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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

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Abstract

Since 2016, A(H5Nx) high pathogenic avian influenza (HPAI) virus of clade 2.3.4.4b has become one of the most serious global threats not only to wild and domestic birds, but also to public health. In recent years, important changes in the ecology, epidemiology, and evolution of this virus have been reported, with an unprecedented global diffusion and variety of affected birds and mammalian species. After the two consecutive and devastating epidemic waves in Europe in 2020–2021 and 2021–2022, with the second one recognized as one of the largest epidemics recorded so far, this clade has begun to circulate endemically in European wild bird populations. This study used the complete genomes of 1,956 European HPAI A(H5Nx) viruses to investigate the virus evolution during this varying epidemiological outline. We investigated the spatiotemporal patterns of A(H5Nx) virus diffusion to/from and within Europe during the 2020–2021 and 2021–2022 epidemic waves, providing evidence of ongoing changes in transmission dynamics and disease epidemiology. We demonstrated the high genetic diversity of the circulating viruses, which have undergone frequent reassortment events, providing for the first time a complete overview and a proposed nomenclature of the multiple genotypes circulating in Europe in 2020–2022. We described the emergence of a new genotype with gull adapted genes, which offered the virus the opportunity to occupy new ecological niches, driving the disease endemicity in the European wild bird population. The high propensity of the virus for reassortment, its jumps to a progressively wider number of host species, including mammals, and the rapid acquisition of adaptive mutations make the trend of virus evolution and spread difficult to predict in this unending evolving scenario.

Keywords: high pathogenic avian influenza A(H5) viruses; phylodynamics; Europe; reassortments; spatial spread.

Introduction

In 1996, high pathogenic avian influenza (HPAI) A(H5Nx) virus of the A/goose/Guangdong/1/1996 (Gs/Gd) lineage was identified for the first time in poultry in China. In late 2003 and early 2004, multiple outbreaks were reported almost simultaneously in poultry in several East Asian countries (Li et al. 2004). The situation dramatically changed in spring 2005, when, following a mass die-off of wild migratory birds infected with A(H5N1) in Qinghai Lake in central China (Chen et al. 2006), this strain spread over long distances reaching for the first time Europe and Africa during the wild bird autumn migration (Olsen et al. 2006). Since then, the HPAI A(H5Nx) virus of the Gs/GD lineage has widely spread across Asia, Europe, Africa, and North America (Fusaro et al. 2019; Lycett et al. 2020), becoming a serious global threat for poultry, endangered wild bird species, as well as representing a zoonotic risk for human health, with 962 HPAI A(H5Nx) zoonotic infections reported up to 14 July 2023 (World Health Organization 2023). The global circulation of this lineage has led to the diversification of the hemagglutinin (HA) gene segment into ten first-order clades and multiple second to fifth-order subclades (Smith and Donis 2015). Since 2014, subclade 2.3.4.4, which originated in China in 2008, has dominated the global scene and has been responsible for all the Gs/Gd epidemic waves reported in Europe. According to the World Health Organization (WHO), this clade has evolved into eight subclades (a–h) (World Health Organization 2021), with 2.3.4.4b being responsible for the largest HPAI epidemic waves recorded in Europe in 2016–2017, 2020–2021, and 2021–2022, as well as for the most recent one in 2022–2023. The 2016–2017 epidemic was predominantly caused by HPAI A(H5N8) viruses, which affected most European countries with a significant impact on both wild (1,563 cases) and domestic birds (1,218 outbreaks) (Adlhoch et al. 2023). Over the following years, 2017–2018 and

2019–2020, two minor incursions were reported, with a limited number of countries affected during the winter seasons. Since October 2020, consecutive epidemic waves have been reported in Europe (Floyd et al. 2021; Bordes et al. 2023; Tammiranta et al. 2023; Vreman et al. 2023), which heavily affected poultry and wild and captive birds, with occasional spill over into mammalian species. The first epidemic wave lasted from October 2020 to September 2021, with 3,792 HPAI A(H5Nx) detections (2,407 in wild birds and 1,385 in domestic birds) across thirty-one European countries. This was followed by an even larger second wave, which lasted from October 2021 to September 2022, counting a total of 6,707 HPAI A(H5Nx) detections (3,936 in wild and 2,771 in domestic birds) reported across thirty-seven countries. In 2022, for the first time the virus caused several mass mortality events in wild birds during the summer months, likely due to the virus spreading to seabird breeding colonies, a category of wild birds, which had very rarely been affected before by HPAI A(H5Nx) (Adlhoch et al. 2022). The 2020–2021 and 2021–2022 waves were characterized by the circulation of different subtypes and genotypes, as a result of reassortment events with low pathogenicity avian influenza (LPAI) viruses (Lewis et al. 2021; Briand et al. 2022; Engelsma et al. 2022; Grant et al. 2022; Nagy, Černíková, and Stará 2022; Pöhlmann et al. 2022). While the A(H5N8) subtype mainly drove the first (2020–2021) epidemic wave, A(H5N1) was responsible for the majority of the 2021–2022 and 2022–2023 outbreaks (Adlhoch et al. 2023).

These epidemics did not affect only Europe, but had global impact. Since January 2021, an A(H5N1) related to the 2020–2021 European viruses had been circulating in West Africa, and soon reached South African countries (Abolnik et al. 2022; Letsholo et al. 2022; Lo et al. 2022; Makalo et al. 2022; Ouoba et al. 2022; Sanogo et al. 2022). From late 2021, A(H5N1) viruses of clade

2.3.4.4b have been detected in South and East Asia (Isoda et al. 2022; Sagong et al. 2022; Ye et al. 2022). In December 2021, the A(H5N1) clade 2.3.4.4b closely related to the A(H5N1) identified in Northern Europe during the 2020–2021 epidemic season was introduced into North America (Caliendo et al. 2022). At the beginning of 2022, a new introduction into North America via the Pacific flyway of an A(H5N1) related to the viruses circulating in Japan was identified (Alkie et al. 2022). Since then, the virus has spread throughout North America, undergoing reassortment events with LPAI viruses of the American lineages (Alkie et al. 2022). In October 2022, A(H5N1) viruses spread for the first time to Mexico and soon after to South America, with mass mortality events reported in wild birds (Adlhoch et al. 2023), wild mammals, and poultry farms.

In this study, we characterized the spatiotemporal patterns of the A(H5Nx) virus spread to/from and within Europe during the 2020–2021 and 2021–2022 epidemic waves, providing important insights on the regions at high risk of virus incursions. We demonstrated the high genetic diversity of the circulating viruses, which underwent frequent reassortment events, and suggested a nomenclature to describe the circulating genotypes. Importantly, we described the relevant changes between the two epidemic waves in terms of spatiotemporal dynamics of virus spread and host range, which have dramatically modified the disease epidemiology in Europe with serious consequences for the poultry industry, wild bird populations (such as sea birds), and farmed carnivores (e.g. minks and foxes), as well as concerns around the increased zoonotic risk for public health.

Materials and methods

Dataset

During the 2020–2021 and 2021–2022 European epidemic waves, sequencing analyses of the complete genome of HPAI A(H5Nx) viruses were performed by the European Union Reference Laboratory (EURL—IZSVe), by National Reference Laboratories (NRLs), and by some other academic partners, for a total of 1,956 complete viral genomes. The samples originated from thirty-two European countries with 875 being collected during the 2020–2021 wave and 1,081 during the 2021–2022 wave—(up to August 2022). All the sequences were deposited in the GISAID EpiFlu database (<http://www.gisaid.org>, Supplementary Table S1).

Among avian hosts, we identified five different host categories: domestic birds (Galliformes, Anseriformes, and others), waterfowls (mainly Anseriformes and Charadriiformes), raptors, colony breeding seabirds (mainly Laridae and Sulidae), and others (Supplementary Table S1).

Phylogenetic analysis and genotype identification

Sequences of the eight gene segments were aligned in MAFFT v7 (Kato and Standley 2013) and compared to the most related sequences determined using the Nucleotide Basic Local Alignment Search Tool (BLAST) search available in the GISAID EpiFlu™ database (accessed on 7 September 2022). Maximum likelihood (ML) phylogenetic trees of all gene segments were generated in IQTREE v1.6.6 (Nguyen et al. 2015; Thi Hoang et al. 2017), performing an ultrafast bootstrap resampling analysis (1,000 replications). ModelFinder, available in IQTREE, was used to select the best-fit model of sequence evolution. FigTree v1.4.4 was used to visualize the phylogenetic tree of each gene segment (<http://tree.bio.ed.ac.uk/software/figtree/>).

The identification of the genotype was based on the phylogenetic tree topology. Monophyletic clusters for genotype assignment were identified for all the trees, using the following criteria:

(1) each HPAI A(H5Nx) cluster had to be supported by ultrafast bootstrap values >90, and (2) they had to show a higher identity with low or (rarely) high pathogenic influenza viruses (the possible donors of the gene segment) than the one observed with the other 2020–2022 HPAI A(H5Nx) clusters. These donors, located at the base of each recognized cluster, were identified from the BLAST search of the 2020–2022 HPAI A(H5Nx) viruses under study. A reference sequence was selected among the donors for each gene segment and each cluster, to represent the potential progenitor virus of that cluster. An identification (ID) number (cluster ID) was assigned to each cluster and the combination of the eight IDs (one for each gene segment) defined a genotype (Fig. 1, Supplementary Figs. S1–S13).

HA datasets' refinement

To investigate the spatial spread of the 2.3.4.4b HPAI A(H5Nx) viruses during the 2020–2021 and 2021–2022 epidemic waves, we generated three different HA datasets: a global dataset and two European datasets.

For the global analysis, we downloaded from the GISAID EpiFlu™ database (accessed on 18 January 2024) all the available A(H5Nx) HA sequences at least 1,600 nucleotides long belonging to clade 2.3.4.4b from Africa, Asia, and North America, sampled in a time frame ranging from January 2018 to August 2022. Sequences were phylogenetically analysed together with the HA gene sequences of the European viruses; all the HA sequences that shared a common progenitor with the European viruses were retained for the following analyses (about 3,700 sequences). Six discrete geographic regions—Africa, North America, East Asia, Central Asia, Europe, and the Middle East—were defined. To mitigate sampling bias and ensure a balanced dataset, a ML phylogenetic tree using IQTREE v1.6.6 (Nguyen et al. 2015; Thi Hoang et al. 2017) was generated for each geographic trait (Africa, North America, East Asia, Central Asia, and Europe), except for the Middle East, in which case all the forty-seven available sequences were retained. The Phylogenetic Diversity Analyzer (PDA) tool (Chernomor et al. 2014) was then used to select about 150 sequences from each tree based on their phylogenetic diversity, making sure to include the first detected viruses from each geographic region. For each trait (geographic area), the number of sequences ranged from 47 (the Middle East) to 165 (Europe), for a total of 806 sequences.

To explore the dynamics of virus spread within Europe, we generated two datasets, one for each epidemic wave. Seven discrete regions were defined: North-Central Europe (the Czech Republic, Denmark, Germany, Norway, Poland, and Sweden), North-Eastern Europe (Estonia, Finland, Latvia, and Lithuania), North-Western Europe (Belgium, the Republic of Ireland, Luxembourg, the Netherlands, and the UK), Central Asia (Russia and Kazakhstan), South-Central Europe (Albania, Austria, Croatia, Greece, Hungary, Italy, Kosovo, Slovakia, Slovenia, and Switzerland), South-Eastern Europe (Bulgaria, Moldova, and Romania), and South-Western Europe (France, Portugal, and Spain). To reduce possible bias associated with different sequences availability from each country, we calculated the percentage of sequenced outbreaks for each epidemic wave in each geographic area: North-Central Europe (16 per cent W1, 11 per cent W2), North-Eastern Europe (17 per cent W1, 17 per cent W2), North-Western Europe (71 per cent W1, 15 per cent W2), South-Central Europe (47 per cent W1, 51 per cent W2), South-Eastern Europe (100 per cent W1, 42 per cent W2), and South-Western Europe (2 per cent W1, 7 per cent W2). For the regions with <20 per cent of characterized outbreaks or with a number of sequences ≤10, all the genetic

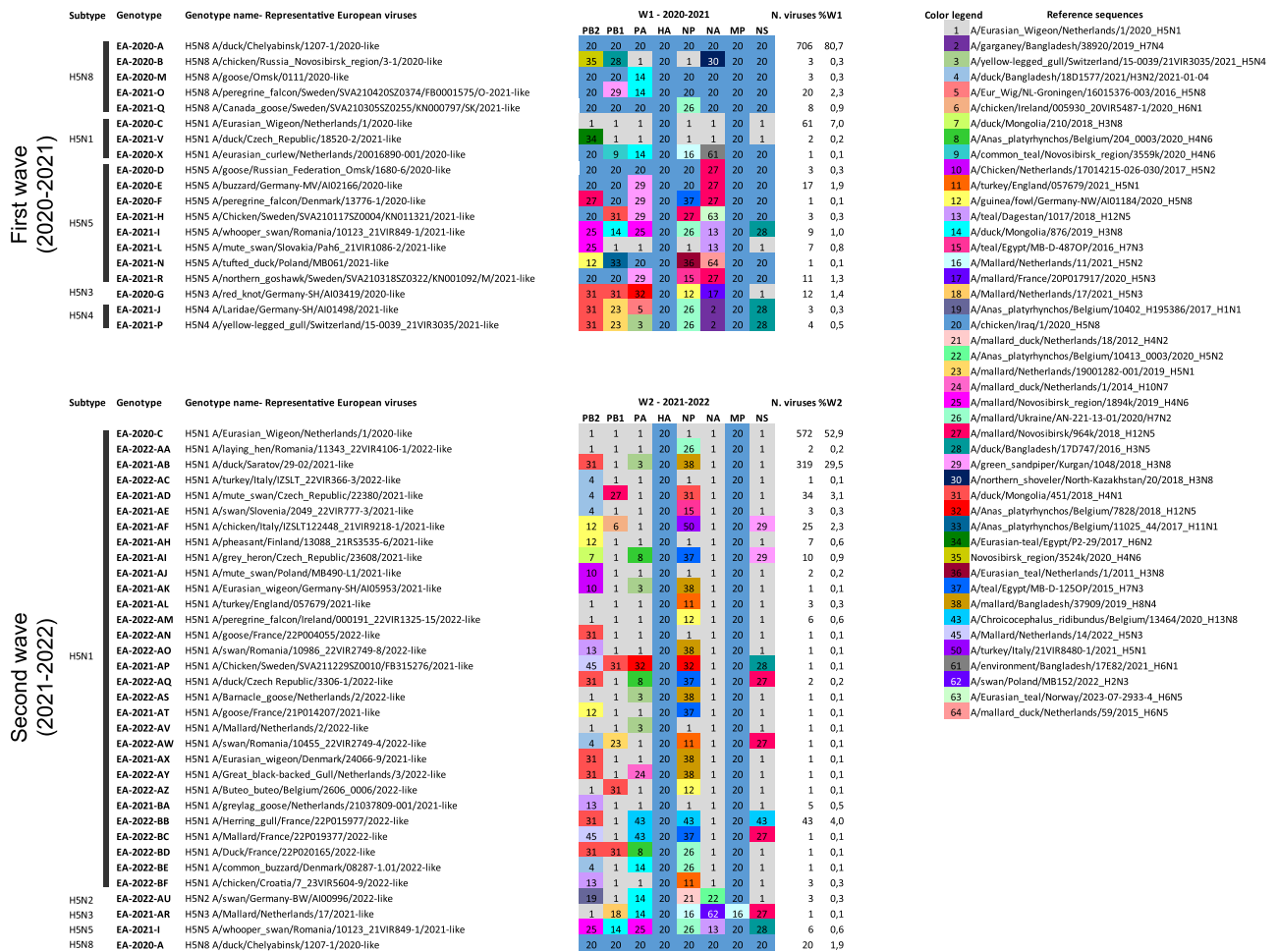


Figure 1. Graphical representation of the A(H5Nx) genotypes identified in Europe during the first (2020–2021) and second (2021–2022) epidemic wave. A number and associated colour code is assigned to each gene based on its genetic clustering in the phylogenetic trees. The reference sequences represent the possible progenitor virus for each specific gene.

Alt text: List of the European HPAI A(H5Nx) genotypes and their gene composition.

data were retained. For the remaining geographic areas, a number of sequences corresponding to 20 per cent of the reported outbreaks were selected using the PDA tool, starting from ML phylogenetic trees generated for each geographic area. The European dataset of the first epidemic wave (2020–2021) included 617 sequences, whereas the one generated for the second epidemic wave (2021–2022) included 838 sequences collected up to August 2022.

Evolutionary and phylogeographic analyses

Markov chain Monte Carlo (MCMC) analyses of the HA gene segment were performed using BEAST v1.10.4 in combination with the BEAGLE library (Suchard et al. 2018). We employed an uncorrelated lognormal relaxed molecular clock, the coalescent constant population size model, and the SRD06 substitution model (HKY85 + Γ_4 with two partitions—first + second positions vs third position—base frequencies and Γ -rate heterogeneity unlinked across all codon positions) (Shapiro, Rambaut, and Drummond 2006). MCMC chains were run for 400–700 million iterations and convergence was assessed using Tracer v1.7.1 (Shapiro, Rambaut, and Drummond 2006; Drummond and Rambaut 2007; Suchard

and Rambaut 2009; Shapiro et al. 2011). The maximum clade credibility (MCC) trees were summarized using TreeAnnotator v1.10.4 and visualized in FigTree v1.4.4 (<http://beast.bio.ed.ac.uk/TreeAnnotator/>, <http://tree.bio.ed.ac.uk/software/figtree/>).

To explore the pattern of spatial diffusion among the geographic regions, discrete phylogeographic analyses using location as a trait were performed (Lemey et al. 2009). We assumed an asymmetric non-reversible transition model and incorporated Bayesian stochastic search variable selection (Lemey et al. 2009). Spread D3 v0.9.6 (Bielejec et al. 2016) was used to visualize the spatial dispersal of the viral strain through time and to assess Bayes factor (BF) supports for individual transitions between discrete locations. We interpreted the strength of statistical support as positive for $5 < BF \leq 20$, strong for $20 < BF \leq 150$, and very strong for $BF > 150$.

We employed the Markov jump counts to measure the number of viral movements along the branches of the phylogeny and estimated the Markov rewards to quantify the time the virus spent in each geographical region (Minin and Suchard 2008). We also counted all jumps ‘from’ and all jumps ‘to’ each geographic region, to evaluate which were the main donor and recipient sites of the virus movements.

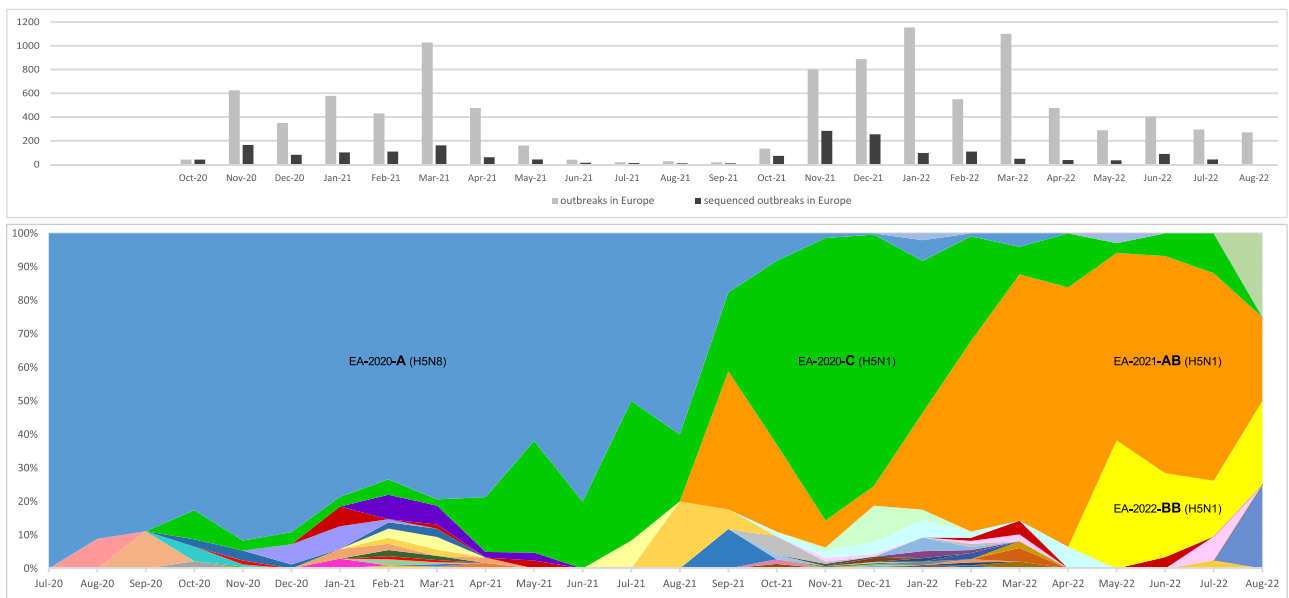


Figure 2. Upper panel: distribution of total number of notified (grey) and genetically characterized (black) HPAI A(H5Nx) viruses in Europe by month of detection. Lower panel: monthly distribution of the A(H5Nx) genotypes identified in Europe between July 2020 and August 2022. Each colour represents a different genotype. The four main genotypes EA-2020-A, EA-2020-C, EA-2021-AB and EA-2022-BB are marked in the graph.

Alt text: Two time plots displaying the proportion of the genetically characterized HPAI A(H5Nx) viruses and the frequency of the genotypes in Europe.

Results

Genetic variability of the European viruses and shift of the circulating genotypes between the two waves

ML phylogenetic analysis of the eight gene segments was used to assess the number and variety of co-circulating genotypes, each defined by a unique gene composition inferred from tree topologies and inconsistencies among the genetic clustering of the eight gene segments (Supplementary Figs. S1–S13). The first identified A(H5Nx) viral genome showing a particular gene constellation was used as a reference strain for a specific genotype (e.g. H5N1-A/Eurasian_Wigeon/Netherlands/1/2020-like). In order to make communication easier and to distinguish the genotypes identified during each epidemic wave, we named them with the indication of the lineage (e.g. EA, Eurasian), the year of identification and with letters—a single letter (i.e. A, B, C) for the genotypes which had originated during the 2020–2021 wave and two letters (i.e. AA, AB, AC) for the genotypes of the 2021–2022 wave (e.g. EA-2020-C, EA-2021-AB).

Overall, we defined fifty different A(H5Nx) genotypes from multiple inter- and intra-subtype reassortment events (Fig. 1). The highest genetic diversity in terms of number of co-circulating genotypes (from 13 to 25) was observed during the winter months in both waves: January–March 2021 and November 2021–February 2022 (Fig. 2). In most cases (73 per cent) gene segments had been acquired by reassortments with LPAI viruses circulating in wild birds. Of note, the frequency of reassortment was not homogeneous among the eight gene segments. While the HA and matrix (M) gene segments were stable, multiple switches were observed for the polymerase basic (PB) 2, PB1, polymerase acidic (PA), and nucleoprotein (NP) segments that were classified into 14, 12, 11, and 16 clusters, respectively.

During the 2020–2021 epidemic wave, we identified nineteen genotypes belonging to the A(H5N8) ($N=5$), A(H5N5) ($N=8$), A(H5N1) ($N=3$), A(H5N4) ($N=2$), and A(H5N3) ($N=1$) subtypes. The great majority of the viruses (80.7 per cent) belonged to genotype

EA-2020-A (H5N8-A/duck/Chelyabinsk/1207-1/2020-like), identified for the first time in July 2020 in central Russia, close to the Kazakh border, followed by the EA-2020-C genotype (H5N1-A/Eurasian_Wigeon/Netherlands/1/2020-like) (7 per cent). EA-2020-A (H5N8), together with EA-2020-C (H5N1), were the first two genotypes identified in Europe (The Netherlands) in mid-October 2020. As genotype EA-2020-C (H5N1) had never been identified before October 2020 in other areas outside Europe, it was assumed to have emerged in Europe from the A(H5N8) subtype through a single reassortment event involving six gene segments (PB2, PB1, PA, NP, neuraminidase (NA), and non-structural (NS)), although it is not possible to discount that this occurred in a step-wise manner involving un-sampled intermediates including outside Europe. The EA-2020-A genotype appeared to dominate over all the other genotypes until August 2021. With the beginning of the second wave in Europe in October 2021 and the rise in the number of A(H5Nx) virus detections, we witnessed a dramatic shift in the genetic composition of circulating subtypes and genotypes (Fig. 2). While in 2020–2021, A(H5N8) had been the most prevalent subtype, the 2021–2022 wave was dominated almost exclusively by A(H5N1). Only three of the genotypes identified during the first wave, namely EA-2020-A (H5N8), EA-2020-C (H5N1), and EA-2021-I (H5N5), continued to be detected through the summer months, while all the other genotypes that were detected in the 2021–2022 wave had either emerged from reassortment events with LPAI viruses circulating in wild birds in Europe or were the result of novel virus introductions, such as genotype EA-2021-AB (H5N1-A/duck/Saratov/29-02V/2021-like). This genotype was identified for the first time in September 2021 in the Russian Federation (Saratov oblast) and during October 2021 it was also detected in Germany, The Netherlands, and Denmark (Supplementary Table S1). Unfortunately, the scarcity of genetic data from countries outside Europe makes it impossible to assess for certain the geographic source area of the different genotypes. Overall, thirty-four different genotypes, thirty belonging to the A(H5N1) subtype, were characterized during the 2021–2022 wave, indicating that the

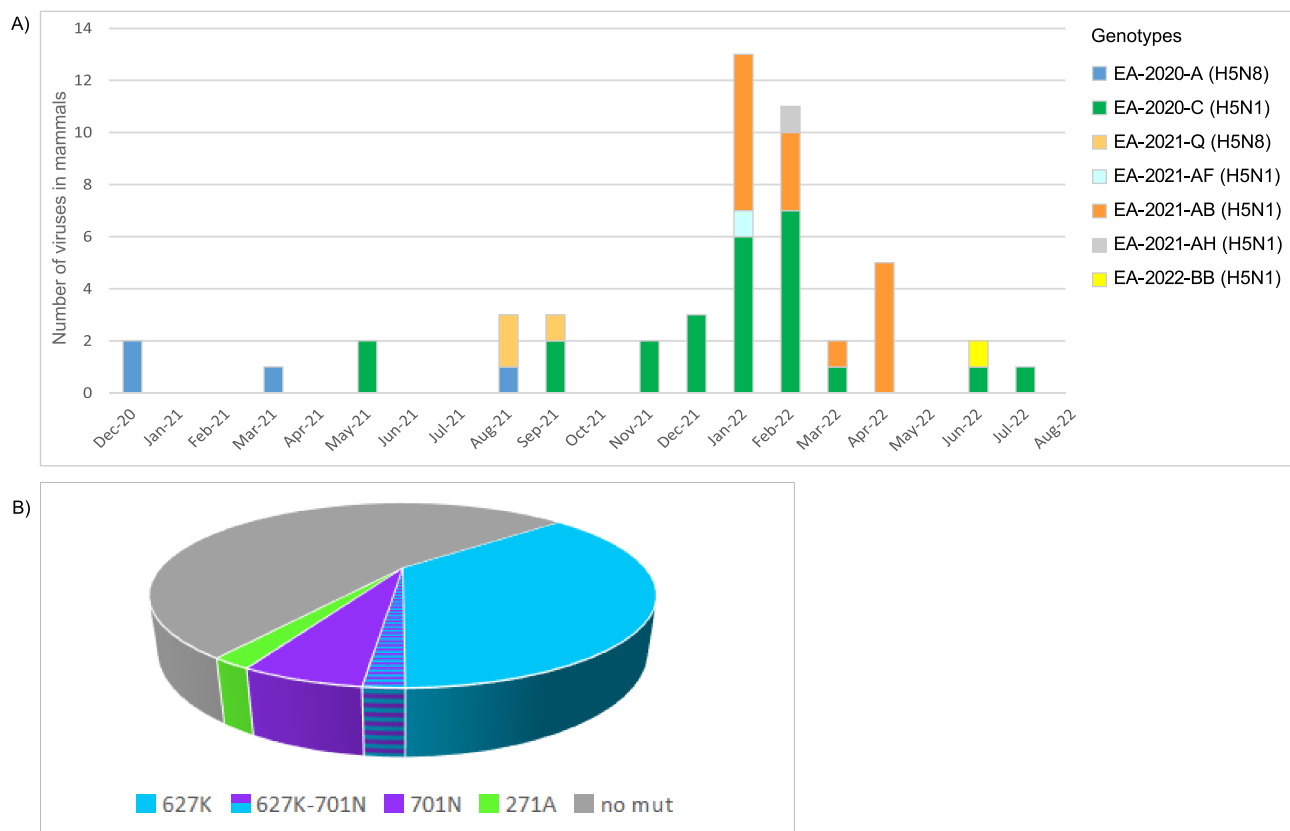


Figure 3. Panel A—bars represent the distribution of the genetically characterized (complete genome) A(H5Nx) viruses collected from mammals in Europe from December 2020 to August 2022, by month of detection, coloured according to the genotype. Panel B—frequency of the molecular markers of mammalian adaptation in the PB2 protein (271A, 627K, 701N) identified in viruses collected from mammalian species in Europe between October 2020 and August 2022.

Alt text: A bar-plot displaying the European HPAI A(H5Nx) genotypes detected over time in mammals and a pie-chart showing the frequency of specific mutations in the PB2 protein.

diversity of genotypes detected was even greater than in the previous wave. With a frequency of 52.9 per cent and 29.5 per cent, the A(H5N1) genotypes EA-2020-C and EA-2021-AB were the most prevalent in 2021–2022. At the beginning of this epidemic wave, a rise of genotype EA-2020-C was observed in Europe, likely as a result of a combination between an increase in the number of local cases and novel virus incursions (probably from Russia) of the same genotype. Indeed, the topology of the phylogenetic trees revealed that the 2021–2022 European viruses of the EA-2020-C genotype split into two sub-clusters, one comprising the A(H5N1) viruses collected in European countries since October 2020, the other including A(H5N1) collected in Russia since the beginning of October 2021 and subsequently (end of October) in Europe (Supplementary Figs. S1–S13). This genotype remained the most prevalent (frequency ranging from 46 per cent to 84 per cent) from October 2021 to January 2022. In February 2022, it was overtaken by genotype EA-2021-AB, which in February–August 2022 reached a frequency of 33 per cent to 78 per cent, thus becoming the most frequent and widespread (No. of countries = 19) genotype in Europe (Fig. 2), based on the available data.

In May 2022, a new A(H5N1) genotype likely emerged from reassortment with A(H13) LPAI viruses, a subtype primarily found in gulls, from which it acquired the PA, NP, and NS gene segments. This new genotype, named EA-2022-BB (H5N1-A/Herring_gull/France/22P015977/2022-like), was identified for the first time in a herring gull in France and, subsequently, in the Netherlands and Belgium. It circulated extensively during

the summer months, being the second most frequently detected genotype (22 per cent of the viruses sequenced between June and August 2022), mainly in gulls (86 per cent), with the European herring gull as the most commonly affected species (70 per cent).

During the 2020–2021 and 2021–2022 epidemic waves, multiple spillover events from birds to ten different mammalian species were reported (Adlhoch et al. 2023). Sequences were generated for a total of forty-nine viruses collected during the 2020–2021 ($N = 10$) and 2021–2022 ($N = 39$) waves, in eleven countries from nine different species: red fox (*Vulpes vulpes*) ($N = 34$), harbour seal (*Phoca vitulina*) ($N = 5$), grey seal (*Halichoerus grypus*) ($N = 1$), Eurasian otter (*Lutra lutra*) ($N = 2$), ferret (*Mustela furo*) ($N = 1$), European polecat (*Mustela putorius*) ($N = 4$), lynx (*Lynx lynx*) ($N = 1$), European badger (*Meles meles*) ($N = 1$), and porpoise (*Phocoena phocoena*) ($N = 1$). The analysed sequences belonged to seven different A(H5N8) and A(H5N1) genotypes—A, C, Q, AF, AB, AH, BB—with 52 per cent and 31 per cent of the viruses belonging, respectively, to the A(H5N1) genotypes EA-2020-C and EA-2021-AB, the two most widespread in Europe during the 2021–2022 wave, when most of the cases in mammals were reported (Fig. 3A).

Geographic and host distribution of the different genotypes

The fifty genotypes identified during the 2020–2021 and 2021–2022 waves are likely the result of novel virus incursions during the fall migration of wild birds, and/or may have emerged *de novo*

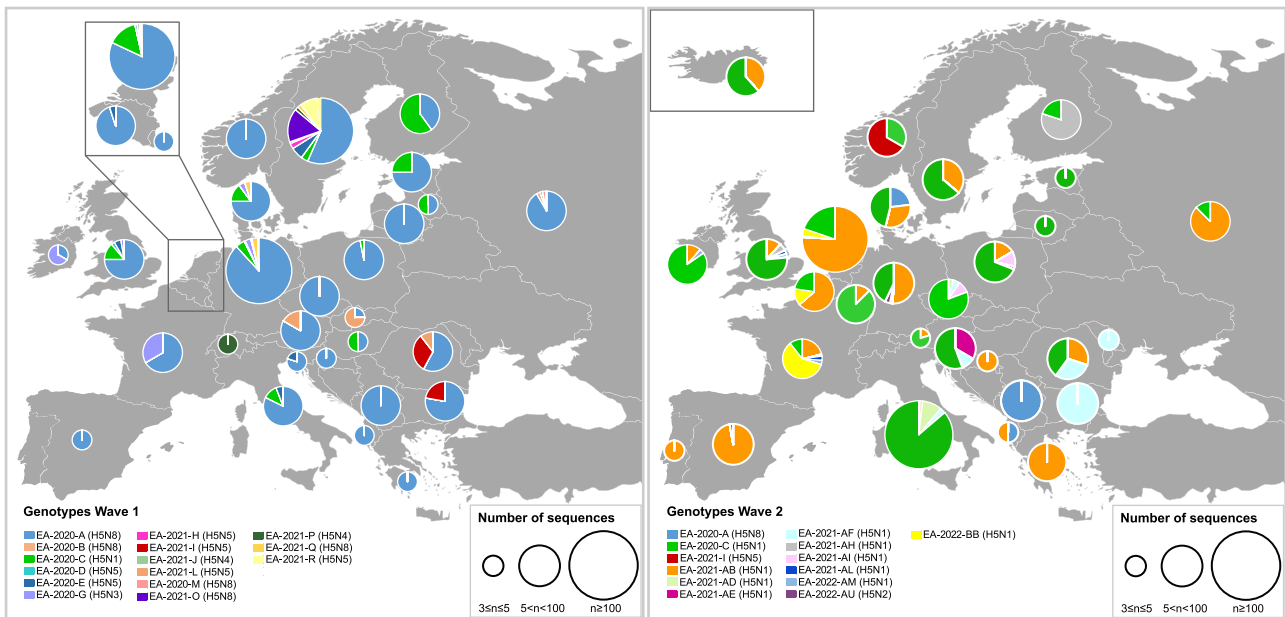


Figure 4. Geographic distribution of the major A(H5Nx) genotypes (represented by at least three viruses) in Europe during the first (2020–2021, left panel) and second (2021–2022, right panel) epidemic wave. The pie charts display the frequency of the genotype in each country. The size of the circle is proportional to the number of sequences analysed.

Alt text: Maps of Europe with pie-charts showing the HPAI A(H5Nx) genotype frequency per country.

from reassortment events with viruses circulating in wild birds in Europe. Three genotypes, specifically genotypes EA-2020-C (H5N1), EA-2020-A (H5N8), and EA-2021-I (H5N5), persistently circulated during both epidemic waves. With respect to the first wave (2020–2021), in 2021–2022, the EA-2020-A and EA-2021-I genotypes reduced their geographic spread, which was limited to three countries (Kosovo, Denmark, and Albania) for EA-2020-A and to one country (Norway) for EA-2021-I. Conversely, during the second wave, genotype EA-2020-C showed a remarkable geographical expansion (twenty-one countries in 2021–2022 vs eleven countries in 2020–2021) (Fig. 1, Fig. 4, Supplementary Tables S2 and S3).

The highest genotype diversity was observed in the Netherlands (fifteen genotypes), France (twelve genotypes), Sweden (eleven genotypes), and Germany (ten genotypes), followed by Belgium, Croatia, Czech Republic, Denmark, Ireland, Italy, Poland, Romania, Slovenia, and the UK, with five to ten circulating genotypes (Fig. 4, Supplementary Tables S2 and S3). However, bias due to the number of available sequences from each country cannot be excluded, as surveillance activities and genetic characterization efforts widely differ (the proportion of sequenced virus samples in each country ranges from 0 per cent to 100 per cent). Specifically, during the 2020–2021 wave, the highest genotype diversity (5–9 genotypes) was detected in Northern Europe (Denmark, Germany, the Netherlands, Sweden, and the UK), while during the 2021–2022 wave, a more widespread genotype diversity was observed, with the Czech Republic, France, Germany, Italy, the Netherlands, Poland, Romania, and the UK having four to nine genotypes each.

About 43 per cent of the genotypes were identified only in wild birds, 20 per cent only in domestic birds, and 37 per cent being detected in both wild and domestic birds. Domestic birds (both Galliformes and Anseriformes) and waterfowl (Anseriformes) are the host categories harbouring the highest genetic diversity among the detected viruses, while the lowest was observed in seabirds (Fig. 5). In this latter group, the frequency of the detected

genotypes also differed considerably from what was noted in the other host categories (domestic, waterfowl, raptors), showing a higher level of circulation of genotype EA-2020-C during the 2020–2021 wave and of genotype EA-2022-BB during the 2021–2022 wave compared to other host categories (Fig. 5).

Global analysis—the origin and global spread of clade 2.3.4.4b HPAI H5 viruses affecting Europe in 2020–2022

To attempt to define the origins of the HPAI A(H5Nx) viruses of clade 2.3.4.4b introduced in Europe in October 2020, and in order to reconstruct their spatial spread, a discrete phylogeographic analysis was performed on the HA gene of 806 representative HPAI A(H5Nx) viruses from six distinct geographical regions, namely East Asia, Africa, North America, Europe, the Middle East, and Central Asia (Russia/Kazakhstan/Georgia) (Fig. 6). According to the most probable location at the root of the MCC tree of the HA gene, the Middle East seems to be the most likely geographical source of this virus, which, between the second half of 2019 and the beginning of 2020, reached Central Asia (Supplementary Fig. S1), which agrees with earlier studies (Lewis et al. 2021). Our analysis indicates that Central Asia, which represents one of the most important breeding sites for multiple wild bird species, was a key source of the virus for most of the other geographic locations (52.42 per cent Markov jumps), including East Asia, the Middle East, and Europe (Fig. 7). Of note, the estimated tMRCAs (Supplementary Fig. S14) indicated that the viruses had been introduced from Central Asia to the different geographic areas over the same time period (summer–autumn) during both epidemic waves. Europe proved to be the major source of the virus for Africa and North America. In Africa, two different virus incursions from Europe of the A(H5N8) and A(H5N1) subtypes belonging to European genotypes EA-2020-C (H5N1) and EA-2020-A (H5N8) (Lo et al. 2022; Meseke et al. 2023) had occurred at the end of 2020. In North America, a first virus introduction of an A(H5N1) closely

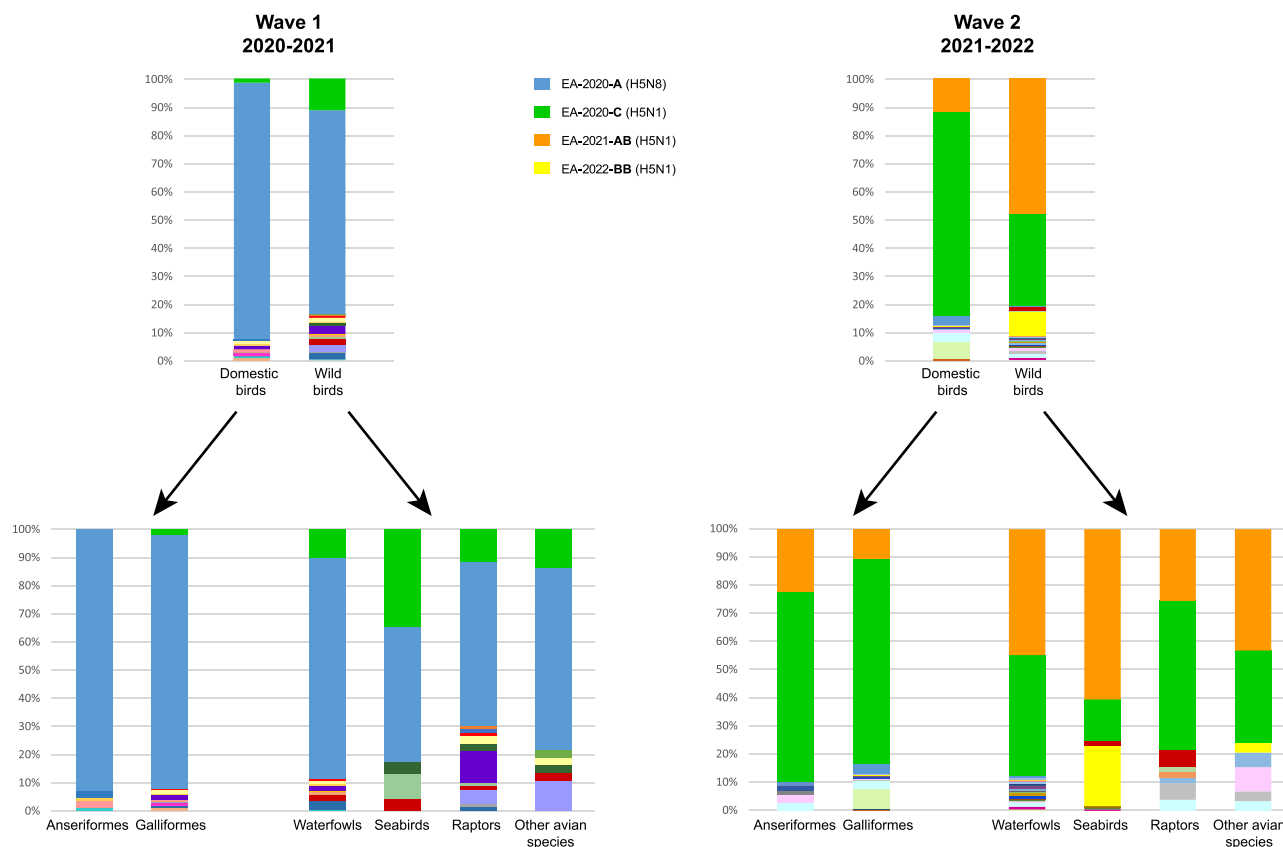


Figure 5. Distribution of the different genotypes by bird host categories during the first (2020–2021, left panel) and second (2021–2022, right panel) epidemic wave. The colours assigned to the four major genotypes are reported in the figure legend.

Alt text: Bar-plots of the HPAI A(H5Nx) genotypes frequency per host species.

related to the viruses detected in Europe (genotype EA-2020-C, corresponding to genotype A in Youk et al. (2023) had been identified at the end of 2021 along the eastern coast, while a second transpacific virus incursion of A(H5N1) viruses closely related to the ones circulating in East Asia had occurred in early 2022 along the western coast, confirming the results from previous studies (Alkie et al. 2022). Markov rewards, which estimate the proportion of time the A(H5Nx) virus spent in each geographical area, revealed that at a global level the virus spent most of the time in East Asia (52.5 per cent) and Europe (48.7 per cent) and similar amounts of time in Central Asia (35.6 per cent), Africa (35.1 per cent), and North America (32.8 per cent) (Fig. 7), although bias due to the number of available sequences from each geographic area cannot be excluded.

European analysis—Clade 2.3.4.4b HPAI A(H5Nx) virus spread within Europe, 2020–2022

To explore the pattern of virus spread within Europe during the 2020–2021 and 2021–2022 epidemic waves, a discrete phylogeographic analysis of the HA gene was undertaken by dividing the dataset into seven geographic regions: North-Central Europe, North-Eastern Europe, North-Western Europe, South-Central Europe, South-Eastern Europe, South-Western Europe, and Central Asia. Analyses confirmed Central Asia as being the major source of the virus for the whole of Europe during both epidemic waves (Fig. 8, Supplementary Fig. S15). North-Central

Europe likely acted as the main point of entry (Markov Jumps from Central Asia to North-Central Europe: 15.06 per cent—first wave, 8.58 per cent—second wave) (Fig. 7) and played a key role in the virus spread to the rest of Europe during both epidemic waves (first wave—53.85 per cent Markov jumps; second wave—75.07 per cent Markov jumps), with the largest number of Markov jumps (>10 per cent) estimated for viral flow from North-Central Europe to North-Western Europe (20.93 per cent and 29.19 per cent Markov Jumps for the first and the second epidemic waves, respectively), South-Central Europe (first wave—13.25 per cent Markov jumps; second wave—28.01 per cent Markov Jumps), North-Eastern Europe (first wave—13.11 per cent Markov jumps), and South-Western Europe (second wave—10.87 per cent Markov Jumps). North-Western and South-Western Europe as well as South-Central Europe and South-Eastern Europe were also connected in one or both waves (Fig. 7), indicating multiple directions of virus spread within Europe: among the northern regions, from northern to southern regions, and—to a lesser extent—also among the southern regions (Fig. 7). Estimation of the time the A(H5Nx) had remained in each region using Markov rewards demonstrated that the virus had spent most of the time in North-Central Europe (43.3 per cent) in 2020–2021, while in 2021–2022 a comparable amount of time across North-Central Europe (30.5 per cent) and North-Western Europe (26.6 per cent) (Fig. 7). A(H5Nx) spent the least amount of time in the South-Eastern and North-Eastern regions of Europe, although bias due to different intensity of genomic surveillance in each geographic region cannot be excluded.

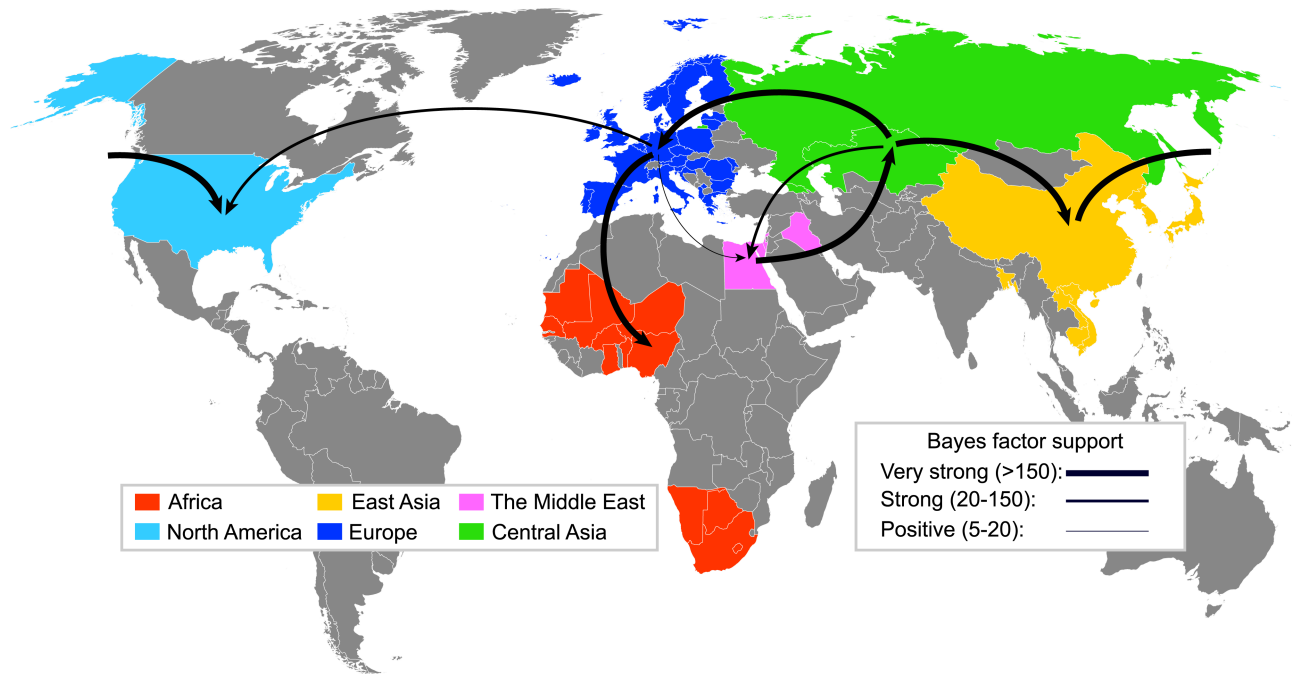


Figure 6. Global migration rates among the geographic regions of clade 2.3.4.4b (2019–2022). The thickness of the lines representing the rates is proportional to the relative strength by which rates are supported: very strong (BF > 150, thick lines) and positive (5 < BF < 20, thin lines).

Alt text: World map with arrows showing the virus movements between the distinct geographic regions

Molecular markers affecting the biological characteristics of the European HPAI A(H5Nx) viruses

Molecular analyses of the European A(H5Nx) viruses investigated in this study (October 2020–August 2022) indicate that they retained the avian-type-receptor-binding signature (222Q and 224G (H5 numbering)) in the HA receptor binding site (Stevens et al. 2006). However, we identified several mutations with a varying frequency, which have been previously demonstrated to affect the biological characteristics of avian influenza viruses (Suttie et al. 2019) (Table 1). These include mutations associated with (1) enhanced polymerase activity and replication in mammals or mammalian cells, (2) increased virulence in mammals or avian species, (3) antiviral drug resistance, and (4) increased *in vitro* binding to human-type receptors alpha-2,6-linked sialic acids. The phenotypic effect of most of these mutations on the biological characteristics of the viruses is still unknown and further studies are needed to improve existing knowledge. Among the mutations in the HA protein that have proved to increase *in vitro* binding to human-type receptors, some (i.e. S133A, S154N, T156A) have been identified in the majority of the A(H5N1) viruses circulating in Europe in the period under study (October 2020–August 2022), while others (i.e. D94N, S155N, T188I, V210I, E251K, and S123P-496K) have only been sporadically detected. Moreover, we identified 84 viruses carrying mutations in the second sialic acid-binding site contact residues (2SBS) of the NA, which can affect the binding and cleavage of receptors and virus replication. Of note, all the viruses belonging to the BB genotype contained mutation S369I (N1 numbering) in the NA. A single mutation in this position of the N1 protein has been demonstrated to disrupt 2SBS and negatively affect N1 activity (Du et al. 2018). This mutation has been observed in all N1 sequences from human (seasonal) and H1N1pdm09 pandemic viruses and in several other viruses adapted to mammalian host species. Similarly, mutation K432E in

the NA, which is also proposed to have an impact on 2SBS, has been detected only among viruses of the EA-2021-AB genotype (12 per cent of the EA-2021-AB viruses).

Molecular markers associated with increased replication and/or virulence in mammals were rarely detected in birds. Specifically, the well-known molecular markers of mammalian adaptation E627K and D701N (Subbarao, London, and Murphy 1993; Hatta et al. 2001, 2007; Shinya et al. 2004; Salomon et al. 2006; Labadie et al. 2007; Mehle and Doudna 2008; Fornek et al. 2009; Li et al. 2009; Rameix-Welti et al. 2009; Steel et al. 2009) were observed in sixteen A(H5Nx) viruses collected from birds (N = 14 E627K, N = 2 PB2-D701N, all domestic/captive) in four European countries, which represented 0.8 per cent of the analysed sequences from avian species. Despite these markers being only sporadically detected in birds, it is worth noting that eleven out of the seventeen of the sequences showing the PB2-627K were detected in viruses collected from a cluster of outbreaks in poultry in Germany, as previously described (King et al. 2022). This suggests that viruses with increased zoonotic potential may retain the capability to infect and be transmitted among poultry farms. The same markers (E627K and D701N) as well as mutation PB2-T271A were detected in about half of the viruses characterized from mammalian species (21/45), with the majority of them (17/21) bearing mutation PB2-627K (Fig. 3B).

Mutations associated with antiviral resistance were identified occasionally. Specifically, thirteen viruses contained mutations associated with reduced sensitivity to amantadine and rimantadine, eleven viruses showed mutations causing reduced susceptibility to neuraminidase inhibitors (oseltamivir/zanamivir), and six viruses displayed mutations able to reduce the virus sensitivity to baloxavir (Table 1).

Moreover, almost all the A(H5N1) viruses belonging to the A(H5N1) genotypes EA-2022-BB and EA-2020-C contained the mutation NP-Y52N/H, which allows the evasion of

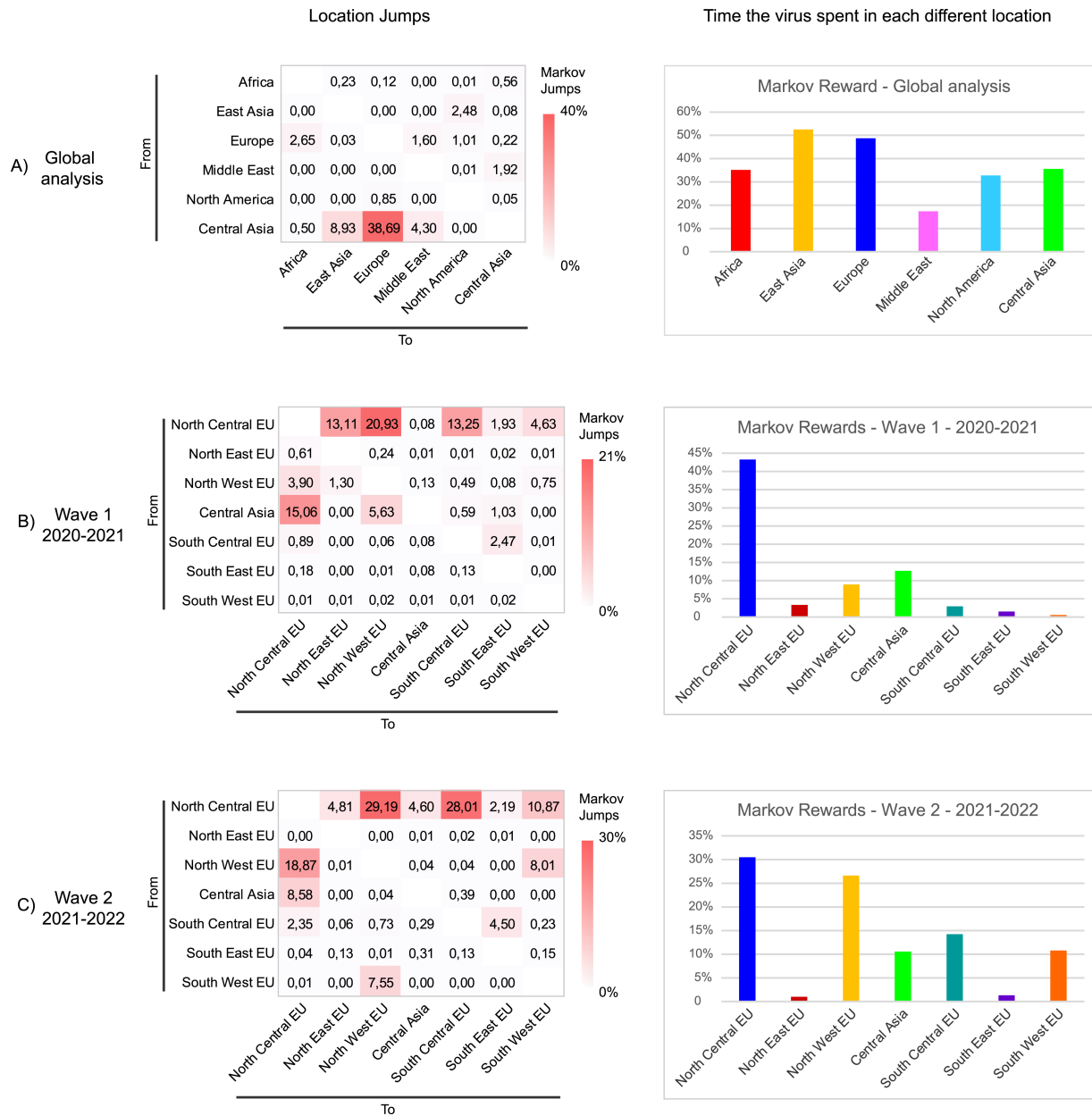


Figure 7. Pattern of geographic jumps and hotspots obtained from the analyses of (A) global dataset, (B) European dataset—first wave (2020–2021), and (C) European dataset—second wave (2021–2022). The three heatmaps on the left indicate the frequency of transitions between locations estimated using a discrete trait phylogenetic model. The number of location transitions was determined using Markov jumps. The bar charts on the right indicate the proportion of time the virus spent in each location.

Alt text: Heat maps of the Markov jumps and bar plots of the Markov rewards

human butyrophilin subfamily 3 member A3 (BTN3A3) protein. BTN3A3 is a major histocompatibility complex associated protein constitutively expressed in human airways and is a potent inhibitor of avian but not human influenza A viruses (Pinto et al. 2022).

Discussion

Since October 2020, Europe has witnessed an important change in the ecology, epidemiology, and evolution of the HPAI A(H5Nx) viruses of clade 2.3.4.4b, exemplified by the following: (1) the geographic expansion of this virus that for the first time reached countries (i.e. Norway, Iceland, and Svalbard Islands) (Madslie

et al. 2021) that had never before been affected by the Gs/GD lineage; (2) the variety of affected wild bird species (Adlhoch et al. 2023); (3) the high number of mammalian infections with sporadic episodes where field data suggested a potential mammal-to-mammal transmission, as reported in Spain (Agüero et al. 2023), but also in other countries outside Europe (Leguia et al. 2023); (4) the unprecedented number of reassortment events; (5) the virus persistence throughout summer 2022, with a severe impact on birds breeding in colonies in northern European countries (Adlhoch et al. 2022); and (6) the first reports of clade 2.3.4.4b A(H5Nx) in humans in countries (i.e. Russia, the UK, the USA, Chile, and Ecuador) where the HPAI the Gs/GD A(H5Nx) virus is not endemically circulating in poultry (Bruno et al. 2023;

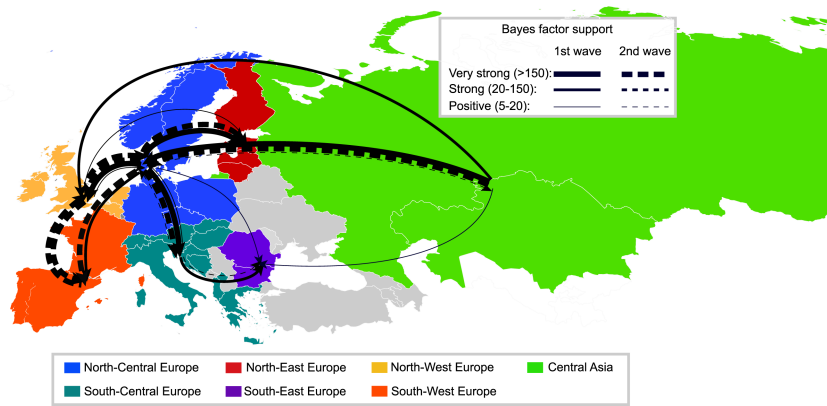


Figure 8. Migration rates between European regions of clade 2.3.4.4b during the first (continuous lines) and second (dashed lines) epidemic wave. The thickness of the lines representing the rates is proportional to the relative strength by which rates are supported: very strong ($BF > 150$), strong ($20 < BF < 150$, medium lines), and positive ($5 < BF < 20$, thin lines).

Alt text: European map with arrows showing the virus movements between the distinct geographic regions

Human Infection caused by Avian Influenza A (H5N1) - Chile, n.d.; Threat Assessment Brief: First identification of human cases of avian influenza A(H5N8) infection, n.d.; U.S. Case of Human Avian Influenza A(H5) Virus Reported| CDC Online Newsroom| CDC, n.d.; Oliver et al. 2022).

In this study, we explored the changing pattern of the HPAI virus evolution between 2020 and 2022 in Europe, one of the most affected continents in terms of geographical spread, number of poultry outbreaks, and number of affected wild bird species. The complete data from the 2022–2023 wave were not available at the time of our analyses; therefore, they have not been included in the present paper. In agreement with previous studies (Lewis et al. 2021; Hagag et al. 2022; Xie et al. 2023), our spatial analyses identified the Middle East as the place where most likely the A(H5N8) virus responsible for the 2020–2021 epidemic wave in Europe originated. In Egypt, the A(H5N8) virus has been endemically circulating in poultry since 2016 (Hagag et al. 2022; Salaheldin et al. 2022). In spring 2020, viruses closely related to those collected in poultry in the Middle East were first identified in wild birds in Central Asia and then in Europe, which suggests that the virus may have jumped from the domestic to the wild bird population, although unsampled ancestry makes definitive assessment challenging. However, where this jump occurred—in the Middle East, Russia, or elsewhere—cannot be ascertained based on the available data. A similar scenario was observed in 2019, when one A(H5N8) reassortant virus of clade 2.3.4.4b, containing six gene segments of sub-Saharan African origin and two genes from LPAI viruses identified in wild birds in Western Siberia, was detected in eastern Europe (Swieton et al. 2020). Since the first transcontinental spread of the Gs/GD in 2005–2006, Eastern Asia has been recognized as the primary source location of A(H5Nx) clades for Europe (Sonnberg, Webby, and Webster 2013; Lycett et al. 2016; Verhagen, Fouchier, and Lewis 2021; Zhang et al. 2023). These two recent incursions in Europe of viruses originating from areas south of the Mediterranean Sea highlight a possible shift in the diffusion dynamics of the Gs/GD lineage and the emergence of new pathways for virus introductions in Europe. Despite data indicating that new hotspots (i.e. the Middle East and North-Central Europe) for virus evolution and subsequent spread may exist, this study confirms the main route of virus introduction into Europe through wild bird migrations from breeding sites in Central Asia, as previously reported (Lee et al. 2017; More et al. 2017; Fusaro et al. 2019; Lycett et al. 2020).

At the European level, North-Central Europe emerged as the major source of A(H5Nx) viruses for the rest of Europe and the area where the virus was detected for the longest period of time. The coastal area of North-Central Europe (i.e. the Wadden Sea) is of particular importance for migratory birds, which use this region for wintering or as a stop-over site on their annual migrations between southern wintering and northern breeding area, with about 10–12 million waterbirds spending at least part of their annual life cycle on these coasts (Scheiffarth 2003). A caveat of this study is that phylodynamic analyses rely on the availability of sequences; big efforts were made to increase wild bird surveillance and to generate sequencing data. The structure, design, and implementation of surveillance for wild birds across Europe are highly variable (i.e. target species, numbers examined, and number of geolocations within country). Furthermore, additional bias cannot be excluded given the different resources and sequencing capabilities of the different countries.

Clade 2.3.4.4b viruses have a high propensity to undergo multiple reassortment events, as observed during the European 2016–2017 epidemic wave (Verhagen, Fouchier, and Lewis 2021). However, compared to the previously described epidemic waves in Europe (Bragstad et al. 2007; Lycett et al. 2020), we detected an unprecedented genetic diversity, with the identification of nineteen and thirty-four distinct genotypes during the 2020–2021 and 2021–2022 epidemic waves, respectively. The greater genetic diversity in the second wave may be explained by the unprecedented number of cases and geographical extent (thirty-seven European affected countries) observed in 2021–2022, which represents the most devastating HPAI A(H5N1) epidemic affecting Europe to date. The highest number of reassortment events were observed for the gene segments encoding for the polymerase complex (PB1, PB1, and PA) and the nucleoprotein gene, while the HA and M genes—during the first wave—and the HA, NA, and M genes—during the second wave—were very stable. Previous studies demonstrated the crucial role of the M gene in virus morphology, transmission, and HA/NA balance (Bourmakina and Garcia-Sastre 2003; Elleman and Barclay 2004; Campbell et al. 2014). It has recently been suggested that the M gene of clade 2.3.4.4b is associated with a more filamentous morphology, which may provide a selective advantage *in vivo* (Leyson et al. 2021). An M-HA effective cooperation has been demonstrated to be necessary for efficient virus replication (Webster, Kawaoka, and Bean 1989; Scholtissek et al. 2002; Leyson et al. 2021). Moreover, Campbell et al. (2014) showed that co-adapted

Table 1. Mutations with a described biological effect identified in the European HPAI A(H5Nx) viruses, 2020–2022.

| Effect | Protein | Mutation | N. viruses | N. viruses from mammals | References | | |
|---|------------------------------------|--------------------------------------|------------------------------------|-------------------------|--|---|-------------------------|
| Enhance polymerase activity and replication in mammals or mammalian cells | PB2 | D253N | 1 | – | (Zhang et al. 2018) | | |
| | | T271A | 1 | 1 | (Bussey et al. 2010) | | |
| | | I292V | 798 | – | (Gao et al. 2019) | | |
| | | K389R | All/almost all viruses (N = 1,637) | – | (Hu et al. 2017b) | | |
| | | K482R | 19 | 3 | (Yamayoshi et al. 2018, Yamayoshi et al. 2014) | | |
| | | K526R | 4 | – | (Song et al. 2014) | | |
| | | A588V | 4 | – | (Xiao et al. 2016) | | |
| | V598T/I | E627K | All/almost all viruses (N = 1,945) | – | (Hu et al. 2017b) | | |
| | | | 32 | 18 | (Bortz et al. 2011; Fornek et al. 2009; Hatta et al. 2001, Hatta et al. 2007; Herfst et al. 2012; Kim et al. 2010; Manzoor et al. 2009; Richard et al. 2017; Shinya et al. 2004) | | |
| | | | 6 | 4 | (Gao et al. 2009; Le et al. 2009; Li et al. 2005; Steel et al. 2009; Taft et al. 2015) | | |
| | PA | V63I | T97I | 16 | 1 | (Hu et al. 2017a, Hu et al. 2016) | |
| | | | K158R | 2 | – | (Taft et al. 2015) | |
| | | | K356R | 7 | – | (Elgendy et al. 2017) | |
| | | | K497R | 3 | – | (Xu et al. 2016) | |
| 118 | | | – | (Yamayoshi et al. 2018) | | | |
| NP | I41V | N319K | 29 | 3 | (Zhu et al. 2015) | | |
| | | | 36 | 5 | (Gabriel, Herwig, and Klenk 2008, Gabriel et al. 2005) | | |
| NS1 | I106M | C138F | All/almost all viruses (N = 1,954) | – | (Ayllon et al. 2014) | | |
| | | | All/almost all viruses (N = 1,943) | – | (Li et al. 2018) | | |
| <i>In vitro</i> increase binding to human-type receptors alpha2,6-SA | HA ^a | D94N (D101N) | 6 | – | (Su et al. 2008) | | |
| | | S133A (S137A) | All/almost all viruses (N = 1,952) | – | (Yang et al. 2007) | | |
| | | S154N (S158N) | All/almost all viruses (N = 1,931) | – | (Wang et al. 2010) | | |
| | | S155N (S159N) | 5 | – | (Wang et al. 2010) | | |
| | | T156A (T160A) | All/almost all viruses (N = 1,939) | – | (Gao et al. 2009; Wang et al. 2010) | | |
| | | T188I (T192I) | 1 | – | (Yang et al. 2007) | | |
| | | V210I (V214I) | 3 | – | (Watanabe et al. 2011) | | |
| | | E251K (E255K) | 7 | – | (Chen et al. 2012) | | |
| | | S123P-496K (S128P-R496K (HA2-R167K)) | 4 | – | (Yamada et al. 2006) | | |
| | | Increase virulence in mice | PB2 | NS1 | D9N | 2 | – |
| P42S | All/almost all viruses (N = 1,922) | | | | – | (Jiao et al. 2008) | |
| L103F, I106M | All/almost all viruses (N = 1,919) | | | | – | (Kuo and Krug 2009; Spesock et al. 2011) | |
| Enhance replication in mammalian cells, decrease IF response | NS1 | K55E, K66E, C138F | 1,115 | – | (Jackson et al. 2008; Soubies et al. 2010) | | |
| | | | 1,087 | – | (Li et al. 2018) | | |
| Resistance toward antiviral drugs: \$ Oseltamivir and/or Zanamivir § Laninamivir # Baloxavir ^ Amantidine-Rimantidine | NA ^b | I117T ^{\$} | 1 | – | (Kode et al. 2019) | | |
| | | | I117V ^{\$} | 2 | – | (Adams et al. 2019) | |
| | | | R152K ^{\$} | 1 | – | (Gubareva et al. 2017) | |
| | | | I223T ^{\$} | 1 | – | (Samson et al. 2014) | |
| | | | S247R ^{\$} | 1 | – | (Gubareva et al. 2017) | |
| | PA | S247N ^{\$} | 34R [#] | 5 | – | (Hurt et al. 2011) | |
| | | | | 199G [#] | 1 | – | (Govorkova et al. 2022) |
| | | | | 5 | – | (Govorkova et al. 2022; Kiso et al. 2020) | |
| | | | | 4 | – | (Abed, Goyette, and Boivin 2005; Cheung et al. 2006; Ilyushina, Govorkova, and Webster 2005; Lan et al. 2010) | |
| | | | | 1 | – | (Chizhnikov et al. 2003) | |
| M2 | V27A [^] | V27I [^] | 1 | – | (Cheung et al. 2006; Ilyushina, Govorkova, and Webster 2005) | | |
| | | | 1 | – | (Cheung et al. 2006; Ilyushina, Govorkova, and Webster 2005) | | |
| | | | 7 | – | (Buranathai et al. 2007; Cheung et al. 2006; He et al. 2008; Ilyushina, Govorkova, and Webster 2005; Lan et al. 2010; Puthavathana et al. 2005) | | |

(continued)

Table 1. (Continued)

| Effect | Protein | Mutation | N. viruses | N. viruses from mammals | References |
|--|-----------------|---------------------------------------|---------------|-------------------------|--|
| Decrease antiviral response in ferrets | NS1 | N205S (with NS2: T47A) | 1,112 | – | (Imai et al. 2010) |
| Disruption of the second sialic acid binding site (2SBS) | NA ^b | S369I S369C/N/R K432E/N | 44 3 38 | – – – | (Du et al. 2021, Du et al. 2018) (Du et al. 2021, Du et al. 2018) (Du et al. 2021, Du et al. 2018) |
| Evasion human BTN3A3 | NP | Y52N | 43 | 1 | (Pinto et al. 2023, Pinto et al. 2022) |
| Adaptation to domestic birds/increased virulence | NA | 22-AA deletion in the NA stalk region | 7 | – | (Hoffmann et al. 2012; Munier et al. 2010; Sorrell et al. 2010; Zhou et al. 2009) |

^aH5 numbering (H3 numbering).

^bN1 numbering.

M, NA, and HA segments are required to provide optimal transmissibility to the A(H1N1) 2009 pandemic virus and revealed the significant impact of the M gene on the increase of NA activity and contact transmission in the 2009 A(H1N1) pandemic virus (Campbell et al. 2014).

Of note, reassortments of the NA gene, which were frequently detected during the first wave and resulted in the co-circulation of five different NA subtypes clustering into seven distinct genetic groups, were extremely rare during the second wave, in which the A(H5N1) subtype was the most prevalent one (97 per cent of the sequences). The A(H5N1) subtype (genotype EA-2020-C) emerged in autumn 2020, most likely within Europe, through reassortment events, preserving only the HA and M segment from the A(H5N8) subtype. This novel A(H5N1) subtype was sporadically detected in Europe during the A(H5N8)-dominated 2020–2021 wave. However, it spread globally and, in the subsequent epidemic wave, it became clearly dominant in Europe, which suggests that it may have had a higher fitness advantage than the other subtypes.

Among the characterized genotypes, EA-2022-BB A(H5N1)—which had emerged in spring 2022 from reassortment events with H13 LPAI viruses, a subtype circulating primarily in gulls, with unique species-specific gene signatures emerging for prolonged genetic isolation and co-evolution with this species (Olsen et al. 2006)—is of particular concern as it is associated with several mass mortality events in *Laridae*. In the summer 2022, this genotype had been detected mainly in European herring gulls in Northern Europe (France, Belgium, the Netherlands, and occasionally in the UK). However, starting from autumn 2022, a southward spread of this genotype to Spain and then Italy, Switzerland, and Austria, followed by an eastward and northward spread to Poland, the Czech Republic, Croatia, Sweden, Norway, and Denmark, was reported (Adlhoch et al., 2023a, 2023b). The ongoing and widespread circulation of this genotype in the gull population, with black-headed gulls representing the most affected species since winter 2022–2023, highlights the serious threat posed by this variant to the sea-bird population, with mass mortality events persistently reported in several colonies of gulls and terns from different countries (Adlhoch et al., 2023a). Of note, the association of this genotype with the infection in *Laridae* may indicate it is well adapted to the members of this family. This genotype possesses the PA, NP, and NS gene segments of A(H13). Gull-specific clades have previously been demonstrated for the PB2, NP, and NS gene segments, regardless of the geographical region. This suggests an intra-host evolution and adaptation of these virus genes that may favour virus replication in and/or transmission between gulls (Fouchier et al. 2005; Wille et al. 2011) and related host taxa,

as well as species in shared habitats with gulls. Moreover, the NP and PA genes have been shown to share a common evolutionary history in different host species, which suggests they tend not to reassort independently (Webster et al. 1992). Viruses of the A(H13) subtype do not readily infect ducks when they are inoculated experimentally (Fouchier et al. 2005), which may explain why in the viruses under study the EA-2022-BB genotype was not identified in Anseriformes and was detected only once in Galliformes (domestic chicken) during the 2021–2022 epidemic wave.

Of note, this genotype possesses mutations in the NP and NA proteins that can contribute to increase its zoonotic potential (Du et al. 2018; Pinto et al. 2023). The EA-2022-BB genotype has also been associated with infections in mammals, as in the outbreak reported in a mink farm in Spain in October 2022—where mink-to-mink transmission was suggested (Agüero et al. 2023)—in the outbreaks in fur farms in Finland in July 2023 (Lindh et al. 2023), as well as in some asymptomatic human detections in the UK ('Investigation into the risk to human health of avian influenza (influenza A H5N1) in England: technical briefing 4 - GOV.UK', n.d.). Whether the zoonotic potential of this genotype is higher than that of the other genotypes is unknown. Based on the available data, the majority of the infections in mammalian species have been caused by genotypes circulating with the highest frequency in birds at the time of the infection, suggesting direct and separate spillover events from birds to scavenging mammals in particular. Of note, our data indicate that mutations in the polymerase proteins associated with adaptation to mammalian species can be rapidly acquired by the virus during replication in a mammalian host, which seems to indicate that mammals may represent an important source of viruses with an increased zoonotic potential. However, recent serological evidence of A(H5Nx) infections in wild and domestic mammals, including pets reared or living in farms where poultry outbreaks were reported, clearly suggests that we have likely uncovered only the tip of the iceberg (Chestakova et al. 2023; Moreno et al. 2023; Rosone et al. 2023) and further studies are needed to increase our knowledge on the extent of virus circulation in mammals. Furthermore, this study, jointly with the recent infection cases reported in cats in Poland (Domańska-Blicharz et al. 2023) and in fur farms in Finland (Lindh et al. 2023), highlights the compelling need to adapt surveillance and to include mammals among the species posing a considerable risk for zoonotic spread of HPAI. This would provide health authorities with adequate information for a proper management of outbreaks in species belonging to this class.

For the first time, this study presents a complete overview of the multiple genotypes that had been circulating in Europe from

October 2020 to August 2022, providing information on their geographic and host distribution. However, such data should always be interpreted taking into account the sampling bias within wild bird surveillance towards sick or dead birds, rather than resilient or asymptomatic birds. We assigned each genotype a name to simplify and encourage discussion between European laboratories. Although this work focuses on the 2020–2021 and 2021–2022 epidemic waves, it is worth mentioning that genotypes EA-2021-AB and EA-2022-BB (Adlhoch et al., 2023b)—that emerged in September 2021 and May 2022, respectively—were the main genotypes that had been circulating in Europe throughout the latest 2022/2023 epidemic.

We have also provided evidence of the emergence of a new genotype with gull adapted genes (EA-2022-BB), which offered the virus the opportunity to occupy new ecological niches and to persist during the summer months, causing massive mortality events in *Laridae* and multiple spill over events in farmed carnivores (Agüero et al. 2023; Lindh et al. 2023). This changing ecology, accompanied by viruses endemically circulating in wild birds in Europe in a progressively wider number of hosts, may change both the hot spots and the target species for virus surveillance, making trends of virus evolution and spread difficult to predict.

Supplementary data

Supplementary data is available at *VEVOLU Journal* online.

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Data Availability

All sequences are published in the GISAID EpiFlu database and sequence accession numbers are available in [supplementary material \(Supplementary Table S1\)](#); xml, log, and trees files generated from the BEAST analyses are available on request.

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