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Genetic diversity of A(H5N1) avian influenza viruses isolated from birds and seals in Russia in 2023

Anastasia S. Panova, Natalia P. Kolosova, Svetlana V. Svyatchenko, Natalia I. Goncharova, Alexey V. Danilenko, Nikita D. Boldyrev, Kiunnei N. Shadrinova, Natalia N. Vasiltsova, Marina L. Egorova, Galina S. Onkhonova, Andrey S. Gudymo, Maria K. Bragina, Alexander B. Ryzhikov & Vasiliy Yu. Marchenko✉

Thousands of outbreaks of the highly pathogenic avian influenza A(H5N1) virus in birds and an increasing number of mammal infections are registered annually. In 2023, multiple avian influenza outbreaks were registered among wild birds, poultry and seals in Russia. The genetic characterization of seventy-seven avian viruses and three viruses from seals showed that they belonged to the 2.3.4.4b clade and represented four distinct reassortant genotypes. The majority of viruses represented genotype BB, which was widespread in Europe in 2023. Viruses from seals and four viruses from birds, isolated from outbreaks in the Far East region, belonged to the G1 (A3) genotype and had the amino acid substitution N319K in the NP protein, previously associated with an increased virulence for mammals. In addition, one virus of the G10 genotype and two viruses, representing a previously undescribed genotype (designated as Ru-23-G4) were identified. The viruses analyzed showed normal inhibition by neuraminidase inhibitors. Seven viruses had genetic markers of amantadine resistance. All the influenza A(H5N1) viruses studied showed a binding preference for α2-3-linked sialic acids, suggesting a low risk of transmission among humans. Nevertheless, monitoring of reassortment and mammalian adaptation mutations is essential for the timely identification of viruses with increased pandemic potential.

Recent years have seen an unprecedented spread of clade 2.3.4.4b A(H5N1) highly pathogenic avian influenza viruses (HPAIV). These viruses emerged by reassortment of the 2.3.4.4b clade A(H5N8) viruses with other avian influenza virus variants, and were first detected in Netherlands in October, 2020[1]. They rapidly spread worldwide, becoming predominant in various countries by the end of 2021[1–3].

Since its emergence, multiple reassortment events were registered among influenza A(H5N1) viruses from the 2.3.4.4b clade. It was noted that reassortment with low pathogenic avian influenza viruses (LPAIV) of wild birds resulted in the formation of various genotypes described in different classifications. These include the genotypes G1-G16[2], as well as several genotypes identified in Europe such as AB and BB[4,5]. In addition, genotypes of reassortant A(H5N1) viruses containing gene segments from the viruses of the Eurasian group together with the segments of the viruses belonging to the North American group have been described[6].

In 2023, outbreaks of HPAIV in poultry and wild birds were reported in many countries in Europe, Asia, Africa, North and South America. More than 650 outbreaks were reported in poultry in 29 countries, resulting in the death or destruction of about 19 million birds[5,7–11]. At the same time, a high incidence of influenza in wild birds was observed, with about 3000 detected outbreaks in 65 countries[7]. The disease was predominantly found in black-headed gulls (*Ichthyaetus melanocephalus*), with bird deaths registered in more than 20 European countries in the spring of 2023[7,9].

In addition, the incidence of mammalian infection with influenza A(H5N1) virus has increased recently[12,13]. For the most part, the virus is detected in terrestrial carnivores. However, outbreaks of HPAI A(H5N1) among marine mammals are increasingly being recorded. Thus, in 2023, a mass death of northern fur seals was registered in Russia, sea lions (*Otaria flavescens*) in Chile, Peru and Argentina[14–16], cases of disease in southern elephant seals (*Mirounga leonina*) and South American fur seals (*Arctocephalus australis*) in Argentina[17], as well as gray (*Halichoerus grypus*) and harbor seals (*Phoca vitulina*) – in the USA[18].

Typically, A(H5N1) viruses isolated from mammals were genetically similar to viruses circulating among birds. However, viruses isolated from dead or sick mammals frequently contained at least one of the mammalian

adaptation mutations in the PB2 protein: E627K, D701N, T271A[8–11]. Mammalian adaptation mutations were also detected in some viruses isolated from rare cases of human infection with HPAIV A(H5N1) clade 2.3.4.4b, including cases of human infection with A(H5N1) virus after exposure to the virus-infected cattle[19].

The substantial threat from the continuous spread of the A(H5N1) viruses and their evolution toward adaptation to mammals highlights the need for continuous monitoring and thorough genetic analysis of circulating viruses. This work undertakes a genetic and virological analysis of influenza A(H5N1) viruses detected in poultry, wild birds and seals in the Russian Federation in 2023.

Results

In 2023, outbreaks of avian influenza were registered in several regions of Russia. A total of 32 collection sets of bird samples from Russia, including 30 collections sets from the European part of Russia and 1 collection sets from the Far East of Russia were analyzed (Table S1). From May to July 2023, mass mortality of gulls was registered, mainly in the European part of Russia. PCR diagnostics were performed for all samples obtained during outbreaks among poultry and wild birds. Genetic material of the influenza A(H5N1) virus was detected in 177 bird samples. A geographically representative set of 77 A(H5N1) avian viruses was studied (Table S1). Whole genome sequences were obtained for 71 virus isolates (cultivated in embryonated chicken eggs, ECE). For 23 of the 71 cultivated viruses, genome data was also obtained using original bird samples. For six of the 77 viruses, genome data was only obtained for original bird samples (Table S1).

In July–August 2023, a mass mortality of marine mammals was registered in the Russian Far East[20]. Animal deaths were recorded mainly on the eastern coast of Sakhalin Island. The Federal Budgetary Institution State Research Center for Virology and Biotechnology "Vector", Rospotrebnadzor within the framework of the Russia wide monitoring of HPAIV A(H5N1), investigated eight specimens collected from four dead northern fur seals (*Callorhinus ursinus*), discovered during the mass death of animals on Tyuleniy Island. Disease progression in the seals during the recorded A(H5N1) outbreak followed as a two-stage process from the onset of symptoms. Observed clinical signs in the first stage included fever, lethargy, confusion, disorientation, and in the second stage, the animals experienced convulsions and died[20]. The results of PCR diagnostics conducted by SRC VB "Vector" revealed the presence of genetic material of the avian influenza A(H5N1) virus in the samples collected from four dead animal. Three A(H5N1) viruses were isolated from three of the four animals.

Genetic analysis of A(H5N1) viruses collected from birds

All the viruses for which the genetic sequences were determined belonged to the A(H5N1) subtype. Phylogenetic analysis showed that all viruses belonged to the clade 2.3.4.4b based on the hemagglutinin (HA) sequences. Analysis of the HA protein of the viruses studied showed a high degree of identity (from zero to six amino acid substitutions (AASs) in the HA1 part of the HA) with the HA of the candidate vaccine viruses (CVV) A/Astrakhan/3212/2020 (H5N8), A/chicken/Ghana/AVL-763_21VIR7050-39/2021 (H5N1) and A/American Wigeon/South Carolina/22-000345-001/2021 (H5N1) of the clade 2.3.4.4b, recommended by the WHO[21] (Table S2).

The genome reassortment analysis of the 77 A(H5N1) avian viruses revealed that the viruses represented four different genotypes (Figs. 1, 2). The genotypes were assigned based on phylogenetic analysis of all eight segments (Supplementary Information). On the phylogenetic trees viruses isolated in Russia in 2023 are indicated by blue, other A(H5N1) 2.3.4.4b viruses are colored in green. Gene sequences of previously characterized genotypes were used as references.

For five viruses, the genotype was determined as G1[2]. According to another classification, this genotype is equivalent to genotype A3 (GISAID, accessed on 28.02.2025) or EA-2020-C[22], and it was designated as Ru-23-G1 in our study. It initially appeared in October 2020 in Europe and then spread widely to Africa, Asia and
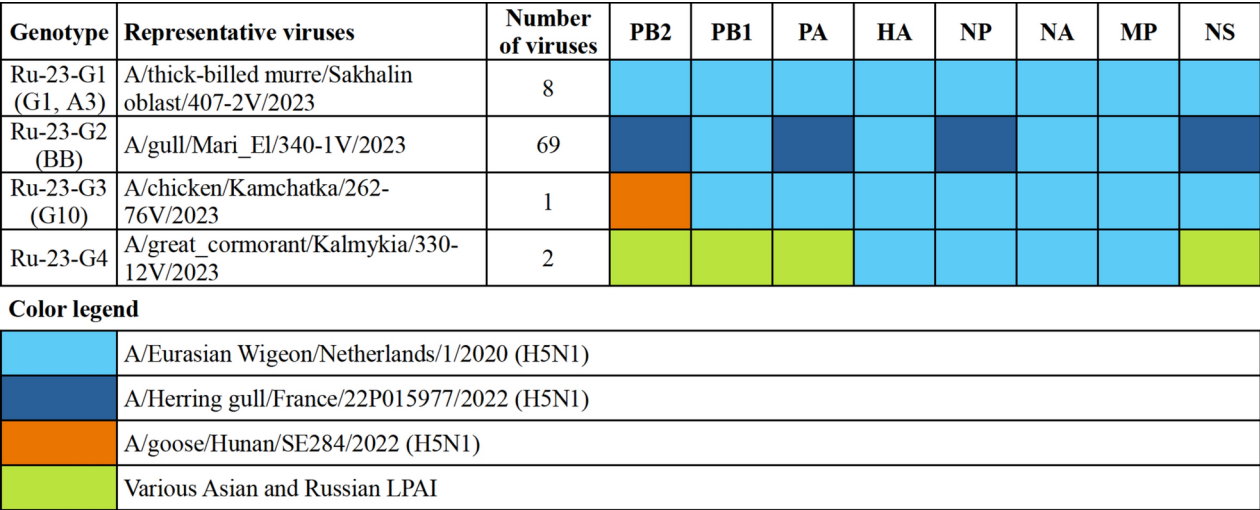
Genotype	Representative viruses	Number of viruses	PB2	PB1	PA	HA	NP	NA	MP	NS
Ru-23-G1 (G1, A3)	A/thick-billed murre/Sakhalin oblast/407-2V/2023	8								
Ru-23-G2 (BB)	A/gull/Mari_El/340-1V/2023	69								
Ru-23-G3 (G10)	A/chicken/Kamchatka/262-76V/2023	1								
Ru-23-G4	A/great_cormorant/Kalmykia/330-12V/2023	2								

Color legend

	A/Eurasian Wigeon/Netherlands/1/2020 (H5N1)
	A/Herring gull/France/22P015977/2022 (H5N1)
	A/goose/Hunan/SE284/2022 (H5N1)
	Various Asian and Russian LPAI

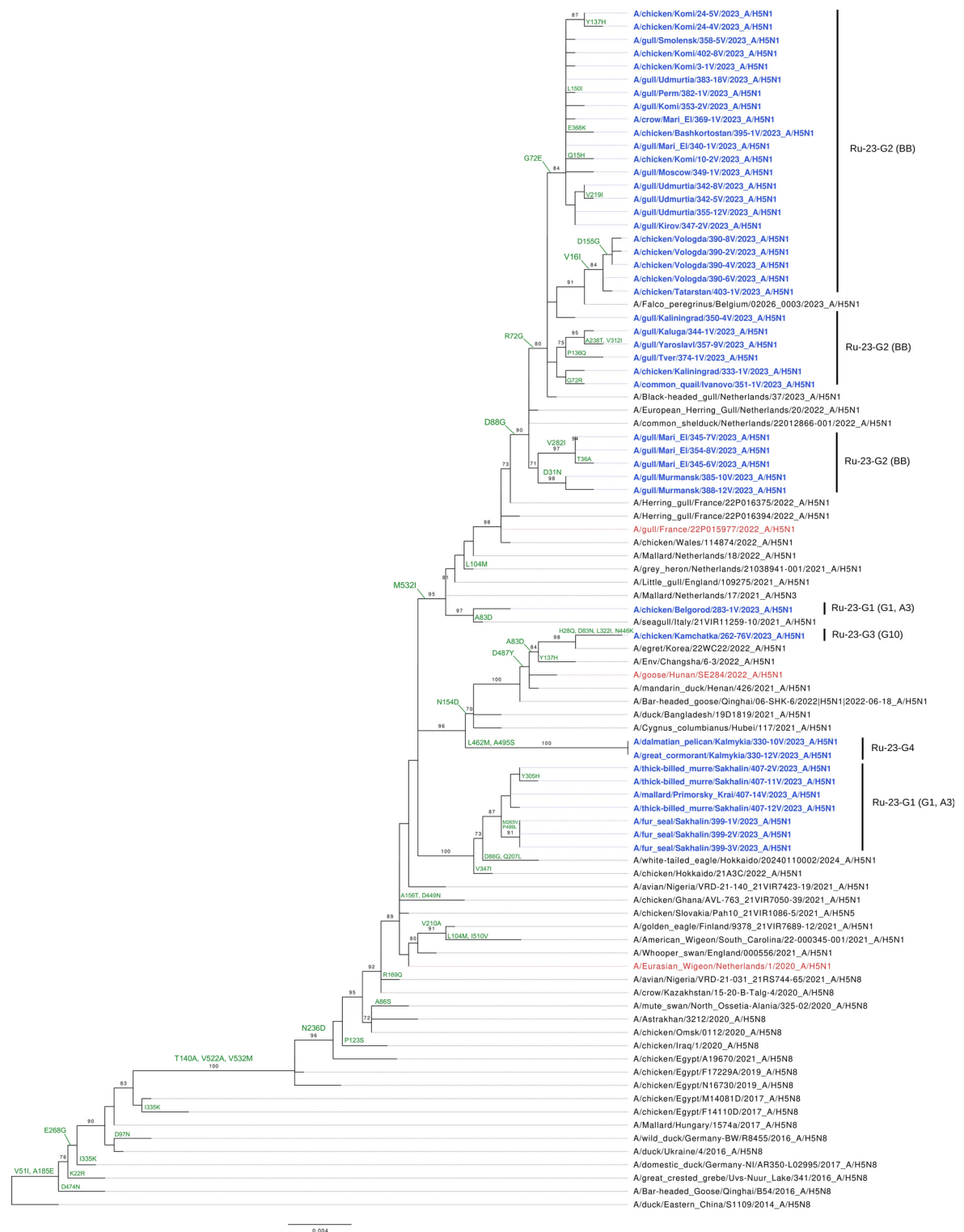**Fig. 1**. Analysis of genotypes of A(H5N1) viruses.

Fig. 2. Phylogenetic tree of the hemagglutinin (HA) gene of A(H5N1) 2.3.4.4b viruses.

North America[1,2]. Most viruses in this group were identical in the amino acid sequence of the HA1 part of the HA protein to the CVV A/Astrakhan/3212/2020 (Table S2). In viruses of the Ru-23-G1 genotype from the Far East of Russia (representative virus A/thick-billed murre/Sakhalin oblast/407-2 V/2023) AAS N319K in the NP protein was detected, previously associated with the increased efficiency of viral replication[23].

For 69 viruses, the genotype was determined as Ru-23-G2 (equivalent to the genotype BB based on the previously presented classification)[5,24]. Viruses of the Ru-23-G2 (BB) genotype were similar to the Ru-23-G1 genotype by the PB1, HA, NA and MP segments (Fig. 1). Segments PA, NP and NS were closely related to

influenza A(H13) viruses circulating in gulls[22]. Viruses of the Ru-23-G2 genotype (representative virus A/gull/Mari_El/340-1V/2023) were detected in the European part of Russia (the easternmost point of detection was western slope of the Urals). Out of these, 38 were collected from 19 outbreaks in wild birds (predominantly gulls) and 31 were collected from 10 outbreaks in domestic birds (predominantly chickens). These viruses had AASs D88G, M532I and polymorphism at position 72R/G/E in the HA protein (Table S2).

Virus A/chicken/Kamchatka/262-76V/2023 of the Ru-23-G3 (G10) genotype was isolated in the Far East region of Russia (Fig. 1) and differed from the Ru-23-G1 genotype by the PB2 segment (Fig. 1). The genotype Ru-23-G3 (G10) was previously detected in China and Korea[2,24].

Two A(H5N1) viruses (A/dalmatian pelican/Kalmykia/330-10V/2023 and A/great cormorant/Kalmykia/330-12V/2023) detected in the European part of Russia represented previously undescribed reassortants, designated as Ru-23-G4. These virus genomes consist of HA, NP, NA and MP segments genetically similar to those of Ru-23-G1 (G1, A3) and Ru-23-G3 (G10) genotypes, while other four segments are genetically similar to the genes of various Asian and Russian LPAI viruses (Fig. 1, Supplementary Phylogenetic Trees).

According to the results of the genetic analysis, AASs H28Q, A83N, N154D, L322I, N446K and D487Y were detected in the HA of A/chicken/Kamchatka/262-76V/2023 virus (genotype Ru-23-G3) when compared to A/Astrakhan/3212/2020. Viruses with the Ru-23-G4 genotype were identical in the amino acid sequence of HA1 with CVV A/Astrakhan/3212/2020 (Table S2) and had AASs L462M and A495S in the HA2.

All viruses analyzed had 123P, 133A, 156A amino acids in the hemagglutinin, which are common for most clade 2.3.4.4b A(H5N1) viruses[22]. The presence of S123P, S133A, T156A substitutions in HA of influenza H5 viruses from other genetic subclades was previously associated with an increased binding to α2-6-linked sialic receptors[25–28]. The AAS HA-N154D, identified in A/chicken/Kamchatka/262-76V/2023 virus (genotype Ru-23-G3), was also previously associated with an increased binding to α2-6-linked sialic receptors[29].

Genetic analysis of A(H5N1) viruses isolated from fur seals in Russia in 2023

A genetic analysis of A(H5N1) viruses identified in samples from fur seals was carried out. A partial genetic sequence (HA gene—91.9%, NA gene—42.7%, PB2 gene—43.4%, NP gene—51.9%) was obtained only from one original specimen from seals (of the four specimens studied) due to low amount of genetic material of the virus in the four studied samples.

The complete genome sequences were obtained from the three isolates (including one isolate from the sequenced original specimen). The three viruses belonged to subtype A(H5N1) and clade 2.3.4.4b. The viruses isolated from fur seals were genetically similar to viruses identified in wild birds (thick-billed murre and mallard) in august 2023 in the Russian Far East Region (HA gene nucleotide sequence homology 99.6–99.8%) (Table S1).

The strains isolated from seals shared a high degree of hemagglutinin (HA) protein identity (1–2 AAS in the HA1 part of the HA protein) with the candidate vaccine strains A/Astrakhan/3212/2020 (H5N8), A/chicken/Ghana/AVL-763_21VIR7050-39/2021 (H5N1) and A/American Wigeon/South Carolina/22-000345-001/2021 (H5N1) from the clade 2.3.4.4b, recommended by the WHO[21]. As a result of analysis of the genome sequences of A(H5N1) strains isolated from fur seals, the amino acid substitution M265V was identified in the HA protein (H5 numbering, without signal peptide) when compared to A/Astrakhan/3212/2020 CVV (Table 1). The HA-M265V substitution has not been described in the literature and has not been identified in other influenza A(H5N1) viruses of the clade 2.3.4.4b circulating among birds and mammals according to the Global Initiative on Sharing All Influenza Data (GISAID) database (accessed 01.06 2024).

In the three viruses studied, amino acids 123P, 133A, 156A, previously associated with an increased binding of the virus to α2,6 receptors[25–28] and typical for A(H5N1) viruses of the 2.3.4.4b clade, were identified.

In all viruses isolated from fur seals, the N319K mutation was found in the NP protein, which can increase the efficiency of virus replication, thereby increasing its virulence for mammals[23].

Single nucleotide polymorphism (SNP) analysis of minor viral populations of the obtained sequence data from the original material did not reveal any additional mutations associated with mammalian adaptation.

Based on the phylogenetic analysis, for all viruses isolated from fur seals genotype G1 (A3) was determined (Supplemental phylogenetic trees).

Receptor specificity

The receptor specificity of the 36 A(H5N1) representative viruses from birds available for characterization and three viruses from fur seals were evaluated by means of biolayer interferometry using influenza virus receptor analogs 3′-Sialyl-N-acetyllactosamine (α2-3-SA) and 6′-Sialyl-N-acetyllactosamine (α2-6-SA). The virus binding to the α2-3-SA receptor analog was detected and the equilibrium dissociation constant was determined, while the virus binding for the α2-6-SA receptor analogue was not detected (Table S3).

Susceptibility to neuraminidase inhibitors

A genetic analysis of neuraminidase (NA) of the 77 A(H5N1) viruses from birds and three viruses from seals showed that the NA of all tested viruses contained no molecular markers of reduced susceptibility to neuraminidase inhibitors, according to the list of markers provided by the WHO[30]. In one virus, A/chicken/Belgorod/283-1V/2023, an AAS NA-Y253H (H5N1 2.3.4.4b numbering) was detected, which was previously associated with hypersensitivity to oseltamivir[31–33].

The PA gene nucleotide sequences of 77 influenza A(H5N1) viruses from birds and three viruses from seals were analyzed for mutations associated with reduced susceptibility to baloxavir marboxil (AASs: A37T, I38F/M/T)[34]. None of the viruses had markers associated with reduced susceptibility to baloxavir marboxil.

Analysis of the M2 protein of the A/chicken/Komi/10-2V/2023 virus revealed a V27A substitution, which was absent in all other studied viruses. In addition, an AAS A30S was detected in 6 viruses. V27A and A30S substitutions have been shown to reduce the susceptibility of viruses to amantadine and rimantadine[35,36].

Antigenic site									A		B	B	B	B		D						
Mature HA1		16	28	31	36	72	83	88	104	136	150	154	155	156	210	219	238	265	282	312	322	325
A/chicken/Ghana/AVL-763 21VIR7050-39/2021 CVV		V	H	D	T	R	A	D	L	P	L	N	D	T	V	V	A	M	V	V	L	K
A/Astrakhan/3212/2020 CVV		.	.	.	.	.	.	.	.	.	.	.	.	A	V	.	.	.	.	.	.	.
A/American Wigeon/South Carolina/22-000345-001/2021 CVV		.	.	.	.	.	.	.	M	.	.	.	.	A	A	.	.	.	.	.	.	.
A/fur seal/Sakhalin/399-1V/2023	1	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	V	.	.	.	.
A/fur seal/Sakhalin/399-2V/2023	2	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	V	.	.	.	.
A/fur seal/Sakhalin/399-3V/2023	3	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	V	.	.	.	.

Table 1. Amino acid substitutions in the HA of A(H5N1) viruses isolated from fur seals compared to CVVs.

In order to assess phenotypic susceptibility, 30 representative of collection sets (at least one per set, if available for characterization) influenza A(H5N1) viruses from birds and two viruses from seals were tested in fluorescence neuraminidase inhibition assay with oseltamivir and zanamivir. The median IC_{50} values (half-maximum inhibitory concentration) of oseltamivir and zanamivir calculated for the A(H5N1) subtype (N = 32) were 1.74 and 0.39 nM respectively. None of the tested A(H5N1) viruses had IC_{50} values significantly exceeding the subtype median: maximum fold-changes between an IC_{50} value determined for an individual strain and the subtype median were 1.4 and 2.7 for oseltamivir and zanamivir respectively. The median IC_{50} values of the A(H5N1) subtype exceeded IC_{50} values of the control susceptible seasonal influenza A(H1N1)pdm09 virus 7.6 times for oseltamivir and 1.2 times for zanamivir (Table S4).

A virus bearing NA-Y253H substitution was not available for phenotypic susceptibility analysis.

Discussion

In 2023, HPAI viruses affected vast areas, being detected in many countries all over the world. Most outbreaks in poultry and wild birds were caused by HPAIV A(H5N1) of the clade 2.3.4.4b. The A(H5N1) virus of the BB genotype predominantly affected gulls and mass deaths of these birds were observed in March–April 2023 in Belgium, the Netherlands, Italy, France, Poland, and the United Kingdom[7,8]. Mass deaths of gulls were registered from May to July 2023 in the European part of Russia and several poultry outbreaks were registered during the same period in the same geographical area (Table S1). All viruses detected in the gulls belonged to the A(H5N1) subtype and clade 2.3.4.4b and were similar in their HA gene to viruses circulating in Europe and worldwide[21,37].

In 2023, mammalian mortality from influenza A(H5N1) was observed worldwide. Mass mortality of mammals was mainly observed in regions where outbreaks of A(H5N1) in birds were also reported[5,7–11]. A large outbreak of A(H5N1) in seals in the Far East of Russia was observed in July–August 2023[20] and co-occurred with the outbreak of A(H5N1) in wild birds in Sakhalin (Table S1). The viruses from the outbreaks in wild birds and seals investigated in this study were closely genetically related (nucleotide identity of HA from 99.6 to 99.8%). The A(H5N1) viruses were isolated from seabirds thick-billed murre and mallard, common in the Far East coastal region of Russia. Birds of these species may participate in the transmission of influenza virus via environmental contacts with marine mammals and can potentially spread the virus over large distances via migration[38,39].

Minor diversification of the HA gene of the 2.3.4.4b A(H5N1) viruses was observed in Russia and worldwide. Most viruses were antigenically similar to A(H5Nx) 2.3.4.4b CVVs[21].

The importance of genotype analysis for clade 2.3.4.4b A(H5N1) influenza viruses has been increasing in recent years due to emergence of many new reassortant viruses in circulation. According to previously reported analysis, 13 genotypes were identified in circulation in Europe in 2020–2022, of which G1 (equivalent to genotype A3) was reported as predominant[2]. Recent analysis available in GISAID identified four genotypes (A5, A3, A2 and B3.2) of A(H5N1) 2.3.4.4b for viruses detected in Europe in 2021–2023, while for most of the viruses the genotype has not been reported. In Russia, A(H5N1) 2.3.4.4b genotype G1 viruses have been circulating in the Russian Far East since 2022 (GISAID, accessed 28.02.25).

This study presents one of the first large scale genotype analysis of 2.3.4.4b clade A(H5N1) influenza viruses in Russia. Genetic analysis of the 77 A(H5N1) viruses from birds and three viruses from seals, detected in Russia in 2023, revealed that they represent four distinct genotypes. Most viruses (69 strains) represented the BB genotype first detected in May, 2022 in France. This genotype presumably acquired PA, NP and NS segments from influenza A(H13) viruses detected in gulls which probably made this genotype well-adapted for circulation in *Laridae* family[22]. In summer 2022 it was detected among sea birds in Northern Europe. However, it later spread to Western, Southern and Eastern Europe and became the most-frequently detected genotype in wild birds in Europe during the period February–August 2023 as well as a predominant genotype registered in poultry in Europe during the summer 2023[5]. According to our data, this genotype reached the European part of Russia in the spring of 2023, causing multiple outbreaks both in gulls and poultry.

The genotype G1[2] also described as A3 or EA-2020-C[22] was identified for five viruses from birds and three viruses from seals. This genotype was first detected in Netherlands in October 2020 and became one of the most prevalent during the 2021–2022 epidemic wave in Europe[22]. This genotype also spread to other regions including Africa, Asia[2] and North America[1].

The remaining three identified viruses were reassortants of the G1 genotype viruses with various LPAI viruses and represented two genotypes: G10 (also described as Ru-23-G3), and novel reassortant variant designated as Ru-23-G4.

Previous studies identified differences in the virulence of A(H5N1) viruses of different genotypes in mammals[2,6,40]. Hence, the ongoing monitoring of various reassortants in circulation is important for pandemic potential assessment. Limited availability of data on circulating genotypes and complexity of required phylogenetic analysis for genotype classification underline the importance of further research and development of a unified genotype classification for circulating 2.3.4.4b A(H5N1) influenza viruses.

In addition to reassortment data, a variety of molecular markers related to virus pandemic potential may have an effect on pandemic risk of circulating clade 2.3.4.4b A(H5N1) viruses[28].

This study showed presence of several molecular markers, previously associated with adaptation to mammals in HA and NP proteins of the investigated A(H5N1) viruses (Table 2).

All influenza A(H5N1) viruses analyzed in this study had 123P, 133A and 156A amino acid residues in HA. Initially, the phenotypic effect of these substitutions was described in previously circulated H5 viruses which did not belong to the 2.3.4.4b genetic clade[25,41,42]. The majority of the currently registered 2.3.4.4b A(H5N1) viruses have HA-123P, 133A, 156A genotype. It is not fully clear what impact this molecular signature has on the receptor specificity of 2.3.4.4b A(H5N1) viruses. In addition, the AAS HA-N154D was identified in the studied A/chicken/Kamchatka/262-76V/2023 virus. Previously it was shown that the AAS HA-N154D reduces

Segment	AAS (H5 numbering)	Phenotype	Subtypes	Clade	References
HA	S123P	Increased virus binding to α2-6-linked sialic receptors	H5N6	2.3.4.4c	[30]
HA	S133A	Increased virus binding to α2-6-linked sialic receptors	H5N1	2.3.4.4	[30,31]
HA	A133W	Increased virus binding to α2-6-linked sialic receptors	H5N1	2.3.4.4b	[48]
HA	T156A	Increases virus binding to α2-3- and α2-6-linked sialic receptors	H5Nx	2.3.4.4	[29,32]
HA	N154D	Reduces virus binding to α2-3-linked sialic receptors Increases virus binding to α2-6-linked sialic receptors	H5N1	1.1	[32]
NP	N319K	Increases virus virulence for mammals	H7N7		[49,50]

Table 2. Molecular markers in the HA and the NP associated with adaptation to mammals.

binding to α2-3-linked sialic receptors and increases binding to α2-6-linked sialic receptors[29]. Some studies show that HA-T156A substitution can probably be associated with a dual α2-6- and α2-3-receptor specificity in closely related 2.3.4.4 clade[29,32]. Dual receptor specificity was also shown for a bovine 2.3.4.4b A(H5N1) virus[43]. Nevertheless, another study failed to confirm this result and showed preferential binding to α2-3-receptors for an analogous strain[44]. Our study also showed a preferential binding of A(H5N1) 2.3.4.4b viruses to avian-type receptors with the absence of human-type receptor binding. In our study, the AAS HA-S123P, S133A, T156A did not result in a detectable increase in α2-6-linked sialic receptor binding, despite being associated with an increase in α2-6-linked sialic receptor binding for viruses of other clades. Thus, further studies, including both in vitro and in vivo research, should elucidate the phenotypic significance of the previously described genetic markers of altered receptor specificity for viruses with the 2.3.4.4b genetic background. For instance, HA-A133W substitution at previously described 133 position have been shown to enhance 2.3.4.4b A(H5N1) virus cell-entry via α2-6-receptor[45].

It should be noted that the adaptation of viruses during observed mass infection of mammals may lead to changes in receptor specificity towards "human"-type receptors. Due to the substantial increase in the cases of illness and death of mammals from influenza A(H5N1), constant monitoring of receptor specificity is important for the timely detection of pandemically dangerous viruses.

The genetic analysis of 77 influenza A(H5N1) viruses from birds and three viruses from seals carried out in our study did not reveal mutations in the PB2 protein associated with increased pathogenicity and adaptation to mammals. In viruses isolated from wild birds and seals in the eastern regions of Russia in 2023, which belonged to G1 genotype, the AAS N319K was detected in the NP protein, previously associated with increased virulence of the viruses for mammals[46,47]. However, the occurrence of this AAS is currently low in broad circulation based on the analysis of the available A(H5N1) 2.3.4.4b clade virus sequences in the GISAID database (2.9%, by 19.04.2024 date). This mutation is not found in other previously reported A(H5N1) 2.3.4.4b clade viruses isolated from marine mammals (based on genetic analysis of data available in GISAID, accessed on 21.02.2025). A previous study of A(H7N7) virus showed that mammalian adaptation resulted in accumulation of NP-N319K AAS together with additional mutation in PB2 which enhanced the virulence. The N319K mutation in the NP protein provides adaptation to the human importin-α1 and -α7 isoforms, supporting efficient replication in human lung cell lines, especially in combination with the PB2 D701N or E627K mutations[48,49]. An A(H5N1) virus from seals with such a combination of AASs (NP-N319K and PB2-E627K) was detected in another study of the seal outbreak in Russia in 2023[20]. This indicates that reassortment and adaptive mutations may quickly lead to mutually enhancing AAS combinations and fast mammalian adaptation. The lack of any reported studies of the effect of the NP-N319K mutation on virulence of A(H5N1) 2.3.4.4b clade viruses warrants additional research.

The AAS V27A and A30S in the M2 protein, associated with a decreased sensitivity to amantadine and rimantadine[35,36], were detected in one and six out of 77 A(H5N1) viruses studied isolated from birds, respectively (9% of the studied viruses). This data suggests that adamantanes may potentially be used for the avian influenza virus infection treatment against susceptible viruses with timely monitoring of the viruses drug susceptibility.

The A(H5N1) viruses analyzed in our study did not have genetic markers of reduced susceptibility to neuraminidase inhibitors and baloxavir. Viruses bearing markers of reduced susceptibility to these drug classes have also been rarely seen globally[50,51]. One virus in our analysis had AAS NA-Y253H, previously associated with an increased susceptibility to oseltamivir[31–33]. This virus was not available for phenotypic analysis.

It is known that influenza viruses bearing neuraminidases of different types and subtypes (or of various genetic lineages of the same subtype) can differ in their susceptibility to neuraminidase inhibitors. Hence, it is important to assess typical levels of neuraminidase inhibitors' concentrations which reduce a neuraminidase activity by half (IC_{50}) for a particular subtype, especially if it was not studied extensively previously. A determination of a median inhibition level for the subtype is a key step in the establishment of a sustained phenotypic monitoring of sensitivity to neuraminidase inhibitors since criteria of normal, reduced or highly reduced drug inhibition is based on comparison of IC_{50} values determined for an individual strain with the subtype median[52]. Based on these criteria, all A(H5N1) viruses tested in our study were normally inhibited by oseltamivir and zanamivir since none of them had IC_{50} values significantly exceeding the median.

Additionally, comparison of typical IC_{50} values between different subtypes can provide valuable information. In our study the median zanamivir IC_{50} determined for the A(H5N1) subtype was comparable to an IC_{50} of a susceptible seasonal A(H1N1)pdm09 strain. There is an extensive amount of data regarding clinical efficacy of neuraminidase inhibitors against seasonal influenza. Median IC_{50} values of these drugs have been continuously monitored for seasonal viruses for several decades. Even though results of the neuraminidase inhibition assay

cannot directly predict clinical efficacy, the similarity in zanamivir IC_{50} values between influenza A(H5N1) viruses and a seasonal susceptible A(H1N1)pdm09 strain suggests that this drug could possibly be useful in the influenza A(H5N1) treatment. At the same time, in our study neuraminidases of influenza A(H5N1) viruses were less well inhibited by oseltamivir than a susceptible A(H1N1)pdm09 virus. Nevertheless, oseltamivir can still be considered a potentially useful drug in the influenza A(H5N1) treatment since its median IC_{50} for the A(H5N1) subtype is 1.74 nM, which is lower than IC_{50} determined for a susceptible seasonal influenza B virus.

Despite the large number of outbreaks of A(H5N1) clade 2.3.4.4b in animals worldwide, relatively few cases of human infection with this A(H5N1) variant have been reported[53]. Since its emergence in 2020, there have been no documented cases of human-to-human transmission of HPAI A(H5N1) virus of the clade 2.3.4.4b. The overall risk to humans remains low; however, individuals whose occupations place them in contact with infected animals are at increased risk.

The broad geographical circulation of the A(H5N1) viruses, their ability to cross species barriers and infect mammalian species, fast evolution, including continuous reassortment and accumulation of mammalian adaptation mutations, indicate the importance of pandemic preparedness measures against these viruses. Timely, broad and thorough monitoring of A(H5N1) and other HPAIV is necessary for optimizing anti-epizootic and anti-epidemic measures. This is essential for the timely response and prevention of virus spreading, thus substantially reducing the risk of infection among people.

Materials and methods

Samples from wild birds and poultry were collected during the National avian influenza virus surveillance program, which supports sample collection for animal, avian or zoonotic influenza diagnostics from all regions of Russia. Samples from birds included cloacal or oropharyngeal swabs, and internal organs fragments. In 2023, a total of 177 samples from wild birds and poultry (31 collection sets) and one collection set of eight samples from four seals were analyzed during avian influenza virus surveillance of HPAI outbreaks (Table S1). In this study we report the characterization of 80 HPAI viruses (all of which were initially identified as H5) isolated from the outbreaks, associated with the death of birds or seals in different regions of Russia.

If collection set had less than 5 samples per animal species, then all samples were used for virus isolation, and all isolated strains were studied further. If collection set contained more than 5 samples per animal species, and more than 5 isolates per animal species were successfully isolated in ECE, then a subset of 5 to 10 strains per animal species were studied further.

Names of influenza A virus strain name include collection location and collection set code, which follows after the region (or city) name (e.g. in A/chicken/Kamchatka/262-76/2023, "Kamchatka" is a geographical region; number 262—is the code of a collection set from an outbreak, and 76 is a sample identifier in the 262 collection set). Virus names and collection dates are provided in Table S1 (Supplementary materials).

Biological samples including swabs from cloaca and trachea, as well as fragments of internal organs, were collected from dead poultry and wild birds in avian influenza outbreak settings. The Copan Universal Transport Medium (UTM-RT) System (Copan, Italy) was used for sample collection. Swabs and organ homogenates were prepared as previously described[54].

Influenza virus propagation and titration

Influenza A(H5N1) viruses were isolated in 10-day-old ECE at 37 °C for up to 48 h. Viral titers were determined by injecting 0.1 mL of tenfold dilutions of virus into the allantoic cavities of 10-day-old ECE and then calculating the 50% embryo infectious dose (EID_{50}) by the method of Reed and Muench[55]. The virus titer was measured for a representative subset of isolated viruses. The studied A(H5N1) viruses demonstrated a high reproduction level when passaging in ECEs. The virus titer in allantoic fluid ranged from 7.7 to 9.7 log10 EID50/ml (Table S5). All experiments with the viruses were conducted in the Biosafety level 3 (BSL 3) containment laboratory.

Isolation of influenza virus RNA

RNA was isolated from original biological samples or allantoic fluid using the AmpliSens RIBO-prep kit (Central Research Institute of Epidemiology of Rospotrebnadzor) or MagnoPrime Uni kit (NextBio LLC, Russia) according to the manufacturer's instructions. For reverse transcription, AmpliSens Reverta-L kit (Central Research Institute of Epidemiology of Rospotrebnadzor) was used.

Typing of influenza virus

Viral RNA was isolated using RIBO-sorb RNA/DNA Extraction Kit (InterLabService, Moscow, Russia) according to the manufacturer's instruction. Reverse transcription was performed using "Reverta-L kit" (Interlabservice, Russia) with random hexamer-primer. Real-time RT-PCR typing and subtyping for H5 was performed using "AmpliSens Influenza virus A/B-FL kit", "AmpliSens Influenza virus A H5N1-FL kit", "AmpliSens Influenza virus A-type-H5, H7, H9-FL kit" (Interlabservice,Russia) and RotorGene 6000 thermal cycler with the programs recommended in the manufacturer's manual of the RT-PCR kits.

Sequence analysis of influenza viruses

Sequencing was carried out at the Federal Budgetary Research Institute State Research Center of Virology and Biotechnology "Vector", Rospotrebnadzor (SRC VB "Vector"). Reverse transcription was carried out with a mixture of primers (Uni12, Uni12.4, and Uni13)[56] using the RT-M-MuLV-RH reagent kit (LLC BIOLABMIX, Russia). PCR amplification of cDNA was performed using the BioMaster LR HS-Taq PCR kit (2×) (LLC BIOLABMIKS, Russia) according to the manufacturer's instructions. NGS sequencing was performed on an Illumina MiSeq using the MiSeq reagent kit v3 (Illumina, San Diego, CA, USA). The full-length genomes were assembled by the alignment of reads to known references with bwa-0.7.15[57]. The obtained nucleotide sequences

were deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database. Maximum likelihood phylogenetic analysis was performed in IQ-TREE v2.0.7[58] applying the best-fitted nucleotide substitution model selected by ModelFinder[59] with 500 bootstrap replicates and visualized in FigTree v1.4.4[60]. Reference strains genome sequences were used from GISAID. Amino acid substitutions have been identified using Treesub[61].

Receptor Binding Assay

Virus-containing fluid inactivated by β-propiolactone was centrifugated at $9000 \times g$ for 1 h and filtered through 0.45 μm filter. Virus particles were next concentrated by centrifugation at $40,000 \times g$ for 3 h and resuspended in phosphate-buffered saline (PBS). Size-exclusion chromatography was performed using Sepharose CL-4B resins (GE Healthcare). Finally, influenza A virions were concentrated using Amicon® Ultra-15 Centrifuge Filters Ultracell® 100KDa (Merck Millipore).

The concentrations of purified viruses were determined by HA assays. Concentrations determined as number of hemagglutination units per mL (HAU/mL) were converted to the nmol/L (nM) measurement units (taking into consideration that one HAU typically corresponds to 10^6 viral particles, and 1 viral particle has approximately 1000 HA glycoprotein spikes; concentration measured in HAU/mL was converted to spikes/mL, and then converted to nmol/mL (by dividing by Avogadro constant) as previously described[62].

The kinetics of virions binding to receptor analogues was studied by biolayer interferometry on Octet RED96e (ForteBio). 3'-Sialyl-N-acetyllactosamine-biotin (3'SLN, Lectinity, Moscow) and 6'-Sialyl-N-acetyllactosamine-biotin (6'SLN, Lectinity, Moscow) were used as α2-3 and α2-6 receptor analogues. 3'SLN and 6'SLN were loaded onto streptavidin biosensors at a concentration of 0.5 μg/mL and then viruses at 30 nM were added. Oseltamivir was used to inhibit neuraminidase activity. Equilibrium dissociation constants were calculated using ForteBio Data Analysis 12.0 software.

Phenotypic analysis of neuraminidase inhibitors susceptibility

Fluorescence neuraminidase inhibition assay with 2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA) substrate was used to characterize susceptibility of avian influenza viruses to oseltamivir and zanamivir[63].

Data availability

Virus genome sequences generated in the study are available in GISAID (https://gisaid.org/: EPI_ISL_17995852–EPI_ISL_17995857, EPI_ISL_17995859–EPI_ISL_17995870, EPI_ISL_17995872–EPI_ISL_17995873, EPI_ISL_17995875–EPI_ISL_17995890, EPI_ISL_18462486–EPI_ISL_18462491, EPI_ISL_18462493–EPI_ISL_18462514, EPI_ISL_18462518–EPI_ISL_18462523, EPI_ISL_18525093–EPI_ISL_18525103, EPI_ISL_18525108–EPI_ISL_18525121, EPI_ISL_18525123, EPI_ISL_19157101, EPI_ISL_19157103–EPI_ISL_19157105; Table S1).

References

1. Youk, S. et al. H5N1 highly pathogenic avian influenza clade 2.3.4.4b in wild and domestic birds: Introductions into the United States and reassortments, December 2021–April 2022. *Virology* **587**, 109860. https://doi.org/10.1016/j.virol.2023.109860 (2023).
2. Cui, P. et al. Global dissemination of H5N1 influenza viruses bearing the clade 2.3.4.4b HA gene and biologic analysis of the ones detected in China. *Emerg. Microbes Infect.* **11**(1), 1693–1704. https://doi.org/10.1080/22221751.2022.2088407 (2022).
3. CDC. Highlights in the History of Avian Influenza (Bird Flu) Timeline—2020–2024. https://www.cdc.gov/flu/avianflu/timeline/avian-timeline-2020s.htm. Accessed August 2024.
4. Lindh, E. et al. Highly pathogenic avian influenza A(H5N1) virus infection on multiple fur farms in the South and Central Ostrobothnia regions of Finland, July 2023. *Euro Surveill.* **28**(31), 2300400. https://doi.org/10.2807/1560-7917.ES.2023.28.31.2300400 (2023).
5. European Food Safety Authority; European Centre for Disease Prevention and Control; European Union Reference Laboratory for Avian Influenza; Adlhoch, C. et al. Avian influenza overview June – September 2023. *EFSA J.* **21**(10), e08328. https://doi.org/10.2903/j.efsa.2023.8328 (2023).
6. Kandeil, A. et al. Rapid evolution of A(H5N1) influenza viruses after intercontinental spread to North America. *Nat Commun.* **14**(1), 3082. https://doi.org/10.1038/S31467-023-38415-7 (2023).
7. WAHIS. Quantitative data dashboard. https://wahis.woah.org/#/dashboards/qd-dashboard. Accessed August 2024.
8. European Food Safety Authority; European Centre for Disease Prevention and Control; European Union Reference Laboratory for Avian Influenza; Adlhoch, C. et al. Avian influenza overview December 2022–March 2023. *EFSA J.* 21(3), e07917. https://doi.org/10.2903/j.efsa.2023.7917 (2023).
9. European Food Safety Authority; European Centre for Disease Prevention and Control; European Union Reference Laboratory for Avian Influenza; Adlhoch, C. et al. Avian influenza overview March–April 2023. *EFSA J.* 21(6), e08039. https://doi.org/10.2903/j.efsa.2023.8039 (2023).
10. European Food Safety Authority; European Centre for Disease Prevention and Control; European Union Reference Laboratory for Avian Influenza; Adlhoch, C. et al. Avian influenza overview April–June 2023. *EFSA J.* 21(7), e08191. https://doi.org/10.2903/j.efsa.2023.8191 (2023).
11. European Food Safety Authority; European Centre for Disease Prevention and Control; European Union Reference Laboratory for Avian Influenza; Adlhoch, C. et al. Avian influenza overview September–December 2023. *EFSA J.* 21(12), e8539. https://doi.org/10.2903/j.efsa.2023.8539 (2023).
12. Agüero, M. et al. Authors' response: Highly pathogenic influenza A(H5N1) viruses in farmed mink outbreak contain a disrupted second sialic acid binding site in neuraminidase, similar to human influenza A viruses. *Euro Surveill.* **28**(7), 2300109. https://doi.org/10.2807/1560-7917.ES.2023.28.7.2300109 (2023).
13. Tammiranta, N. et al. Highly pathogenic avian influenza A (H5N1) virus infections in wild carnivores connected to mass mortalities of pheasants in Finland. *Infect. Genet. Evol.* **111**, 105423. https://doi.org/10.1016/j.meegid.2023.105423 (2023).
14. Pardo-Roa, C. et al. Cross-species transmission and PB2 mammalian adaptations of highly pathogenic avian influenza A/H5N1 viruses in Chile. bioRxiv [Preprint]. 2023.06.30.547205. https://doi.org/10.1101/2023.06.30.547205v1 (2023).

15. Leguia, M. et al. Highly pathogenic avian influenza A (H5N1) in marine mammals and seabirds in Peru. *Nat. Commun.* **14**(1), 5489. https://doi.org/10.1038/S31467-023-41182-0 (2023).
16. Rimondi, A. et al. Highly Pathogenic Avian Influenza A(H5N1) Viruses from Multispecies Outbreak, Argentina, August 2023. *Emerg. Infect. Dis.* **30**(4), 812–814. https://doi.org/10.3201/eid3004.231725 (2024).
17. Uhart, M. M. et al. Epidemiological data of an influenza A/H5N1 outbreak in elephant seals in Argentina indicates mammal-to-mammal transmission. *Nat. Commun.* **15**(1), 9516. https://doi.org/10.1038/s41467-024-53766-5 (2024).
18. Puryear, W. et al. Highly Pathogenic Avian Influenza A(H5N1) Virus Outbreak in New England Seals, United States. *Emerg. Infect. Dis.* **29**(4), 786–791. https://doi.org/10.3201/eid2904.221538 (2023).
19. Nguyen, Th. -Q. et al. Emergence and interstate spread of highly pathogenic avian influenza A(H5N1) in dairy cattle. bioRxiv [Preprint]. 2024.05.01.591751. https://doi.org/10.1101/2024.05.01.591751v1 (2024).
20. Sobolev, I. et al. Highly pathogenic avian influenza A(H5N1) virus clade 2.3.4.4b infections in Seals, Russia, 2023. *Emerg. Infect. Dis.* **30**(10), 2160–2164. https://doi.org/10.3201/eid3010.231728 (2024).
21. WHO. Genetic and antigenic characteristics of zoonotic influenza A viruses and development of candidate vaccine viruses for pandemic preparedness (February 2024). https://cdn.who.int/media/docs/default-source/influenza/who-influenza-recommend ations/vcm-northern-hemisphere-recommendation-2024-2025/202402_zoonotic_vaccinvirusupdate.pdf?sfvrsn=70150120_4. Accessed August 2024.
22. Fusaro, A. et al. High pathogenic avian influenza A(H5) viruses of clade 2.3.4.4b in Europe-Why trends of virus evolution are more difficult to predict. *Virus Evol.* **10**(1), ceae027. https://doi.org/10.1093/ve/veae027 (2024).
23. Gabriel, G. et al. Differential polymerase activity in avian and mammalian cells determines host range of influenza virus. *J. Virol.* **81**(17), 9601–9604. https://doi.org/10.1128/JVI.00666-07 (2007).
24. Kang, Y. M. et al. Introduction of multiple novel high pathogenicity avian influenza (H5N1) virus of clade 2.3.4.4b into South Korea in 2022. *Transbound Emerg Dis.* **2023**, 8339427. https://doi.org/10.1155/2023/8339427 (2023).
25. Yang, Z.-Y. et al. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. *Science* **317**, 825–828. https://doi.org/10.1126/science.1135165 (2007).
26. Gao, R. et al. T160A mutation-induced deglycosylation at site 158 in hemagglutinin is a critical determinant of the dual receptor binding properties of clade 2.3.4.4 H5NX subtype avian influenza viruses. *Vet. Microbiol.* **217**, 158–166. https://doi.org/10.1016/j.vetmic.2018.03.018 (2018).
27. Cao, L. et al. Epidemiological and genetic characteristics of H5 subtype avian influenza virus in Guangzhou, 2014–2019. *Zhonghua Liu Xing Bing Xue Za Zhi* **41**(7), 1115–1120. https://doi.org/10.3760/cma.j.cn112338-20190730-00565 (2020).
28. Yamaji, R. et al. Pandemic potential of highly pathogenic avian influenza clade 2344 A(H5) viruses. *Rev. Med. Virol.* **30**(3), e2099. https://doi.org/10.1002/rmv.2099 (2020).
29. Hu, W. Mutations in hemagglutinin of H5N1 influenza that switch receptor specificity from avian to human types. *Comput. Mol. Biosci.* **3**, 32–37. https://doi.org/10.4236/cmb.2013.32005 (2013).
30. WHO. Summary of neuraminidase (NA) amino acid substitutions associated with reduced inhibition by neuraminidase inhibitors (NAIs) among avian influenza viruses of Group 1 and Group 2 NAs. https://www.who.int/publications/m/item/summary-of-neu raminidase-(na)-amino-acid-substitutions-associated-with-reduced-inhibition-by-neuraminidase-inhibitors-(nais)-among-avia n-influenza-viruses-of-group-1-and-group-2-nas. Accessed July 2024.
31. Collins, P. J. et al. Crystal structures of oseltamivir-resistant influenza virus neuraminidase mutants. *Nature* **453**(7199), 1258–1261. https://doi.org/10.1038/nature06956 (2008).
32. Ilyushina, N. A., Seiler, J. P., Rehg, J. E., Webster, R. G. & Govorkova, E. A. Effect of neuraminidase inhibitor-resistant mutations on pathogenicity of clade 2.2 A/Turkey/15/06 (H5N1) influenza virus in ferrets. *PLoS Pathog.* **6**(5), e1000933. https://doi.org/10.1371/journal.ppat.1000933 (2010).
33. Nguyen, H. T. et al. Antiviral susceptibility of highly pathogenic avian influenza A(H5N1) viruses isolated from poultry, Vietnam, 2009–2011. *Emerg. Infect. Dis.* **19**(12), 1963–1971. https://doi.org/10.3201/eid1912.130705 (2013).
34. Summary of polymerase acidic (PA) protein amino acid substitutions analysed for their effects on baloxavir susceptibility. https://www.who.int/publications/m/item/summary-of-polymerase-acidic-(pa)-protein-amino-acid-substitutions-analysed-for-their-eff ects-on-baloxavir-susceptibility. Accessed July 2024.
35. Abed, Y., Goyette, N. & Boivin, G. Generation and characterization of recombinant influenza A (H1N1) viruses harboring amantadine resistance mutations. *Antimicrob. Agents Chemother.* **49**(2), 556–559. https://doi.org/10.1128/AAC.49.2.556-559.2005 (2005).
36. Ilyushina, N. A., Govorkova, E. A. & Webster, R. G. Detection of amantadine-resistant variants among avian influenza viruses isolated in North America and Asia. *Virology* **341**(1), 102–106. https://doi.org/10.1016/j.virol.2005.07.003 (2005).
37. WHO. Genetic and antigenic characteristics of zoonotic influenza A viruses and development of candidate vaccine viruses for pandemic preparedness (September 2023). https://cdn.who.int/media/docs/default-source/influenza/who-influenza-recomm endations/vcm-southern-hemisphere-recommendation-2024/202309_zoonotic_vaccinvirusupdate.pdf?sfvrsn=e78676a0_5. Accessed August 2024.
38. irdLife International. 2018. *Uria lomvia*. The IUCN Red List of Threatened Species 2018:e.T22694847A132066134. https://www.iu cnredlist.org/species/22694847/132066134. Accessed November 2024.
39. BirdLife International. 2019. Anas platyrhynchos (amended version of 2017 assessment). The IUCN Red List of Threatened Species 2019: e.T22680186A155457360. https://www.iucnredlist.org/species/22680186/84955317. Accessed November 2024.
40. Zhao, W. et al. Virulence and transmission characteristics of clade 2.3.4.4b H5N6 subtype avian influenza viruses possessing different internal gene constellations. *Virulence.* **14**(1), 2250065. https://doi.org/10.1080/21505594.2023.2250065 (2023).
41. Yamada, S. et al. Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. *Nature* **444**, 378–382. https://doi.org/10.1038/nature05264 (2006).
42. Wang, W. et al. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. *J. Virol.* **84**(13), 6570–6577. https://doi.org/10.1128/JVI.00221-10 (2010).
43. Eisfeld, A. J. et al. Pathogenicity and transmissibility of bovine H5N1 influenza virus. *Nature* **633**(8029), 426–432. https://doi.org/10.1038/S31586-024-07766-6 (2024).
44. Chopra, P. et al. Receptor Binding Specificity of a Bovine A(H5N1) Influenza Virus. bioRxiv [Preprint]. 2024.07.30.605893. https://doi.org/10.1101/2024.07.30.605893v1 (2024).
45. Dadonaite, B. et al. Deep mutational scanning of H5 hemagglutinin to inform influenza virus surveillance. bioRxiv [Preprint]. 2024.05.23.595634. https://doi.org/10.1101/2024.05.23.595634v2 (2024).
46. Gabriel, G. et al. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. *Proc. Natl. Acad. Sci. USA* **102**, 18590–18595. https://doi.org/10.1073/pnas.0507415102 (2005).
47. Gabriel, G. et al. Differential use of importin-alpha isoforms governs cell tropism and host adaptation of influenza virus. *Nat. Commun.* **2**, 156. https://doi.org/10.1038/ncomms1158 (2011).
48. Danzy, S. et al. Mutations to PB2 and NP proteins of an avian influenza virus combine to confer efficient growth in primary human respiratory cells. *J. Virol.* **88**(22), 13436–13446. https://doi.org/10.1128/JVI.01093-14 (2014).
49. AbuBakar, U. et al. Avian influenza virus tropism in humans. *Viruses* **15**(4), 833. https://doi.org/10.3390/v15040833 (2023).
50. Nguyen, H. T. et al. Antiviral susceptibility of clade 2.3.4.4b highly pathogenic avian influenza A(H5N1) viruses isolated from birds and mammals in the United States, 2022. *Antiviral Res.* **217**, 105679. https://doi.org/10.1016/j.antiviral.2023.105679 (2023).

51. Andreev, K. et al. Antiviral susceptibility of highly pathogenic avian influenza A(H5N1) viruses circulating globally in 2022–2023. *J. Infect. Dis.* **229**(6), 1830–1835. https://doi.org/10.1093/infdis/jiad418 (2023).

52. WHO. Meetings of the WHO working group on surveillance of influenza antiviral susceptibility—Geneva, November 2011 and June 2012. Wkly Epidemiol Rec. 87(39), 369–374 (2012).

53. WHO. Avian Influenza Weekly Update Number 964. Human infection with avian influenza A(H5) viruses. https://cdn.who.int/media/docs/default-source/wpro---documents/emergency/surveillance/avian-influenza/ai_20240913.pdf?sfvrsn=5f006f99_141. Accessed September 2024.

54. Spackman, E. & Killian, M. L. Avian influenza virus isolation, propagation, and titration in embryonated chicken eggs. in (Spackman, E., eds.) *Animal Influenza Virus. Methods in Molecular Biology*, Vol. 1161. (Humana Press, 2014). https://doi.org/10.1007/978-1-4939-0758-8_12.

55. Reed, L. J. M. & Muench, H. A simple method of estimating fifty percent endpoints. *Am. J. Epidemiol.* **27**, 493–497. https://doi.org/10.1093/oxfordjournals.aje.a118408 (1938).

56. Kawaoka, Y. & Neumann, G. Influenza virus methods and protocols. New York: Humana Press; 2012. (Methods in molecular biology, 865). https://lib.ugent.be/catalog/ebk01:3390000000031639. Accessed August 2024.

57. Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. ArXiv. 16, 1303. https://doi.org/10.48550/arXiv.1303.3997 (2013).

58. Minh, B. Q. et al. IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. *Mol. Biol. Evol.* **37**(5), 1530–1534. https://doi.org/10.1093/molbev/msaa015 (2020).

59. Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A. & Jermiin, L. S. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat. Methods.* **14**(6), 587–589. https://doi.org/10.1038/nmeth.4285 (2017).

60. Rambaut, A. Figtree ver 1.4.4. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh. http://tree.bio.ed.ac.uk/software/figtree (2018).

61. Tamuri, A. U. Treesub: annotating ancestral substitution on a tree. https://github.com/tamuri/treesub (2013).

62. Fei, Y. et al. Characterization of receptor binding profiles of influenza A viruses using an ellipsometry-based label-free glycan microarray assay platform. *Biomolecules* **5**, 1480–1498. https://doi.org/10.3390/biom5031480 (2015).

63. Leang, S.-K. & Hurt, A. C. Fluorescence-based neuraminidase inhibition assay to assess the susceptibility of influenza viruses to the neuraminidase inhibitor class of antivirals. *J. Vis. Exp.* **122**, 1–7. https://doi.org/10.3791/55570 (2017).

Author contributions

P.A.: data interpretation, original draft preparation, writing-reviewing and editing. K.N., G.N.: PCR analysis, N.G.S. experiments, bioinformatics analysis and data interpretation, writing-reviewing and editing. S.S.: drug susceptibility analysis, data interpretation, writing-reviewing and editing. D.A., B.N., S.K., B.M.: PCR analysis, N.G.S. experiments, bioinformatics analysis and data interpretation. V.N., E.M., G.A.: influenza virus propagation and titration, data interpretation. O.G.: receptor binding analysis and data interpretation. R.A.: conceptualization the overall study and coordinated the investigation. M.V.: formulation of overarching research goals and aims, original draft preparation, supervision.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to V.Y.M.

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