequence analysis was performed using Jalview v.2.11.3.2 (<https://www.jalview.org/>). Phylogenetic trees are presented in Supplementary Figures.

Embryonates eggs and animals

SPF chicken embryonated eggs were obtained from the Ptichnoye poultry farm in Moscow, Russia. BALB/c mice were purchased from Lesnoye, Moscow region, Russia, and maintained with unrestricted access to food and water. For pathogenicity analysis, groups of five mice were infected intranasally under light ether anesthesia. Each mouse received 50 µl of either placebo or VAF at the appropriate dilution. Survival and body weight were monitored daily. All experiments were conducted in accordance with the standard for the care and maintenance of laboratory animals, GOST 33215-2014. Seroconversion was done on sera collected from mice at 14 dpi. Mouse sera were collected 14 days after the final administration of the virus. Serum antibodies (AB) to viruses were measured by ELISA as described in [Boravleva et al. \(2024\)](#). Ninety-six-well plates were coated with fetuin, a universal receptor analog for influenza viruses, blocked with 0.5 mg/ml BSA solution, and incubated with VAFs containing the test viruses. After washing, serial dilutions of immune mouse sera in 0.1% Tween-20 with 0.2% BSA in PBS

were added. Normal mouse serum served as a control. Plates were incubated at room temperature for 2 hours, washed, and then incubated with goat peroxidase-labeled antibodies against mouse immunoglobulins (Sigma-Aldrich, Inc., St. Louis, MO, USA) at 100 µl/well and a 1:2000 dilution. After washing, 100 µl of tetramethylbenzidine solution in acetate buffer (pH 5.7) with hydrogen peroxide was added, and plates were incubated at room temperature for 10–15 minutes. The reaction was stopped by adding 50 µl of 5% sulfuric acid per well, and absorbance was measured at 450 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific). Final antibody titers were defined as the highest dilution yielding an optical density at least twice that of the control serum.

Results and discussion

From September to November, duck feces were collected along the banks of reservoirs in the Moscow region and processed for influenza virus isolation. Sample collection locations are shown in [Figure 1](#).

Relationships of viruses isolated in 2022 and 2023 with viruses isolated earlier in Moscow

[Figure 2](#) illustrates the relationships between the 2022-2023 viruses and earlier Moscow isolates. For each gene, viruses within the same evolutionary clade are assigned the same color. Cells in the same column that represent viruses from different clades of a given gene are distinguished by different colors.

The PB2 genes of the 2022-2023 viruses are distributed among four distinct clades. The first clade comprises H3N8 viruses d/6103, d/6104, d/6105, and d/6106, as well as the 2018 d/5586 (H1N2) and 2019 d/5712 (H11N6) viruses. The corresponding cells in the table are dark yellow. These viruses are part of a broader clade that includes European, Asian, and Egyptian viruses. The second clade comprises viruses d/6147 (H3N8), d/6454 (H11N9), and 2015 d/5169 (H3N6), with their table cells colored purple. These viruses are related to Russian, Egyptian, and Western European viruses. The third clade includes d/6130 (H3N8), d/6131 (H3N8), d/6135 (H6N2), and d/6455 (H11N9) from 2023, represented by green cells in the table. According to PB2 analysis, these viruses are related to Ukrainian and Mongolian viruses, and there are no representatives of this clade among earlier Moscow viruses.

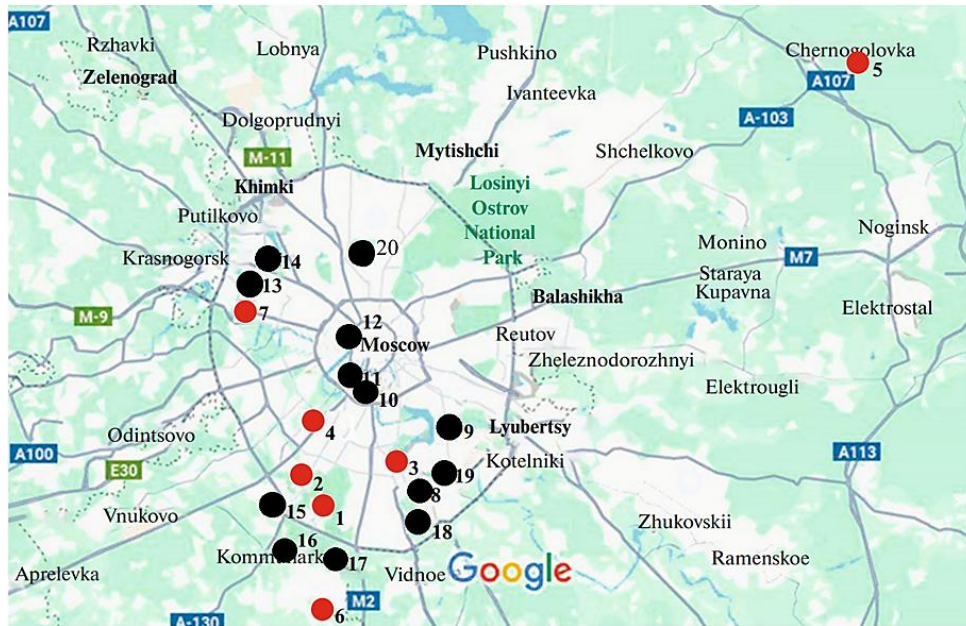


Figure 1: Sample collection sites. Red marks indicate ponds where avian influenza viruses were isolated from 2006 to 2025, while black marks indicate locations where they were not detected.

Table 1: Avian influenza viruses isolated in 2022 and 2023.

Collection date	#*	Strain name		Subtype	GenBank accession numbers
		Isoalte name	Abbreviation		
30.09.2022	7	A/duck/Moscow/6103/2022	d/6103	H3N8	PP897171-PP897178
30.09.2022	7	A/duck/Moscow/6104/2022	d/6104	H3N8	PP897179-PP897186
30.09.2022	7	A/duck/Moscow/6105/2022	d/6105	H3N8	PP897187-PP897194
30.09.2022	7	A/duck/Moscow/6106/2022	d/6106	H3N8	PP897195-PP897202
08.10.2022	5	A/duck/Moscow/6133/2022	d/6133	H4N6	PP897232-PP897239
08.10.2022	5	A/duck/Moscow/6134/2022	d/6134	H3N8	PP897261-PP897268
08.10.2022	5	A/duck/Moscow/6135/2022	d/6135	H6N2	PP897277-PP897284
17.10.2022	5	A/duck/Moscow/6130/2022	d/6130	H3N8	PP897208-PP897215
17.10.2022	5	A/duck/Moscow/6131/2022	d/6131	H3N8	PP897216-PP897223
09.11.2022	1	A/duck/Moscow/6147/2022	d/6147	H3N8	PP754244-PP754251
16.11.2023	6	A/duck/Moscow/6454/2023	d/6454	H11N9	PP897294-PP897301
16.11.2023	6	A/duck/Moscow/6455/2023	d/6455	H11N9	PP897304-PP897311

#* -The number of the pond indicated in Figure 1

The fourth clade comprises d/6134 (H3N8) and d/6133 (H4N6), as indicated by blue cells in the table. These viruses are related to the 2019 and 2021 Moscow viruses, as well as viruses isolated in the Netherlands, Mongolia, and Egypt. The Moscow virus d/4970/2013 (H1N1) is positioned at the base of this clade.

The PB1 genes of the 2022-2023 isolates are grouped into three clades. Viruses d/6103, d/6104, d/6105, and d/6106 (H3N8) possess identical PB1 genes that are related to the 2018 Moscow viruses and the d/5712/2019 virus, all of which are of Asian origin. Viruses d/6130, d/6131, d/6147 (H3N8), d/6135 (H6N2), d/6454, and d/6455 (H11N9) share a PB1

lineage with the earlier Moscow isolate d/4242/2010 (H3N8) and H4N6 viruses isolated in Egypt in 2017. The nearly identical PB1 genes of viruses 6133 (H4N6) and 6134 (H3N8) are related to virus d/4238/2010 and other Moscow viruses from 2009 to 2012, forming a subclade of Asian viruses. The PA genes of the 2022–2023 isolates are divided into two clades. Viruses d/6130, d/6131, d/6147 (H3N8), and d/6135 (H6N2) are related to the Moscow isolate d/5744/2019. The other 2022-2023 viruses are related to the d/5897/2021 virus. The PA genes in all these viruses were introduced to the Moscow region by viruses originating from Southeast Asia and Egypt.

Strain	Subtype	PB2	PB1	PA	HA	NP	NA	M	NS
d/3641/2008	H11N9								
d/3740/2009	H4N6								
d/3720/2009	H6N2								
d/3806/2009	H3N8								
d/3735/2009	H4N6								
d/4238/2010	H3N6								
d/4298/2010	H3N8								
d/4242/2010	H3N8								
d/4643/2011	H4N6								
d/4970/2013	H1N1								
d/5037/2014	H3N8								
d/5169/2015	H3N6								
d/5586/2018	H1N2								
d/5744/2019	H1N1								
d/5712/2019	H11N6								
d/5881/2021	H3N2								
d/5897/2021	H3N8								
d/6103/2022	H3N8								
d/6104/2022	H3N8								
d/6105/2022	H3N8								
d/6106/2022	H3N8								
d/6130/2022	H3N8	>							
d/6131/2022	H3N8	>							
d/6133/2022	H4N6								
d/6134/2022	H3N8								
d/6135/2022	H6N2	>							
d/6147/2022	H3N8								
d/6454/2023	H11N9						>		
d/6455/2023	H11N9	>					>		

Figure 2: Relationships of viruses isolated in 2022 and 2023 with viruses isolated earlier in Moscow. Viruses are identified by strain number and year of isolation. Identical cell colors within each column indicate membership in the same clade on the evolutionary tree. For viruses isolated in 2022–2023, cell color denotes their relationship to strains previously isolated in Moscow. All relevant strains are listed at the top of the table. The > symbol signifies the absence of a related strain for this gene among Moscow viruses isolated prior to 2022.

The H3 gene of all 2022 isolates is related to the 2021 and 2009 isolates and belongs to the Worldwide-1 sublineage (Yang, 2025). On the evolutionary tree, the 2022 Moscow isolates form a compact cluster. The H4 gene of d6133 (H4N6) clusters with viruses from Northwestern Europe isolated between 2016 and 2018. The Eurasian H6 lineage is heterogeneous and includes viruses from the Americas (Cui et al., 2025; Rimondi et al., 2011). Three major clades are identified within this group. Isolate 6135/2022 is assigned to clade III, which is derived from viruses isolated in Jiangxi and Hunan provinces, China (Huang,

2010). In contrast, three other H6 viruses previously isolated in Moscow (2006, 2009, and 2010) were related to Dutch and Swedish viruses, whereas the 6135/2022 virus is related to viruses from Korea and Bangladesh. The H11 gene of the 2023 Moscow viruses belongs to a clade comprising viruses with diverse geographical origins. These viruses are located in the same subclade as A/duck/Moscow/5712U/2019 (H11N6), but are distinct from A/avian/Moscow/3641/2008 (H11N9).

Based on the NP gene, the 2022–2023 isolates

are divided into two groups. Viruses d/6103, d/6104, d/6105, and d/6106 (H3N8) belong to the mixed Euro-Asian clade and are distantly related to the Moscow virus A/avian/Moscow/3641/2008 (H11N9). The remaining viruses are closely related to d/5897/2021 and belong to a clade that includes European and Egyptian viruses.

The neuraminidase (NA) genes of eight H3N8 viruses are distributed between two unrelated clades. In viruses d/6103, d/6104, d/6105, and d/6106, the NA genes belong to the European clade, as does d/4298/2010. In the remaining four viruses, the NA genes are assigned to a clade containing predominantly Asian viruses and Moscow 2021 viruses.

The NA of d/6133(H4N6) virus is related to the d/4643/2011 virus and the NAs of the 2013 Swedish H3N6 viruses. These N6 NAs belong to a large clade containing both European and Asian viruses. The NA gene of isolate 6135/2022 (H6N2) is closely related to those of H3N2 and H1N2 viruses isolated in Moscow during 2018–2021, but is distinct from the neuraminidase genes of other Moscow H6 viruses. Viruses with this NA gene belong to a large, mixed European-Asian cluster. The NA gene of the 2023 H11N9 viruses belongs to a clade of Asian viruses and is unrelated to the NA gene of A/avian/Moscow/3641/2008 (H11N9).

Based on the M protein gene, the 2022–2023 isolates are divided into two groups, consistent with the NP gene analysis. Viruses d/6103, d/6104, d/6105, and d/6106 (H3N8) originated from d/3735/2009 and belong to a clade composed mainly of Mongolian viruses. The remaining viruses are closely related to the 2018–2021 viruses and belong to a clade that includes European, Asian, and Egyptian viruses. The 2023 viruses d/6454 and d/6455 possess the A allele of the NS gene, whereas all 2022 viruses have the B allele. The NS genes of 2022 viruses are distributed between two distinct clades. Viruses d/6103, d/6104, d/6105, and d/6106 (H3N8) are descended from d/4970/2013 and belong to the Korean-Mongolian clade, while the remaining viruses are related to Swedish and Dutch viruses, as well as Moscow d/5744/2019 and d/3735/2009.

Routes of virus dissemination

To assess virus movement, a database search

was conducted to identify viruses with minimal genetic differences relative to the Moscow isolates. These viruses were extracted from the GISAID database using BLAST, with searches performed for each of the eight genes. The results are presented in [Figure 3](#). For viruses nearly identical across all genes (d/6103, d/6104, d/6105, d/6106, d/6130, and d/6131), only one representative is listed. Numbers in the table cells indicate the percentage difference for each gene between the Moscow isolate and the BLAST-identified variant. For comparison, results of a similar analysis for viruses from 2008–2010 are shown at the top of the table.

Viruses closely related to the 2022–2023 isolates were primarily detected in Kazakhstan, Egypt, India, Bangladesh, China, and Korea. In contrast, viruses closely related to the 2008–2010 isolates were mainly detected in the Netherlands and Sweden, and to a lesser extent in Mongolia and Ukraine. Previous reports indicated a decline in the number of viruses of European origin in Moscow over time. Viruses from Asian evolutionary lineages have begun to circulate in Europe, displacing older European lineages. After 2014, the majority of viruses isolated in Moscow belonged to Asian evolutionary lineages ([Treshchalina et al., 2022; 2024](#)).

Another difference between the 2008–2010 and 2022–2023 viruses is the degree of genetic similarity between Moscow and closely related non-Moscow viruses. The 2008–2010 viruses often differ by less than 1% from their non-Moscow counterparts (see, for example, the 2009 strains for the PB1, PA, HA, and NA genes). These differences are especially low when the compared virus was isolated in the Netherlands or Sweden. Such minimal differences, combined with isolation within the same season, suggest a direct introduction into Moscow by migrating birds.

The 2022–2023 viruses do not show such high similarity to the viruses used for comparison. Even when a closely related virus is isolated in the Netherlands, the percentage differences in the PB2, PB1, PA, HA, and NA genes are typically much higher than 1. This rules out transfer during the same season. It is possible the virus was introduced to Moscow after birds wintered together in Egypt or Central Asia.

Strains	PB2	PB1	PA	HA	NP	NA	M	NS	
d/3556/2008	0,78	1,39	1,44	2,06	1,38	1,56	0,81	0,47	Netherlands
d/3641/2008	2,18	1,28	0,73	1,53	0,98	1,26	0,59	0,46	Sweden,
d/3661/2008	0,83	1,34	1,39	1,01	2	0,63	0,3	0,35	Belgium
d/3720/2009	1,3	1,33	0,77	0,52	0,39	0,35	0,71	0,12	Ukraine
d/3735/2009	1,26	0,17	0,09	0,29	0,19	0,21	0,4	0,23	Kazakhstan
d/3740/2009	0,35	0,26	0,37	0,41	0,77	0,28	0,1	0,11	Egypt
d/3799/2009	0,44	0,17	0,28	0,48	0,77	0,28	0,61	0,11	Mongolia
d/3806/2009	1,31	1,48	0,63	1,4	0,33	0,63	0,2	0,11	China&Korea
d/4031/2010	0,95	0,66	1,05	0,59	0,98	0,37	0,4	1,46	India & Bangladesh
d/4182/2010	1,25	1,39	1,09	1,77	0,71	0,15	0,2	0,8	Japan
d/4203/2010	0,69	0,48	0,41	2,15	0,39	0,55	0,2	0,23	South Africa &
d/4238/2010	0,48	0,7	1,1	2,09	0,39	0,28	0,2	0,23	Australia
d/4242/2010	1,1	1,4	0,72	1,04	1	1,62	0,4	0,69	
d/4298/2010	0,61	0,39	0,59	2,07	1,02	1,40	0,30	1,12	
d/6103/2022	1,24	1,22	1,3	1,17	1,79	1,64	1,11	0,46	
d/6130/2022	1,17	1,45	1,04	1,17	1,09	1,57	0,88	0,67	
d/6133/2022	1,65	1,14	1,39	3,04	1,34	2,1	0,59	1,24	
d/6134/2022	1,65	1,14	1,39	0,99	1,34	1,57	0,88	0,67	
d/6135/2022	1,39	1,5	1,22	0,87	1,22	2,8	0,98	0,9	
d/6147/2022	0,79	1,41	1,04	1,05	1,28	1,53	0,29	0,67	
d/6454/2023	1,58	1,58	1,75	4,77	0,81	1,61	0,79	0,79	
d/6455/2023	1,92	1,58	1,75	4,59	1,24	1,68	0,79	0,79	

Figure 3: Locations of virus isolation with minimal differences relative to the Moscow isolate.

Gene Ontology (GO) enrichment analysis of the closest relatives of the Moscow virus isolates was performed in the GISAID database using BLAST. The table displays the percentage differences for each gene between the Moscow isolate and the BLAST-identified variant. For comparison, results from a similar analysis of viruses collected between 2008 and 2010 are presented at the top of Figure 3.

The pronounced differences in virus circulation likely reflect changes in the migratory behavior of the primary host species. Zoological studies have documented an increase in the number of ducks remaining in urban areas during winter rather than migrating, thereby reducing interpopulation contact on European wintering grounds (Avilova, 2016). As a result, the spread of viruses carried by these ducks has decreased.

Molecular determinants influencing influenza virus pathogenicity

The risk to mammals posed by the studied viruses was evaluated by analyzing key amino acids associated with host switching and pathogenicity. The hemagglutinin (HA) cleavage site structure serves as a critical determinant of influenza virus pathogenicity. In all sequences analyzed from the 2022-2023 viruses, this site

contains a single arginine residue, consistent with the consensus sequence observed in apathogenic viruses of this HA subtype. The receptor binding site in the HA of all studied viruses contains Gln226 and Gly228, both characteristic of avian influenza viruses. These residues facilitate recognition of receptors terminated by sialyl(2-3)galactose moieties (Rogers and Paulson, 1983; Matrosovich et al., 2000). All Moscow isolates possess Glu627 and Asp701 in the PB2 protein, residues specific to avian influenza viruses. At position 66 of the PB1-F2 accessory protein, most isolates contain asparagine, although an N66S substitution was observed in some cases. The PDZ domain ligand in NS exhibits the sequence ESEV in all isolates except for virus d/6135, which contains ESEI. The ESEV variant predominates among avian influenza viruses, while ESEI is less common but still characteristic of these viruses (Obenauer et al., 2006).

Pathogenicity of the viruses in mice

Moscow isolates from 2022 to 2023 belong to the H3N8, H4N6, H11N9, and H6N2 subtypes, which are primarily associated with wild avian populations. However, H3 subtype viruses have repeatedly given rise to evolutionary lineages in

pigs (Stadejek et al., 2023). H4N6 influenza A viruses were isolated in 1999 from pigs with pneumonia in Canada (Karasin et al., 2000). H6 viruses can infect humans (Yan et al., 2023), and H11N3 influenza viruses can efficiently transmit through the respiratory tract in mammalian models (Jiang et al., 2022). The pathogenicity of these viruses in mammals was evaluated using a mouse model. Balb/c mice weighing 12 to 14 grams survived intranasal administration of the virus at a dose of 10^5 EID₅₀ per mouse, although weight loss was observed in some cases. Following double infection, all mice developed a

strong immune response (Table 2). In contrast, younger mice (8 to 10 grams) infected with a higher dose (10^7 EID₅₀ per mouse) exhibited illness. The severity of the disease varied by viral strain. Under these conditions, H11N9 viruses caused only slight weight loss compared to the control group; H6N2 viruses induced significant weight loss followed by recovery in all mice; H4N6 viruses resulted in weight loss and partial mortality; and H3N8 viruses caused severe disease and, in some cases, complete mortality (Table 2).

Table 2: Pathogenicity and immune response of isolated influenza viruses in BALB/c mice

Strains:	Subtype	Low dose groups*		High dose groups**
		d./s./h.	Antibody titers#	d./s./h.
d/6103/2022	H3N8	0/0/5	100000	2/2/1
d/6104/2022	H3N8	0/0/5	100000	3/2/0
d/6105/2022	H3N8	0/0/5	100000	2/0/3
d/6106/2022	H3N8	0/0/5	100000	2/2/1
d/6130/2022	H3N8	0/0/5	50000	5/0/0
d/6131/2022	H3N8	0/0/5	100000	3/1/1
d/6134/2022	H3N8	0/0/5	40000	5/0/0
d/6147/2022	H3N8	ND	ND	1/1/3
d/6133/2022	H4N6	0/0/5	40000	2/2/1
d/6135/2022	H6N2	0/0/5	60000	0/2/3
d/6454/2023	H11N9	ND	ND	0/0/5
d/6455/2023	H11N9	ND	ND	0/0/5
Control	-	0/0/5	ND	0/0/5

*N=5 mice/group, weight 12-14g, dose 10^5 EID₅₀. ** N=5 mice, weight 10-12g, dose 10^7 EID₅₀. d./s./h. - Number of dead, sick, and healthy mice on day 13 post-infection: d= dead, s=sick, h=healthy. #Antibody titer in ELISA following two-fold intranasal challenge with 10^5 EID₅₀ of the virus. ND=Not done.

This study examines avian influenza viruses isolated from fecal samples collected from wild ducks along pond shores in the Moscow region. Eighteen years of monitoring revealed a decline in the number and diversity of influenza viruses isolated from mallards in Moscow ponds. Following 2014, both the frequency and diversity of isolates decreased. A trend toward predominance of Asian viruses replaced the previously dominant European viruses, a pattern confirmed in 2022-2023. The shift from European to Asian virus prevalence may result from changes in mallard migration routes.

Phylogenetic analysis of the 2022-2023 virus genomes demonstrated that all isolates belong to the Eurasian lineage of avian influenza viruses. Most genes are grouped in clades primarily associated with Asian viruses. For each gene, the 12 isolates are divided into two to five evolutionary clades. Four isolates collected on the same day and at the same location are nearly identical, differing only by a few substitutions in

individual genes. Similarly, two isolates (d/6130 and d/6131) collected on the same day represent a single virus. The remaining viruses differ in their combinations of genes from various clades. For instance, in one season, five influenza viruses from four clades were isolated from a single pond, while four additional variants were found in viruses from other ponds. These findings indicate that several variants of each gene are intricately mixed among the isolates. This phenomenon is attributed to genome reassortment, which is particularly prevalent in low-pathogenic avian influenza viruses (Steel and Lowen, 2014). The observed genomic instability aligns with Dugan's concept that "gene segments form transient genomic constellations without selective pressure to maintain associated genomes" (Dugan et al., 2008).

Conclusion

Amino acid analysis related to host shift and pathogenicity revealed no mutations in any of the

viruses that would increase the risk of adaptation to mammals. All isolates exhibited the typical apathogenic genomic structure characteristic of influenza viruses. However, these viruses replicated efficiently in mice, altering body weight curves and significantly increasing antibody levels. Some H3N8 strains caused mortality in young mice. These findings highlight the importance of monitoring influenza virus diversity in wild populations to inform effective control strategies.

Article Information

Funding. This research received no external funding.

Conflict of interest. The authors declared that there is no conflict of interest.

Authors' contribution. All authors contributed equally to work.

Supplementary materials: Phylogenetic trees

Publisher's Note. The claims and data contained in this manuscript are solely those of the author(s) and do not represent those of the GMPC publisher, editors, or reviewers. GMPC publisher and the editors disclaim the responsibility for any injury to people or property resulting from the contents of this article.

References

- Avilova K.V., 2016. Life cycle and population dynamics of the urban mallard population (*Anas platyrhynchos*, Anseriformes, Aves) in Moscow, *Zoological Journal*, 95, 12, p. 1427–1440.
- Boravleva, E., Treshchalina, A., Postnikova, Y., Gambaryan, A., Belyakova, A., Sadykova, G., et al., 2022. Molecular Characteristics, Receptor Specificity, and Pathogenicity of Avian Influenza Viruses Isolated from Wild Ducks in Russia. *International Journal of Molecular Sciences* 23, 10829. <https://doi.org/10.3390/ijms231810829>
- Boravleva, E.Y., Treshchalina, A.A., Gordeeva, D.R., Gambaryan, A. 2024. Development of an inexpensive and simple test system for the differential detection of avian influenza viruses and avian paramyxoviruses in environmental monitoring. *Journal of Research in Veterinary Sciences* 4, 58–58. <https://doi.org/10.5455/JRVS.20240529123341>
- Capua, I., Alexander, D.J. 2004. Avian influenza: recent developments. *Avian Pathol.* 33, 393-404. <https://doi.org/10.1080/03079450410001724085>
- Cui, N., Wang, P., Huang, Q., Yuan, Z., Su, S., Xu, C., Qi, L., 2025. Detection of Avian Influenza Virus in Pigeons. *Viruses* 17, 585. <https://doi.org/10.3390/v17040585>
- Dugan, V.G., Chen, R., Spiro, D.J., Sengamalay, N., Zaborsky, J., Ghedin, E., et al., 2008. The evolutionary genetics and emergence of avian influenza viruses in wild birds. *PLOS Pathogens* 4, e1000076. <https://doi.org/10.1371/journal.ppat.1000076>
- Fereidouni, S., Starick, E., Karamendin, K., Genova, C.D., Scott, S.D., Khan, Y., et al., 2023. Genetic characterization of a new candidate hemagglutinin subtype of influenza A viruses. *Emerging Microbes and Infections* 12, 2225645. <https://doi.org/10.1080/22221751.2023.2225645>
- Horimoto, T., Kawaoka, Y. 2001. Pandemic threat posed by avian influenza A viruses, *Clinical Microbiology Reviews* 14, 129–149. <https://doi.org/10.1128/CMR.14.1.129-149.2001>
- Huang K, Bahl J, Fan XH, Vijaykrishna D, Cheung CL, Webby RJ, Webster RG, Chen H, Smith GJ, Peiris JS, Guan Y., 2010. Establishment of an H6N2 influenza virus lineage in domestic ducks in southern China. *Journal of Virology*, 84(14):6978–86. <https://doi.org/10.1128/JVI.00256-10>.
- Jiang, L., Li, J., Cui, H., Zhang, C., Jin, Y., Fu, Y., et al., 2022. Etiologic characteristics of avian influenza H11 viruses isolated from the live poultry market in southeast coastal region in China. *Frontiers in Microbiology* 13, 1002670. <https://doi.org/10.3389/fmicb.2022.1002670>
- Karasin, A.I., Brown, I.H., Carman, S., Olsen, C.W. 2000. Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. *Journal of Virology* 74, 9322–9327. <https://doi.org/10.1128/jvi.74.19.9322-9327.2000>
- Lomakina, N.F., Gambaryan, A.C., Boravleva, E.Y., Kropotkina, E.A., Kirillov, I.M., Lavrientev, M.V., et al., 2009. The study of the nonpathogenic influenza virus A/gull/Moscow/3100/2006 (H6N2) isolated in Moscow. *Molecular Genetics. Microbiology and Virology* 1, 32-39.
- Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M.R., et al., 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals, *Journal of Virology*, 74, 8502–8512. <https://doi.org/10.1128/jvi.74.18.8502-8512.2000>.
- Obenauer, J.C., Denson, J., Mehta, P.K., Su, X., Mukatira, S., Finkelstein, D.B., et al., 2006. Large-scale sequence analysis of avian influenza isolates. *Science*, 311, 1576–80. <https://doi.org/10.1126/science.1121586>
- Postnikova, Y., Treshchalina, A., Boravleva, E., Gambaryan, A., Ishmukhametov, A., Matrosovich, M., et al., 2021. Diversity and Reassortment Rate of Influenza A Viruses in Wild Ducks and Gulls. *Viruses* 13, 1010. <https://doi.org/10.3390/v13061010>
- Postnikova, Y., Treshchalina, A., Gambaryan, A., Belyakova, A., Ishmukhametov, A., Matrosovich, M., et al., 2023. Evolutionary Dynamics of Avian

- Influenza Viruses Isolated from Wild Birds in Moscow. *International Journal of Molecular Sciences* 24, 3020. <https://doi.org/10.3390/ijms24033020>
- Rimondi, A., Xu, K., Craig, M.I., Shao, H., Ferreyra, H., Rago, M.V., et al., 2011. Phylogenetic analysis of H6 influenza viruses isolated from rosy-billed pochards (*Netta peposaca*) in Argentina reveals the presence of different HA gene clusters. *Journal of Virology* 85, 13354–13362. <https://doi.org/10.1128/JVI.05946-11>
- Rogers, G.N., Paulson, J.C., 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin, *Virology*, 127, 361–373. [https://doi.org/10.1016/0042-6822\(83\)90150-2](https://doi.org/10.1016/0042-6822(83)90150-2).
- Stadejek, W., Chiers, K., Van Reeth, K. 2023. Infectivity and transmissibility of an avian H3N1 influenza virus in pigs. *Veterinary Research* 54, 4. <https://doi.org/10.1186/s13567-022-01133-x>
- Stech, J., Stech, O., Herwig, A., Altmeyen, H., Hundt, J., Gohrbandt, S., et al., 2008. Rapid and reliable universal cloning of influenza A virus genes by target-primed plasmid amplification. *Nucleic Acids Research* 36, e139. <https://doi.org/10.1093/nar/gkn646>
- Steel, J., Lowen, A.C. 2014. Influenza A virus reassortment. *Current Topics in Microbiology and Immunology*, 385, 377–401. https://doi.org/10.1007/82_2014_395
- Subbarao, K., Swayne, D.E., Olsen, C.W. 2006. Epidemiology and control of human and animal viruses. In *Influenza Virology, Current Topics*, Y. Kawaoka, ed. (Norfolk, UK, Caister Academic Press), 229–279.
- Treshchalina, A., Postnikova, Y., Gambaryan, A., Ishmukhametov, A., Prilipov, A., Sadykova, G., et al., 2022. Monitoring of Avian Influenza Viruses and Paramyxoviruses in Ponds of Moscow and the Moscow Region. *Viruses* 14, 2624. <https://doi.org/10.3390/v14122624>
- Treshchalina, E.F., Rodina, A.S., Gambaryan, E.Y., Boravleva, K.V., Avilova, S.P., Kharitonov et al., 2024. Long-Term Dynamics of Different Avian Influenza Viruses in Mallard (*Anas platyrhynchos*) Population in Moscow City and Moscow Oblast: Dependence on the Migration Activity. *Biology Bulletin* 51, 1623–1635. © Pleiades Publishing, Inc., ISSN 1062-3590.
- Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. *Microbiological Reviews* 56, 152–179. <https://doi.org/10.1128/mr.56.1.152-179.1992>
- Yan, Z., Li, Y., Huang, S., Wen, F. 2023. Global distribution, receptor binding, and cross-species transmission of H6 influenza viruses: risks and implications for humans. *Journal of Virology* 97, e0137023. <https://doi.org/10.1128/jvi.01370-23>
- Yang, J., Chen, X., Li, X., Zhang, Y., Liu, J., Tan, M., et al., 2025. Global spread of H3 subtype avian influenza viruses with an accelerated evolution after interspecies transmission. *Journal of Infection* 91, 106542. <https://doi.org/10.1016/j.jinf.2025.106542>
- Yoon, S.W., Webby, R.J., Webster, R.G. 2014. Evolution and ecology of influenza A viruses. *Current Topics in Microbiology and Immunology* 385, 359–75. https://doi.org/10.1007/82_2014_396