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Analysis of the genotypes of Influenza A H5N1 in Bulgaria 2021-2023

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Abstract

Circulating HPAI virus H5N1 can be traced back to the original A/goose/Guangdong/1/96 strain. During the epidemiological years 2022 and 2023, a remarkable number of HPAI H5N1 virus isolates were detected and reported in a diverse range of hosts, including wild and domestic birds, mammals and humans, in more than 28 European countries. Interestingly, the potential for cross-species transmission highlights the complex dynamics of viral spread among these different susceptible species. The persistently emerging outbreaks of HPAI H5N1 in animals and humans demonstrate the need for comprehensive understanding of this virus and its public health implications, as well as for detailed genomic mapping and characterization. With increasing reports of human infections and the potential for devastating economic consequences in industrial livestock production, it is imperative to deepen analyzes of HPAI H5N1 to effectively respond to the continued spread of the virus in animals, prevent future outbreaks and transmission to the people. To provide a comprehensive analysis of the emergence and spread of HPAI H5N1, it is necessary to examine the genetic characteristics, mutations in the viral genome, and the evolution of the virus, and to evaluate the effectiveness of current prevention and control strategies.

Key words: Avian Influenza, genotype, mutations, cranes

Introduction

Genetic diversity analysis of HPAI A(H5N1) viruses in avian species in 2022-2023 in Europe. Avian influenza viruses (AIVs) pose a significant challenge to global human and animal health systems due to their widespread distribution and significant mortality rates among susceptible species. AIV have an eight-segment genome and encode at least 11 different proteins, including hemagglutinin (HA) and neuraminidase (NA) glycoproteins. HA and NA in avian species are classified into 16 and 9 subtypes, respectively. Based on the genetic variations of these two proteins, different subtypes of influenza viruses are defined. AIVs are categorized into two major groups based on their pathogenicity to birds: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses. In recent years, the transmission and spread of HPAIv such as H5N1, H5N8 and H7N9 has posed a significant threat to public health. Among the different HPAIv types, H5N1 is considered the most pathogenic, with high mortality in birds, mammals and humans. Since the initial outbreak of H5N1 highly pathogenic avian influenza back in 1959 among poultry in Scotland and its subsequent transmission to humans in 1997 in Hong Kong, numerous outbreaks of the virus have been reported not only in birds. From 2003 to 14 July 2023, the World Health Organization (WHO) has documented 878 human cases of HPAI H5N1 infection and 458 (52%) deaths in 23 countries (*Buthelezi et al., 2022*).

The viral genome of HPAI H5N1 consists of a single-stranded RNA molecule, approximately 13.5 kilobases in length. The genome is segmented into eight distinct segments, each encoding specific proteins that play a critical role in the virus' life cycle. These proteins include polymerase 1 (PB1, 757 amino acids), polymerase 2 (PB2, 759 amino acids), acid polymerase (PA, 716 amino acids), hemagglutinin (HA, 568 amino acids), nucleoprotein (NP, 498 amino acids), neuraminidase (NA, 499 amino acids), matrix protein 1 (M1, 252 amino acids), matrix protein 2 (M2, 97 amino acids), as well as nonstructural proteins NS1 (225 amino acids) and NS2 (121 amino acids). The HA and NA glycoproteins have the most mutations and the most frequent ones.

HA serves as the primary glycoprotein on the surface of the H5N1 virus and is an integral part of the early stages of viral infection. It is responsible for the binding of the virus to specific receptors on the surface of host cells, facilitating the entry of the virus. In addition, the HA protein plays a critical role in releasing the viral genome into the host cell cytoplasm. On the other hand, NA is involved in the final stages of infection and cleaves sialic acid-containing receptors and glycoproteins. This enzym activity of NA helps to release the virus from cells, allowing it to invade other cells and potentially transmit it to new hosts. NP with the three polymerase subunits - PA, PB1 and PB2 - forms a viral ribonucleoprotein (vRNP). This RNP complex plays a crucial role in various stages of the virus life cycle, including RNA transcription, replication and viral particle packaging. M1 is a critical component of the HPAI H5N1 virus and has been found to have a significant role in the viral replication process and also interacts with vRNP to regulate vRNP transport. The M2 protein of HPAI H5N1, known as the M2 proton channel, maintains pH balance during various stages of the viral life cycle. The NS1 protein of the virus has a critical role in inhibiting interferon production and, through its multifunctional properties, helps to evade the host's immune response and facilitates viral replication (Buthelezi et al., 2022).

The HPAI H5N1 virus is categorized into ten major clades (labeled 0 - 9), accompanied by numerous secondary clades and various subclades. To date, several intercontinental waves of H5 viruses have been observed, including H5N1 clade 2.2 (2005–2006), H5N1 clade 2.3.2.1c (2009–2010), H5N8 clade 2.3.4.4a and H5N1 clade 2.3.2.1c (2014–2015), and H5Ny clade 2.3.4.4b (2016–2017). Between 2020 and 2021, a new wave of highly pathogenic avian influenza virus (HPAIv) H5N1 clade 2.3.4.4b was reported within geographical regions of Europe, Asia and Africa, affecting a wide variety of wild and domestic birds. In November 2021, two new reassortants of HPAIv (H5N1) clade 2.3.4.4b.2 were isolated from dead migratory birds in China. The low antigenic response of this new reassortant to vaccine antiserum indicates high public health risks. In one of the most recent CDC (*Center for Disease Control and Prevention*) reports, outbreaks of HPAIv of clade 2.3.4.4b were observed among seals, resulting in unusual mortality that was observed at the same time as the wave of avian infections. Based on information on ≥2 secondary outbreaks of HPAIv occurring in the seal population within the wave of avian infections and data from Sanger sequencing and subsequent phylogenetic analysis, the researchers concluded that the virus exhibits high adaptation to different hosts. The data suggest that monitoring of both marine mammals and wild shorebirds is essential to characterize the pandemic potential of H5N1 (Buthelezi et al., 2022).

When comparing avian isolates and human isolates, the replication efficiency of H5N1 AIV and the genomic sequence encoding HA was equivalent. Alteration in the sequence encoding NA shows enhanced viral replication in human airway epithelium. In 10 different influenza isolates having group 1 and 2 NAs, the L204 M mutation was found to have the same suppressive effect on NA activity. For example, H5N1 virus isolates from humans do not increase the viral RNA replication potential and do not produce as much NA protein as avian isolates. These isolates also showed reduced virus particle release and increased cell-to-cell spread, in addition to increased NA accumulation at the plasma membrane. The affinity of the virus for human receptors is also enhanced by NA mutations. Although the NA coding sequence mutations studied had no effect on the highly pathogenic nature of H5N1 in birds, a change in the level of virulence and replication of H5N1 AIV

was observed in mice and to a lesser extent in ferrets. Changes in the NA coding sequence in human H5N1 isolates have different effects in mammals, but have no discernible effect on the pathogenicity of the virus or its ability to spread to birds. These facts could be useful in predicting the zoonotic potential of AIV (Buthelezi et al., 2022).

In support of the proposition that mutations in the virus genome differentially affect the etiology and behavior of the influenza virus, in a study by Buthelezi et al. resistance of the H5N1 virus to peramivir was detected in the presence of the double mutation H274Y–I222K. Furthermore, mutations in the PB1, PB2 and PA subunits can have significant consequences for the virulence, transmission and drug resistance of the H5N1 virus subtype. The PB1 subunit is susceptible to mutations that affect levels of virulence and pathogenicity. Of particular significance is the H99Y mutation, which demonstrates increased polymerase activity and increased replication efficiency in mammalian cells. Mutations occurring in the PB2 subunit, the core component of the polymerase complex, lead to significant consequences, including increased replication and host adaptability. The E627K mutation results in a glutamic acid to lysine substitution at position 627. This genetic change plays a key role in enhancing replication in humans. Another genetic mutation, D701 N, also in the PB2 subunit, results in the substitution of aspartic acid for asparagine at position 701. This mutation is associated with increased polymerase activity and increased virulence in mammals. PA, the third subunit in the polymerase complex, functions as the main endonuclease involved in viral mRNA synthesis. Similar to the PB2 and PB1 subunits, extensive studies of mutations in this virus at specific amino acid positions in the PA protein—namely PA-573, PA-383, PA-353, PA-347, PA-343, PA-241, PA- 237, PA-224, PA-185, PA-127, PA-101 and PA-44, play a prominent role in influencing both the replication process and virulence characteristics of the virus (*Buthelezi et al., 2022*).

In 2023 highly pathogenic influenza subtype H5N1, clade 2.3.4.4b, continues to circulate in avian population in Europe. This subtype affects not only birds, but also mammals and humans. The most common genotypes for 2023 are BB, AB and CH. Since February, a rapid increase in the frequency of genotype BB (H5N1-A/Herring_gull/France/22P015977/2022-like) has been observed, which in the period April - August reaches a frequency > 80%. This genotype, which circulates mainly among the family Laridae, has been found in 20 European countries, in the Leningrad region, as well as in West Africa (*EFSA Science Report, 2023*).

Since September 2023, with the decrease in occurrences in seabirds and increased prevalence in wild birds of the family *Anseriformes* and family *Gruidae* (especially common cranes), there has been a sharp decline in the frequency of detection of genotype BB. However, new genotypes were identified, four of which constantly circulated in Europe in the previous epidemiological years, namely BB, AB (H5N1 A/duck/Saratov/29-02/2021-like), CH (H5N1-A/Mallard/Netherlands/ 18/2022-like) and I (H5N5 A/whooper_swan/Romania/10123_21VIR849-1/2021-like) and seven new genotypes that arose as a result of reassortments between low pathogenic viruses circulating in wild birds and H5N1, namely:

DA (H5N1-A/mute_swan/Slovenia/PER1486-23TA_23VIR10323-22/2023-like),

DB (H5N1-A/herring_gull/Germany-NI/2023AI08764/2023-like),

DC (H5N1-A/ Common_Buzzard/Netherlands/23023642-002/2023-like),

DD (H5N1- A/Pheasant/England/113705/2023 - like),

DE (H5N1- A/Chicken/Scotland/114176/2023-like),

DF (H5N1 -A/Sparrowhawk/Scotland/131359/2023-like) and

DG (H5N1-A/chicken/Germany-NI/2023AI08838/2023-like) (*EFSA Science Report, 2023*).

It cannot be concluded with certainty whether these new genotypes originate in Europe or represent new viral reassortants given the limited data available from other countries outside of Europe. Among the genotypes characterized in 2023, five (AB, BB, DA, DB and I) were identified in multiple countries. The new DA genotype was found in wild and captive (zoo) birds, as well as in domestic birds in Eastern and Southern Europe (Austria, France, Italy, Germany, Romania and Slovenia). It should be noted that, based on available genomic data, all viruses isolated from whooping cranes in Europe (Austria, France, Germany and Italy) during this period belonged to the DA genotype (*Fusaro et al., 2023*).

In contrast, the DB genotype was isolated from wild and domestic poultry populations in Northern Europe (Denmark, Germany and the Netherlands). Subtype H5N5, genotype I, was detected in Europe in 2021, and continues to be detected in various countries. In September 2023, viruses of this genotype, genetically close to those from Norway, were identified in Iceland and the United Kingdom, suggesting an expansion of the geographic distribution of this variant (*Fusaro et al., 2023*).

One of the H5N1 viruses from an outbreak in poultry in Poland in October 2023 belonged to the CH genotype. Virus isolates from white stork (*Ciconia ciconia*), chickens, domestic cats (*Felis catus*) and caracal (*Caracal caracal*), again in Poland, in the period June and July 2023, also belong to this subtype. Similar to these viruses circulating in birds and mammals in summer, it has the PB2-E627K mutation. This discovery proves the constant circulation of viruses containing a mutation associated with adaptation of the virus to mammals among the bird population of the country. To date, this genetic variant (CH with PB2-627K mutation) has not been identified in other European countries. In addition to this mutation, other mutations in different domains of the protein have been reported to be associated with mammalian pathogenicity (D9N, I147T, E158G, E192K, A199S, D253N, T271A, K339T, F404L, K526R, A588I, A588T, G590S, Q591R, Q591K, A674T, D 701N, K702R, and S714R, etc.) (Lee et all, 2020; Demirev, A.V. et. Al., 2020).

A possible carrier of a genotypic DA variant of H5N1 - cranes, migration and habitats. The European crane has two subspecies West European crane (*Grus grus grus*) and Transcaucasian (*G.grusarchibaldi*). Transcaucasian cranes are an endangered species, about 250–300 birds, breeding at high altitude in the wetlands of Georgia, Armenia, western Iran and eastern Turkey. They migrate short distances to lower and warmer sites in southern Turkey and Iraq (Nowald et.al,. 2021). Western European cranes number in the tens of thousands. These birds are found in the northern parts of Europe and in front of the Palearctic to Siberia. Their nesting and breeding takes place in southern Europe, smaller numbers in Greece, Romania and Serbia, Denmark and Germany. Larger breeding populations are found in Scandinavia, especially in Finland and Sweden and Russia. The species is a long-distance migrant, mostly wintering in North Africa. Autumn migration is from August to October and spring migration is from March to May. Some birds also winter in southern Europe, mainly Spain, Portugal and France. Most cranes winter in Sudan, Ethiopia, Tunisia and Eritrea. The third major winter region is the Indian subcontinent, including Pakistan. Some birds settle in Burma, Thailand and Vietnam. There are also wintering birds in eastern China (Figure 1) (Nowald et.al,. 2021).

In Europe, the crane mainly prefers mixed forests and predominantly breeds in boreal and taiga forest and mixed forests, from an elevation of sea-level to 2200 meters. Treeless swamps and marshes where there are small lakes or other bodies of water is the crane's breeding ground. High humidity is where the crane feels good. In winter, this species moves to flooded areas, shallow sheltered bays, and swampy meadows. When migrating, it can be seen in areas with savanna-like vegetation, especially in the Iberian Peninsula (Archibald, 2023, Nowald et.al,. 2021).

Cranes in Bulgaria rarely winter in the country, they are more of a passing species. They prefer vast plains near water bodies, swamps in the foothills, meadows, swamps.

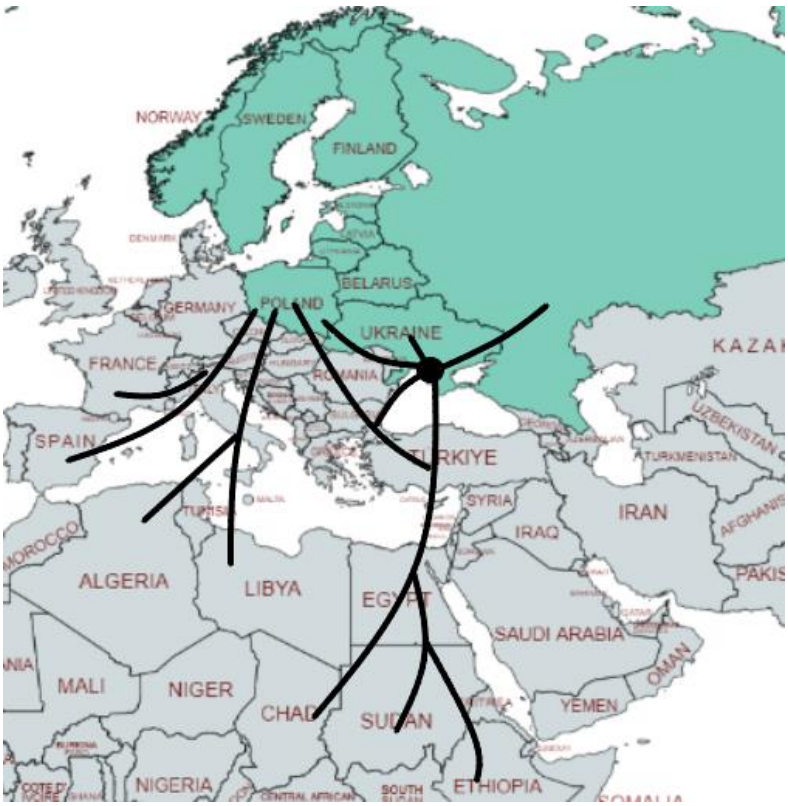


Figure 1. Map of Europe with main Crane's migration paths and the wintering places of the birds (map were created by MapChart, https://www.mapchart.net/)

Materials and Methods.

Sequences of viruses. The used sequences are found at Global database for influenza gene sequences (https://gisaid.org/). For the current analysis, it was used PB2, NP and HA sequences.

FluServer Mutation tool. For comparison of the mutation we use the FluServer https://gisaid.org/database-features/flusurver-mutations-app/.

Evolutionary analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. For HA, NP and PB2 gene the trees with the highest log likelihood (respectively-3010.1, -5114.839, -5726.39) are shown. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA11.The analysis of HA involved 107 nucleotide sequences. There were a total of 1781 positions in the final dataset. The analysis of NP involved 88 nucleotide sequences. There were a total of 3050 positions in the final dataset. The analysis of PB2 involved 107 nucleotide sequences. There were a total of 2341 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al. 1993; Tamura et al. 2023).

Philogenic trees. Graphical view of phylogenic trees is designed by FigTree v1.4.4. software (Rambaut, 2018).

Results.

Genomic characterization of 16 subtype H5N1 viruses identified in Bulgaria for the period from January 2022 to November 2023 shows that the virus isolates are grouped in clade 2.3.4.4b. Three main circulating genotypes have been identified. The virus isolates from April 2022 and March 2023 belong to the AF genotype, as well as the viruses detected in November-December 2021. One

virus isolate from an outbreak in backyard chickens in the village of Feldfebel Denkovo, reg. Dobrich in January 2022, belongs to AP genotype. The outbreak in the quail farm in city Etropole, Sofia region (January 2023) and the virus isolates collected in October-November 2023 belong to a new genotype called DA, which has also been detected in other Member States since September 2023. As previously said, this subtype is new to Europe and is found in wild birds (mainly cranes, swans and storks).

The outbreaks for 2023 in Bulgaria, show that there are small ponds or rivers near the affected farms, a suitable environment for passing migratory birds. The time of onset of the disease coincides with migration: spring and autumn.

Due to the lack of published sequences of the viruses isolated in Bulgaria in 2022-2023, we performed an indirect analysis of the DA genotype. H5N1-A/mute_swan/Slovenia/PER1486-23TA_23VIR10323-22/2023-like is reference strain for the genotype and closest to the isolates in Bulgaria. The phylogenetic tree of the PB2 gene shows strong presence of viruses in cranes (Figure 2). This genotype is also found in swans and storks in Austria, Romania, Slovakia, etc. It can be seen that the cluster of Bulgarian isolates from 2021 is genetically distant. The isolate from Ukraine (A/turkey/Kherson/540-6/2023) is relatively close, as is the presence of quite a few viruses from Korea from 2021–2022.

Figures 3 and 4 show an analysis of the NP and HA genes. In the analysis of the sequences encoding NP, there is a large distance between the viruses isolated in Bulgaria from 2021 and 2023, due to the different genotypic affiliation. When analyzing the HA sequences the isolates appear to be closely related (as it is the same subtype-H5N1 2.3.4.4b). Viruses of genotype CH from Poland are also clustered in the phylogenetic tree. This only shows that regardless of the different genotypes, these viruses are not so different.

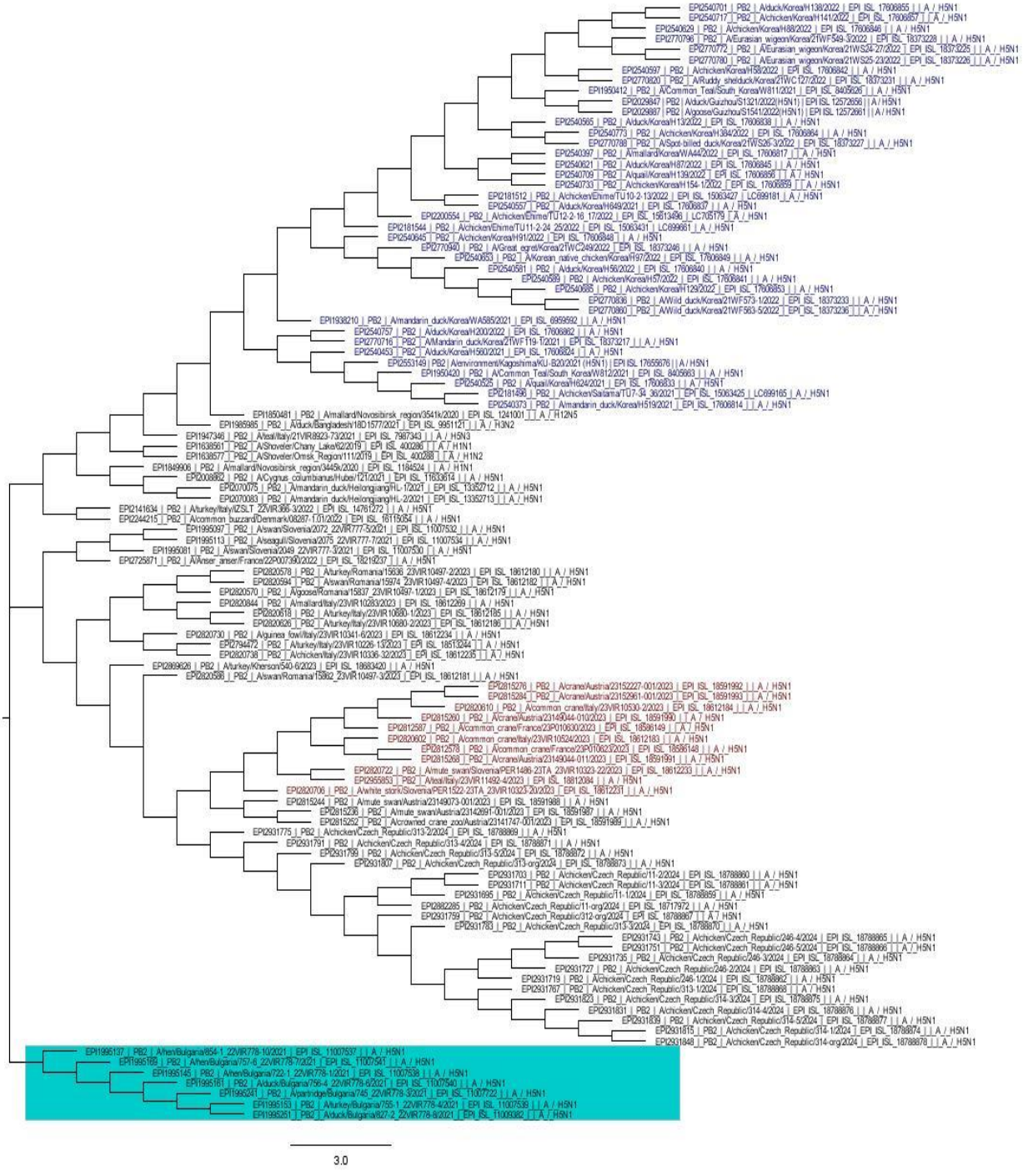


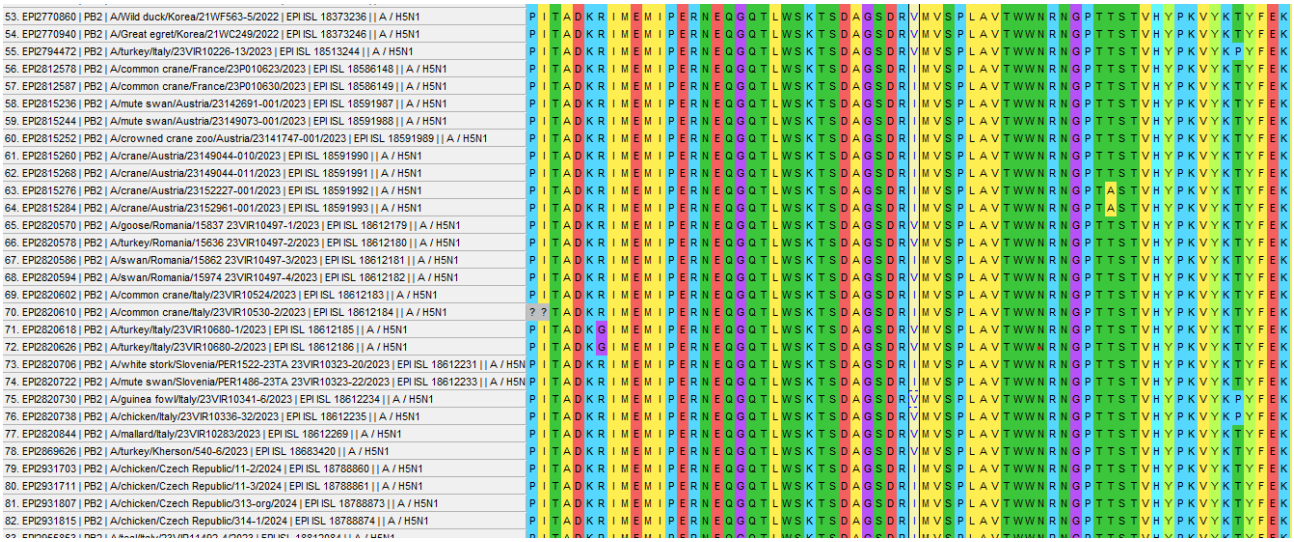
Figure 2. Phylogenetic analysis of the PB2 gene of viruses of genotype DA of subtype H5N1 2.3.4.4b (sequences published in GSAID, as well as Mega 11Fig and Tree1.4.4 software were used to create and build the phylogenetic tree). DA genotype viruses are marked in dark red, those originating from Korea in dark blue, and isolates from Bulgaria from 2021 are marked in the rectangle.

Figure 3. Phylogenetic analysis of the NP gene of H5N1 subtype 2.3.4.4b genotype DA viruses (sequences published in GSAID and Mega 11Fig and Tree1.4.4 software were used to construct and construct the phylogenetic tree). DA viruses are marked in dark red, and isolates from Bulgaria from 2021 are marked in the rectangle.

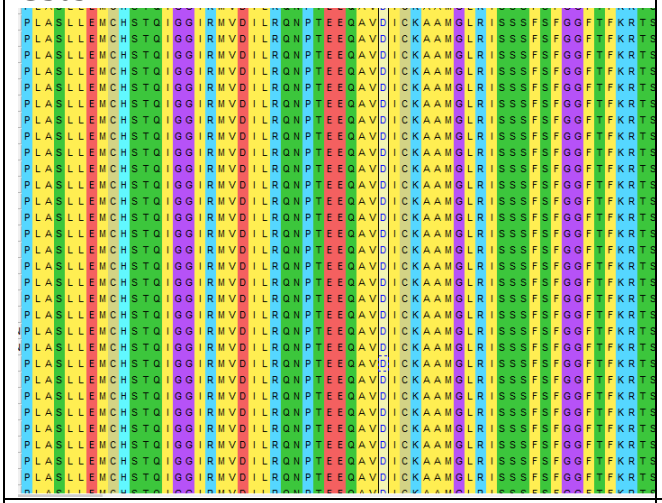
Figure 4. Phylogenetic analysis of the HA gene of viruses of genotype DA of H5N1 subtype 2.3.4.4b (sequences published in GSAID as well as Mega 11Fig and Tree1.4.4 software were used to construct and construct the phylogenetic tree). DA viruses are marked in dark red, and isolates from Bulgaria from 2021 are marked in the rectangle.

Molecular markers with zoonotic potencial indentified in protein PB2 of the analysed viruses are presented in Figure 5. Only in A/common crane/France/23P010623/2023 was found **A674T.**

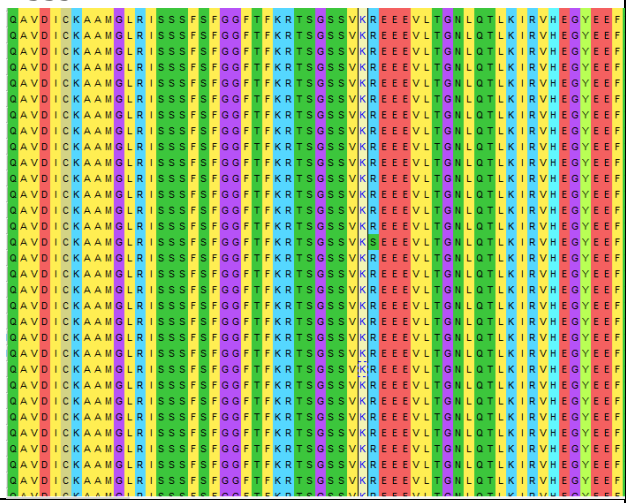
L89V/I*



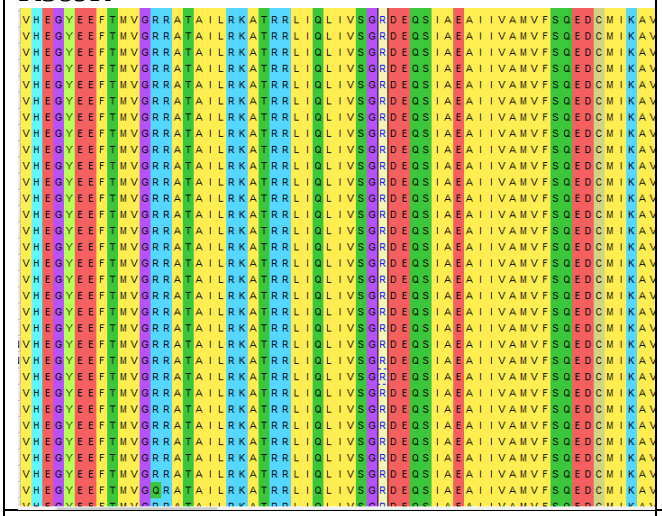
G309D



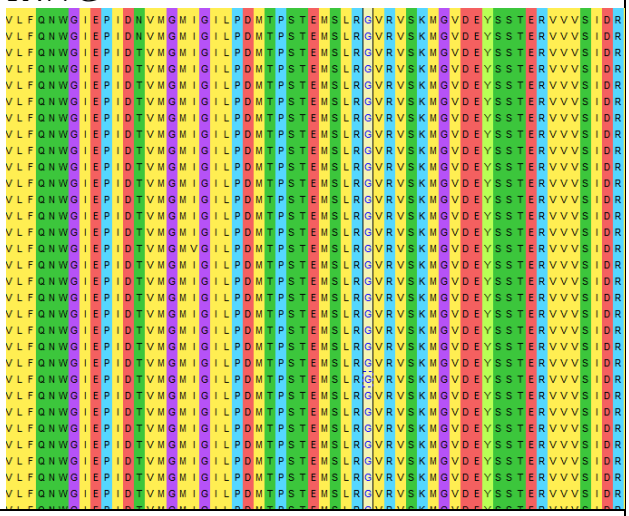
T339K



K389R

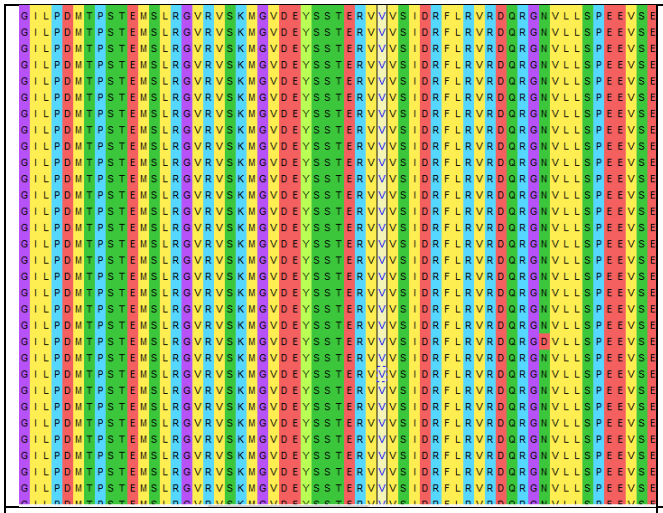


R477G

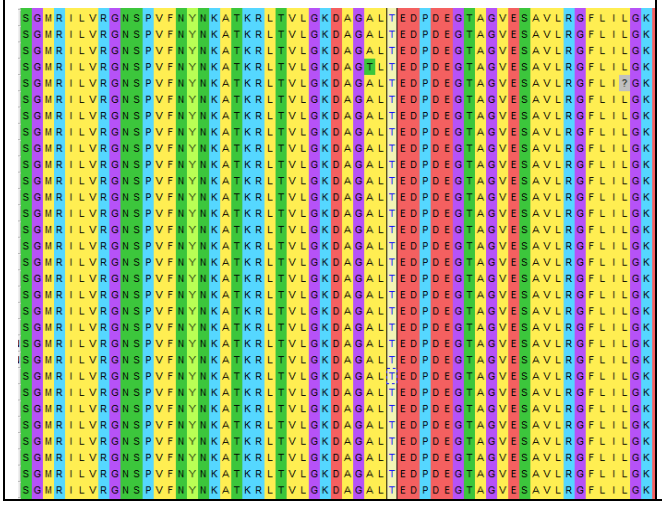


I495V

K627E



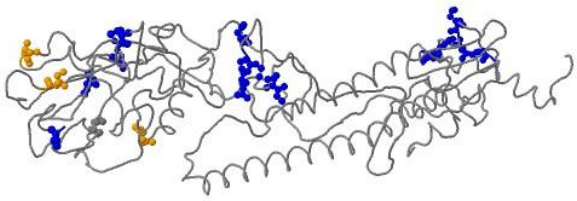
A676T



*L89V/I- In position 89 Leucine is substituted with Valine or Isoleucine. Most of the viruses from genotype DA, including Bulgarian ones have L89V mutation.

Figure 5. Mutations in PB2: L89V, G309D, T339K, R477G, I495V, K627E, A676 (Mega 11)

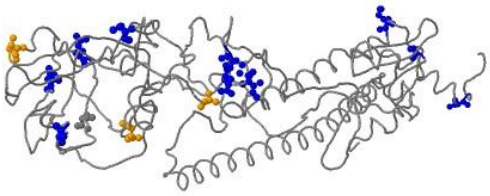
We compared mutations in haemaglutinin and neuramidase in one of Bulgaria viruses from 2021 - A/hen/Bulgaria/757-6_22VIR778-7/2021_11007541 and the reference strane for genotype DA (A/mute_swan/Slovenia/PER1486-23TA_23VIR10323-like). The mutation T156A, which is present in both HA is responsible for increasing the virus binding to α2-6 -linked sialic acid, and transmition in guinea pigs. The mutations in NA in analised viruses are not associate wuth antiviral resistanse (Figure 6.). Other presented mutations in Ha and NA are not significant.



FluSurver-JSmol

Sample: HA_A/hen/Bulgaria/757-6_22VIR778-7/2021_11007541;

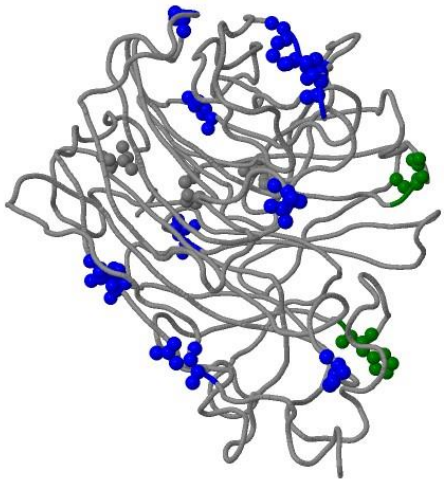
Mutation(s): *K3N*, *I10T*, *G16S*, *D59N*, *N110S*, *T139P*, *T156A*, *I167V*, *Q185R*, *V194I*, *A201E*, *N252D*, *E284G*, *M285V*, *I298V*, *K492E*, *R498K*, *Q506H*, *V538A*, *I547M*, *V548I*



FluSurver-JSmol

Sample: HA_A/mute_swan/Slovenia/PER1486-23TA_23VIR10323-like

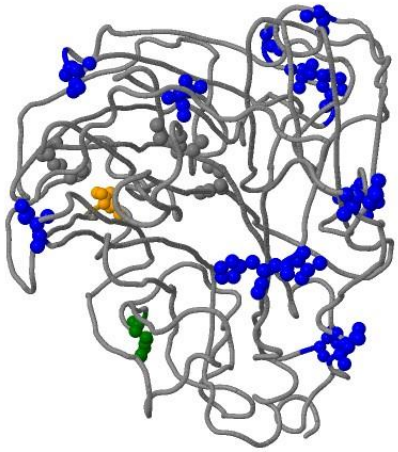
Mutation(s): *K3N*, *G16S*, *A99D*, *N110S*, *T139L*, *T156A*, *Q185R*, *V194I*, *A201E*, *N252D*, *E284G*, *M285V*, *I298L*, *K492E*, *S519N*, *V538A*, *I547M*, *V548I*



FluSurver-JSmol

Sample: NA_A/hen/Bulgaria/757-6_22VIR778-7/2021_11007541;

Mutation(s): *K6R*, *I10T*, *V17I*, *I20V*, *H44Y*, *A46P*, *T76A*, *K78Q*, *A81T*, *V99I*, *H100Y*, *H155Y*, *T188I*, *N222K*, *M258I*, *T289M*, *G336S*, *V338M*, *P340S*, *N366S*, *G382E*, *S405T*, *I418M*, *S434N*, *D460G*



FluSurver-JSmol

Sample: NA_A/mute_swan/Slovenia/PER1486-23TA_23VIR10323:

Mutation(s):*K6R*, *I10T*, *V16I*, *V17I*, *I20V*, *H44Y*, *A46P*, *F74C*, *T76A*, *K78Q*, *A81T*, *V99I*, *H100Y*, *H155Y*, *T188I*, *N200D*, *M258I*, *T289M*, *Q308K*, *G336S*, *V338M*, *P340S*, *N366S*, *G382E*, *S405T*, *I418M*, *S434N*, *D451N*, *D460G*

Figure 6. Mutations in HA and NA proteins in A/hen/Bulgaria/757-6_22VIR778-7/2021_11007541 and A/mute_swan/Slovenia/PER1486-23TA_23VIR10323-like. Reference: HA_H5N6_Human_2014_Sichuan26221 and NA_H5N1_Goose_1996_Guangdong1. https://gisaid.org/database-features/flusurver-mutations-app/.

Discussion

The genomic characterization of subtype H5N1 viruses identified in Bulgaria for the period from January 2022 to November 2023 shows that the viruses again belong to clade 2.3.4.4b. and belong to 3 genotypes AF, AP and DA. The virus isolates from 2023 in Bulgaria belong to a new genotype for Europe - DA. It has been proven in various wild birds in Europe. Interestingly all infected cranes are carriers this particular genotype. Whether they are the main source of influenza virus for Europe and Bulgaria cannot be proven. But the time of DA genotype appearance is related to the migration of birds and is related to their habitats. Water areas located near the affected sites are the main risk factor for the appearance of the virus in these areas.

Wild birds from northern and central Europe played a key role in spreading the virus to the rest of the continent. In 2023 11 H5N1 genotypes have been identified in Europe: BB, AB, CH, I, DA, DB, DC, DD, DE, DF and DG. This ever-changing scenario highlights the need to closely monitor the evolution of the virus to detect timely signs of adaptation to new species or the emergence of new variants of the virus, which may increase its zoonotic potential (*EFSA Science Report, 2023*).

The predominant genotype in Korea for 2022–2023 was D, accounting for 57.5% of all virus isolates. It has been found in white-fronted goose, white heron and gray heron. Phylogenetic analyzes indicate that the virus was most likely transferred from China to Russia in 2021–2022, then introduced from Russia to South Korea in 2022 (Ye-Ram Seo et all, 2024). Phylodynamic analyzes demonstrate that a key source of the virus for Europe during the 2020-2021 and 2021-2022 epidemic waves is the Russia/Kazakhstan/Georgia region, while Europe appears to be the main source of the virus for North America. Within Europe, wild birds from the north-central region played a key role in spreading the virus to the rest of the continent.

Molecular markers affecting biological characteristics of avian influenza viruses in PB2 protein where very important for understanding of zoonotic potencial. The muttion E627K (substitution of glutamine with lysine in position 627) is the best-known. It is responsible for adaptation to replication in mammalian species. In analised viruses it is not presented, but there are others (L89V, G309D, T339K, R477G, I495V, K627E, A676). They increase viral polymerase activity in mammalian cell lines and increase virulence in mice (Suttie et al., 2019). Mutation A674T, which was found in A/common crane/France/23P010623/2023, occurs commonly in influenza virus isolates from humans and very rarely in avian isolates (Lee et all, 2020; Demirev, A.V. et. Al., 2020). Mutation in HA and NA, showed differences between different genotypes. There are no significant markers for drug resistance, but there are increasing virus binding to α2-6 and transmition in guinea pigs. Avian viruses prefer to bind to α2,3-linked sialic acid, whereas the human prefer to bind to α2,6-linked sialic acid. In human upper respiratory tract α2,6 allow spread of virus through production of aerosols by sneezing and cough.

Rapid analysis of emerging new viruses is needed, which can only be done if they are sequenced in time. This is due to the high variability of the Influenza A virus due to the many mutations in the genome. The appearance of so many genotypes in one year with different characteristics is disturbing. The fact that genotype CH with a PB2-627K mutation has been discovered so far only in Poland should not reassure us, because "tomorrow" it may also appear in our country.

Surveillance of wild birds should be expanded, especially those that serve as reservoirs of the virus in the wild. It is obvious that they have been a major source of infection in recent years. It is necessary to improve and deepen the analysis of the data from epizootological studies in the field and to combine them with the results of the phylogenetic analysis of the isolated Influenza A viruses from Bulgaria. After the sequences of the viruses isolated in Bulgaria in 2023 and 2024 are published, the detailed analysis of the genotype would be possible.

Conclusion

HPAI H5N1 remains a significant global challenge due to its widespread distribution and high mortality rates. Documented cases of HPAI H5N1 infection in humans, together with recent outbreaks of HPAI H5N1 in various animal species, highlight concerns about the transmission and spread of this virus among birds, mammals, and humans. The genetic variation observed among HPAI H5N1 isolates further underscores the need for vigilance and continued research to understand virus etiology, origin, distribution, genome structure, phylogeny, evolution, transmission routes, immune response, pathogenesis, and diagnostic and preventive strategies.

In conclusion, the ongoing challenges posed by HPAI H5N1 on a global scale necessitate proactive actions to address future trends in the spread of the virus. Several key areas require focus, attention and research to effectively manage the spread and impact of this pathogen. They are:

1. Surveillance and early detection: Strengthening global avian influenza surveillance systems is critical for timely identification and surveillance of emerging reassortants, especially those with zoonotic potential. Continuous monitoring of wild and domestic bird populations and in high-risk areas is needed, which will provide early warning signs of virus circulation and facilitate timely action.

2. Genetic variation and viral evolution: The emergence of genetic variation among HPAI H5N1 isolates highlights the need for ongoing genetic analysis and monitoring. Studying mutations and reassortment events in the virus can provide insight into its evolutionary potential, transmission dynamics and pathogenicity.

3. Interspecies transmission and reservoirs: The observed interspecies barrier jumping and transmission between mammalian species and avian populations raises concerns about the establishment of viral reservoirs as a continuing risk to both animals and humans. Future research should be directed at characterizing the mechanisms and factors facilitating interspecific transmission, with particular emphasis on identifying potential reservoir species and understanding transmission dynamics in these populations.

4. Preventive measures and vaccines: The development and application of effective vaccines against HPAI H5N1 is of paramount importance. Recombinant vaccines that have been developed have not been fully tested for efficacy and effectiveness and do not allow to distinguish vaccine from circulating wild-type virus. Research should focus on improving vaccine efficacy, expanding the range of protection across different viral subtypes, and optimizing vaccine delivery strategies.

5. Antiviral therapies and treatment strategies for people: Further investigation of antiviral therapies and treatment strategies is essential for HPAI H5N1 infections. Research should focus on the identification and development of novel broad-spectrum antiviral agents against avian influenza viruses. Additionally, the study of host immune responses and the development of immunomodulatory therapies may help reduce disease burden and improve patient outcomes.

6. International cooperation and preparedness: Strengthening international cooperation and information sharing among countries are vital to address the global challenges posed by HPAI H5N1. Establishing robust communication networks, sharing data from surveillance and molecular testing will facilitate rapid response, adequate and timely decision-making, early containment and effective control of outbreaks.

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