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Genomic signatures and host adaptation of H5N1 clade 2.3.4.4b: A call for global surveillance and multi-target antiviral strategies

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ABSTRACT

The recent report of the first fatality associated with infection by influenza virus H5N1 clade 2.3.4.4b, identified as genotype D1.1, which is distinct from the B3.13 genotype, has sparked fears of a potential human pandemic. However, the genetic relationships between B3.13 and D1.1, as well as their origins, host adaptability, and antiviral resistance, remain poorly understood. Here we conducted a comprehensive phylogenetic and comparative analysis of H5N1 clade 2.3.4.4b across multiple species, in order to identify the molecular characteristics and frequency of resistance mutations in these two genotypes, elucidate their evolutionary trajectories, and assess their implications for public health. Our results demonstrate that B3.13 exhibits mammalian adaptability, while D1.1 retains avian adaptability. Importantly, both genotypes display limited occurrences of human-like signatures, which can help alleviate public anxiety. Additionally, the emergence of the resistance mutations in the clade 2.3.4.4b on the binding sites of antivirals calls for the development of multi-target antiviral strategies to mitigate the risk of resistant strain reassortment.

1. Introduction

The highly pathogenic avian influenza (HPAI) virus H5N1 is a major subtype of HPAI viruses and represents a significant threat to global public health (P Huang et al., 2023; P Plaza et al., 2024). As of 2024, a total of 963 confirmed cases of human infection with H5N1 have been reported, resulting in 465 deaths with a case fatality rate of 48%, rendering it the most lethal subtype of highly pathogenic avian influenza viruses (HPAIVs) known to infect humans to date (RJ Webby et al., 2024; H Song et al., 2025).

The H5N1 clade 2.3.4.4b is currently the predominant strain (S Elbe et al., 2017; P Huang et al., 2023), which has sporadically infected avian species, as well as some mammalian populations, worldwide (Fig. 1 A and Fig. 1 B) (P Huang et al., 2023; R Xie et al., 2023; P Plaza et al., 2024). In March 2024, the United States reported the first outbreak of H5N1 clade 2.3.4.4b, genotype B3.13 in dairy cows (E Burrough et al., 2024; U.S. Department of Agriculture, 2025). Subsequently, the first human infection associated with bovine-origin H5N1 was reported in the Texas panhandle region (TM Uyeki et al., 2024). As of January 5, 2025, over 915 cases of H5N1 infection have been confirmed in dairy cows and 40 human cases associated with dairy cows in the United States (Fig. 1 C) (U.S. CDC, 2024b; U.S. Department of Agriculture, 2025; U.S. CDC, 2025b). The B3.13 genotype appears to be associated with transmission in dairy cows and represents a complex reassortant virus derived from HPAI H5N1 strains from Eurasia and low pathogenic avian influenza (LPAI) strains from North America (TP Peacock et al., 2025). H5N1-infected cows exhibit an array of symptoms, such as decreased feed intake, reduced lactation, and production of abnormally thick yellow milk, in addition to increased mortality rates (LC Caserta et al., 2024; E Burrough et al., 2024; AL Baker et al., 2025). Human infections with bovine-origin H5N1 (B3.13) typically present with mild symptoms, including conjunctivitis and coughing (TM Uyeki et al., 2024; U.S. CDC, 2024c).

Concurrently, on January 6, 2025, the United States reported its first fatal case involving a person infected with H5N1 who progressed to severe disease, raising widespread concern (U.S. CDC, 2024a; U.S. CDC, 2025a). After analysis, this case was found to be infected with another H5N1 2.3.4.4b genotype, D1.1, which was previously reported to cause

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outbreaks in wild birds and poultry, as well as isolated human infection cases linked to exposure of a backyard flock (MG Ison et al., 2024). A case in Canada involving a D1.1-infected patient initially presented symptoms with bilateral conjunctivitis and fever, but the infection subsequently progressed to respiratory distress and multiple organ dysfunction (AN Jassem et al., 2024), thus indicating the high pathogenicity for humans.

Although no person-to-person transmission of H5N1 has been observed for the currently prevalent 2.3.4.4b genotypes, both B3.13 and D1.1 genotypes demonstrate a broad host range (Fig. 2 A). The molecular features of host adaptation include mutations in both hemagglutinin (HA), neuraminidase (NA) and viral RNP proteins (JK Taubenberger et al., 2010). Adaptive mutations in the receptor binding site (RBS) of influenza virus play a critical role in determining the potential for human-to-human transmission. Unlike avian adaptive influenza viruses, which bind preferentially to α-2,3-linked sialic acid (avian-like) receptors, HPAIVs infecting humans tend to utilize α-2,6-linked sialic acid receptor, which dominantly expressed in the upper respiratory tract (L Yao et al., 2008; HS Leung et al., 2012). Certain mammals, such as pigs (G Neumann et al., 2009), also express the α-2,6-linked sialic acid, which suggests that these mammals may serve as intermediate hosts, generating adaptive mutations in variant strains that could spill over to humans. Additionally, an ongoing arms race exists between the influenza virus and its host. Adaptive changes in the influenza virus, such as the E627K mutation in the polymerase PB2 protein, have been shown to amplify viral replication efficiency in mammalian cells. However, this adaptation remains dependent on the functionality of the host factor ANP32A (L Na et al., 2024). These mutations accumulate in the hosts, ultimately strengthening the virus's pathogenicity and transmissibility. Finally, addressing the emergence of resistance mutations against antiviral drugs, especially within the infected population, requires taking precautionary countermeasures during clinical treatment. Such efforts are essential (J Arino et al., 2009) to ensure the availability of effective medications, particularly in cases of severe illness. However, mutations associated with host adaptation and receptor-binding preferences remain to be elucidated, making it challenging to evaluate the

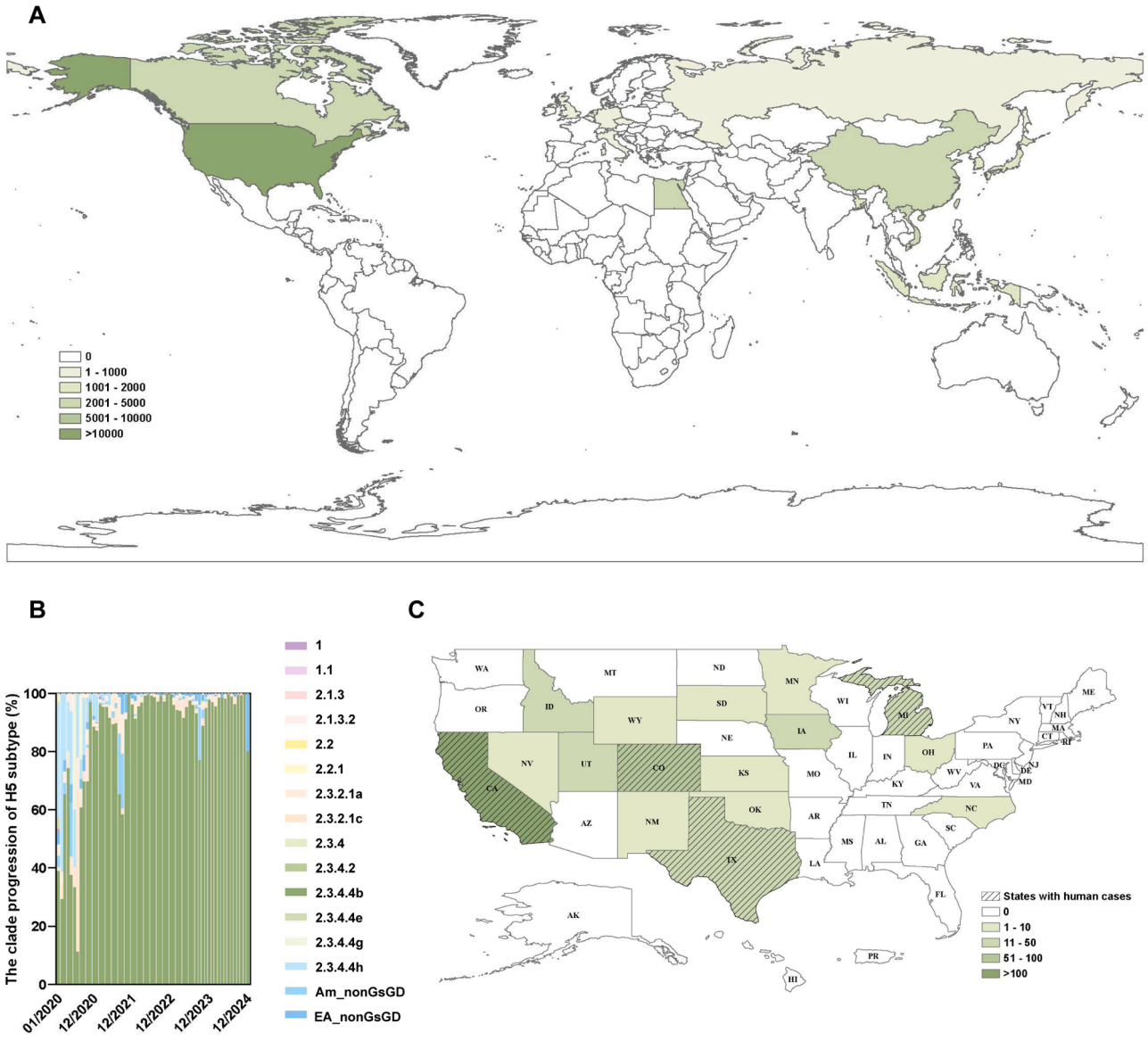


Fig. 1. Geographical distribution of H5N1. (A) Global geographical distribution of the top 15 countries with circulating H5 subtype influenza viruses between 2020 and 2024. Data were obtained from GISAID on December 25, 2024. (B) Clade progression of H5 subtype influenza viruses from January 2020 to December 2024. Data were obtained from GISAID on December 25, 2024. (C) Geographical distribution of H5N1 infection cases in dairy cows and human infection cases associated with dairy cows in the United States. Data were obtained from the USDA and U.S. CDC on January 5, 2025.

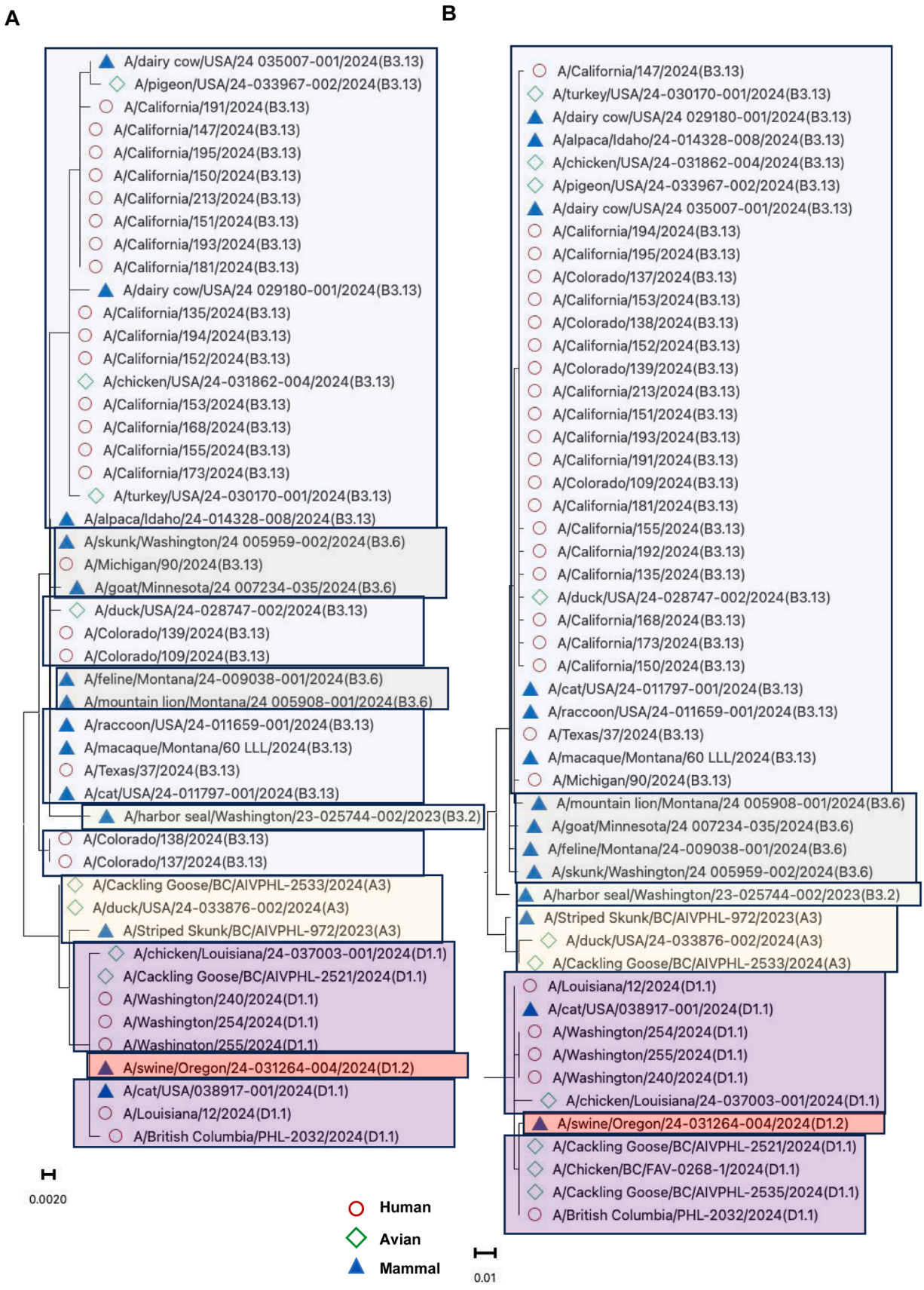


Fig. 2. Phylogenetic analysis of HA and NA amino acid sequences of H5N1 clade 2.3.4.4b in North America. (A) Maximum likelihood phylogenetic analysis of 48 hemagglutinin (HA) proteins and (B) 51 neuraminidase (NA) proteins. Identified sequences detected from avians and mammals (other than human) are labeled with green rhombus and triangle filled with blue separately. Red circles represent sequences detected in human samples.

pathogenicity and potential for human transmission of H5N1 circulating in North America.

In this study, we first conducted an evolutionary analysis of the HA and NA proteins in the North America 2.3.4.4b strain across various species. Additionally, we performed a comparative analysis of receptor binding sites in the B3.13 (2839 strains) and D1.1 (300 strains) genotypes that have been observed. Furthermore, we identified mammalian or avian adaptive mutations in other viral proteins, as well as mutations resistant to antiviral drugs recommended by the U.S. Centers for Disease Control and Prevention (CDC) and other clinically used antivirals. Through genomic signature analysis, we identified the genetic characteristics and human transmission potential of these two viral genotypes prevalent in North America and provided insights to guide the use of antiviral medications in clinical settings.

2. Materials and methods

2.1. Collection of H5N1 sequences

To conduct genomic signature analysis, protein sequences from isolates of influenza virus H5N1 clade 2.3.4.4b from various species in North America were obtained from the GISAID Influenza Database (S Elbe et al., 2017). Additionally, protein sequences of major H5 subtype clade 2.3.4.4b human influenza A viruses (H5N1, H5N6, and H5N8) detected globally between 2020 and 2024 were retrieved from the GISAID Influenza Database.

2.2. Sequence analysis

Protein sequence alignment was conducted using the MEGA 11 (K Tamura et al., 2021) program (version 11.0.13). Key amino acid residues within the proteins were analyzed utilizing ESPript 3.0 (X Robert et al., 2014), and the logo plot was depicted using WebLogo (GE Crooks et al., 2004). Mutation landscapes were examined and visualized using GraphPad Prism. Briefly, A/Aichi/2/1968 (H3N2) was used as the reference strain (H3 numbering and N2 numbering) in HA and NA. As for other proteins (PB2, PB1, PA, NP, M1, M2, NS1, and NS2), A/Goose/Guangdong/1/1996 (H5N1) was set as the reference strain (Xu et al., 1999; A Suttie et al., 2019). Mutations in other human or animal H5N1 clade 2.3.4.4b isolates were annotated by indicating the substituted amino acid residue following the mutation. We retrieved all the sequences of B3.13, D1.1 and H5NX clade 2.3.4.4b with human host from GISAID submitted during Jan. 2023 - Jan. 2025 for mutation frequencies analysis. The mutations frequency was then calculated using the BioPython (PJA Cock et al., 2009) to process the alignment file and count the mutation frequencies.

2.3. Phylogenetic analysis

Using the aligned HA and the NA amino acid sequences of H5N1 clade 2.3.4.4b strains from 2023 to 2024, we conducted a maximum likelihood phylogenetic analysis using the default settings and visualized the results with the MEGA 11 program (version 11.0.13).

2.4. Protein modeling analysis

All protein structures of compound-protein complexes were retrieved from the RCSB PDB (HM Berman et al., 2000) and visualized using PyMOL (version 2.5.0). The side chains of interface residues were highlighted in light pink and represented as both sticks and surface.

3. Results

3.1. Phylogenetic analysis of H5N1 clade 2.3.4.4b in North America

To trace the origin of viruses causing human infections and evaluate their potential for transmission and adaptation in human, we performed a phylogenetic analysis of sequences identified from humans, alongside sequences from mammal, including farm animals, such as dairy cows and wild mammals, such as macaques, and avian, such as farm poultry like chickens and wild birds like cackling geese, in North America. This analysis included a total of 48 HA sequences and 51 NA sequences from the GISAID database. Based on HA and NA segment sequences, our analysis revealed that genotypes, such as B3.13 and B3.6, clustered together, while genotypes D1.1 and D1.2 formed a distinct, separate cluster. This finding suggested that these two groups of viruses may have differing reassortant origins and host adaptation profiles.

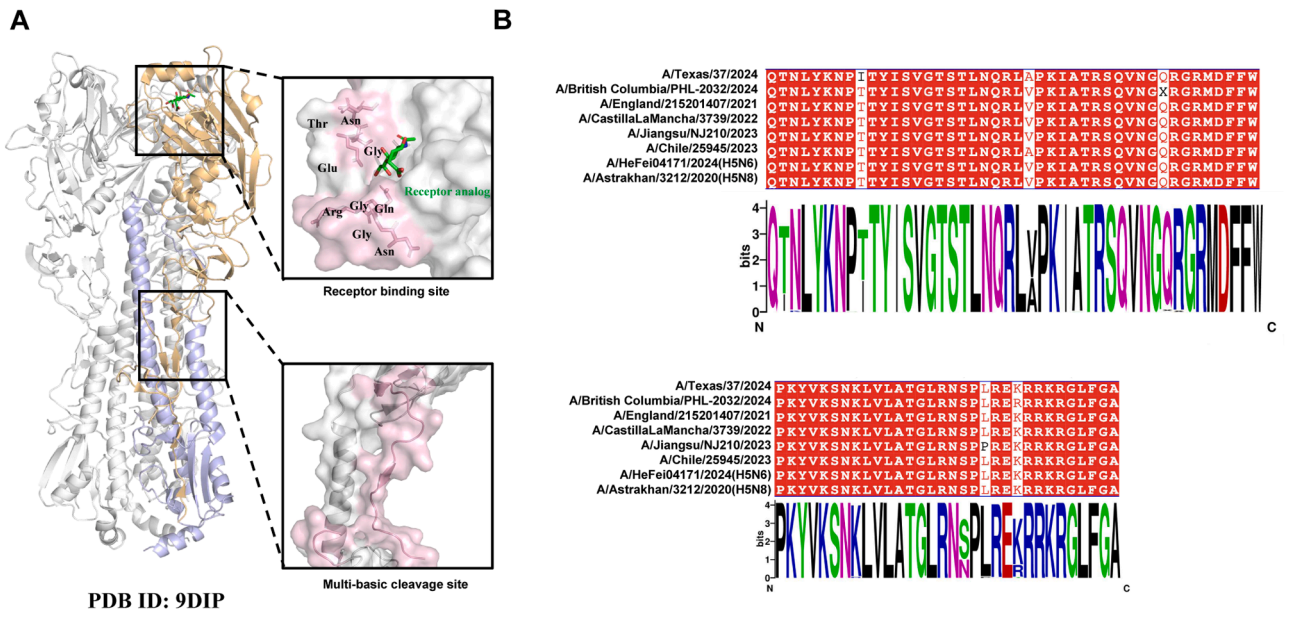
The B3.13 genotype cluster has been widely detected in human samples across the United States (Fig. 1 C), including the states of Texas, California and Colorado, and it has been identified in other mammalian species. Dairy cows serve as the primarily infected host for the B3.13 genotype strain with over 2839 viral sequences of bovine-origin B3.13 uploaded to the GISAID database. Notably, sequence comparisons of HA and NA revealed that strains from monkeys (A/Macaque/Montana/60_LLL/2024) and cats (A/Cat/USA/24–011797–001/2024) also exhibited 100% amino acid sequence similarity to the reference strain (A/Texas/37/2024), suggesting a close genetic relationship and potential cross-species transmission (Fig. 2 A and Fig. 2 B). Besides, only 490 sequences of avian origin were detected as of January 5, 2025, suggesting that the B3.13 genotype may gain adaptations for mammals. In contrast, the D1.1 genotype has been detected in human cases in British Columbia, Canada and Louisiana and Washington in the United States. It may be closely associated with influenza viruses transmitted among avian species in Canada (O Dyer, 2024). In the D1.1 genotype, 9 of 11 sequences from mammalian isolates were derived from felines, while the remaining 2 sequences were derived from ermine and dairy cows relatively. Compared to the relatively few viral sequences identified in mammals (excluding humans), more than 280 sequences of D1.1 have been recorded in avian species. This suggests that the D1.1 genotype may predominantly retain avian adaptability.

California is the state with the highest number of current B3.13 infections in human cases. Data show that 3 mutations, D96G, V135M and S323N, of this HA differed from the reference HA sequence in Texas. Similarly, unlike the severe cases reported in Canada, the cases in Washington exhibit mild symptoms, and 2 more mutations, S271P and K432R, were also found in their NA sequence. The regional differences observed between these two genotypic strains may be attributed to their transmission to humans via infections from different animal hosts. This could also serve as indirect evidence explaining why human-to-human transmission has not yet occurred.

3.2. The signature of receptor binding site and cleavage sites on HA

Using phylogenetic analysis, we have investigated the amino acid sequence similarity and evolutionary characteristics of HA and NA proteins among strains from various host sources. Notably, the key factors influencing host adaptability in influenza viruses are the HA protein and its recognition mechanisms with the host. As previously mentioned, host adaptability by the virus's HA is primarily driven by adaptive mutations targeting the host receptors, specifically the α-2,6-linked sialic acid receptor. The receptor binding site within the head domain forms a hydrophobic groove that facilitates binding to diverse glycan sialic acid receptors (Fig. 3 A).

We compiled and compared amino acid sequences of the RBS (positions 191–234, H3 numbering) from 65 strains of H5NX clade 2.3.4.4b viruses identified in humans between 2021 and 2024. The results are presented as a logo plot (Fig. 3 B) with representative strains selected for sequence comparison. This analysis aims to highlight mutations that may have emerged in clade 2.3.4.4b as adaptations to human-preferring receptors. Molecular characterization of both B3.13 and D1.1 genotypes identified receptor-adaptive signatures in HA, such as HA-137A and HA-160A, while low-frequency receptor-adaptive mutations, including HA-



C

	Mutation numbering	B3.13 (2,839)	D1.1(300)	H5NX 2.3.4.4b (Human, 65)
Receptor binding site (RBS)	HA-133	/	/	L133R (1.5%)
	HA-135	V135M (26.3%), V135A (0.14%), V135E (0.04%)	/	V135M (15.4%)
	HA-137	/	/	A137T (1.5%)
	HA-185	S185P (0.04%)	/	S185P (3.1%)
	HA-189	/	/	E189A (1.5%)
	HA-192	T192A (0.39%)	/	T192I (24.6%), T192R (1.5%)
	HA-193	N193T (0.04%)	/	N193K (3.1%)
	HA-199	/	T199N (0.66%), T199A (0.33%)	T199I (38.5%)
	HA-217	/	I217L (0.66%)	/
	HA-221	S221P (0.07%)	S221P (0.33%)	/
	HA-222	Q222R (0.04%), Q222L (0.04%)	/	/
	HA-226	/	/	Q226L (1.5%)
Cleavage site	HA-323	S323N (52.9%)	S323N (2.3%)	S323N (30.8%)
	HA-324	P324L (0.88%)	/	/
	HA-325	L325P (1.9%)	/	L325P (3.1%)
	HA-326	/	R326I (0.66%)	R326K (1.5%)
	HA-328	/	/	K328R (15.4%), K328T (3.1%)
	HA-330	R330K (0.11%)	R330I (0.66%)	/
	HA-331	K331R (0.32%)	/	/

Fig. 3. The signature of receptor binding site and cleavage sites on HA. (A) Structural modeling of HA trimers of A/Texas/37/2024 (PDB ID: 9DIP) that interacted with receptor analog LSTa. HA1 is shown in wheat, and HA2 is in light blue. Enlarged view highlights interactions between the receptor binding sites (RBS) of HA and LSTa and the lower panel showed the enlarged view of the multi-basic cleavage sites (MBCS). Side chains of amino acids were shown in light pink sticks. (B) Sequence alignment of the binding receptor site and multi-basic cleavage site (MBCS) in representative strains of human-infecting H5N1 clade 2.3.4.4b viruses. Logo plot of amino acid substitution of all 65 strains of human-infected viruses collected globally from 2020 to 2024 visually represents the variability observed within these regions. (C) Mutation frequencies of all detected B3.13, D1.1 and human-infected clade 2.3.4.4b H5N1 viruses on the RBS and MBCS.

101N, were detected in certain strains like A/Dairy cow/USA/24 029180–001/2024. This may explain the sporadic cases of human infections that have appeared across North America.

Notably, mutations in the RBS of H5NX clade 2.3.4.4b, such as HA-V135M, HA-S185P, HA-T192I/R/A, and HA-N193K/T (Fig. 3 C), have also been identified among the 2839 strains of B3.13 viral sequences uploaded. These mutations, located near the RBS, may influence receptor preference and potentially contribute to species-specific receptor binding (H Guo et al., 2017; E Świętoń et al., 2020). Among them, V135M, T192I/R/A, and T199I are the three mutation sites with the highest frequency with mutation rates of 15.4%, 26%, and 38.5%, respectively. At the same time, we also conducted a sequence analysis of all sequences under the same genotype for B3.13 and D1.1. Similar to the sequence of human-derived strain 2.3.4.4b, we found that B3.13 also exhibited high frequency mutations at the V135M/A/E (26.5%) and T192A (0.39%) sites, while the D1.1 strain showed similar mutations on the T199N/A site (1%).

Beyond host adaptability, the multi-basic cleavage site (MBCS) in the HA of HPAI H5 2.3.4.4b viruses is also a key determinant of viral pathogenicity (JM Luczo et al., 2015). For instance, the cleavage site motif of B3.13 genotype strain A/Texas/37/2024 is PL_REKRRKR↓GLF. In contrast, strains within the D1.1 genotype, such as A/British_Columbia/PHL-2032/2024, exhibit the motif PL_RERRRKR↓GLF. The HA sequence of human-derived H5NX 2.3.4.4b strains exhibits notable mutation frequencies at S323N (30.8%) and K328R/T (18.5%). However, greater variability was observed at HA cleavage site region S323N-L325P (0.88–52.9%) in the B3.13 genotype (Fig. 3 C). In contrast, the D1.1 genotype displays such mutations as S323N (2.3%), R326I (0.66%), and R330I (0.66%) in this region. Overall, our analysis reveals that the mutations on the HA of the B3.13 genotype shared a higher similarity to human-derived H5NX 2.3.4.4b sequences in both RBS and cleavage site region, potentially affecting its host selectivity and pathogenicity.

3.3. Species replication-associated amino acid signatures

Although HA and NA proteins have a significant impact on host adaptability and pathogenicity of the influenza virus, other influenza virus proteins also contain important amino acid sites that influence host adaptability. Sequence alignment of 7 proteins (PB2, PB1, PA, NP, M1, M2, NS1 and NS2) from the 2.3.4.4b H5N1 strains was conducted using A/Goose/Guangdong/1/1996 (H5N1) as a reference sequence for numbering (X Xu et al., 1999; A Suttie et al., 2019), and we mapped the mutations across different animal hosts, as shown in Fig. 4. To investigate the adaptation of the emerging H5N1 2.3.4.4b to human hosts in North America, we analyzed 47 reported molecular signatures (W Xu et al., 2017) of the human-like signatures (HLS) (G-W Chen et al., 2009) and found that only a low-frequency mutation at position PB2-E627K was specifically identified in the A/Texas/37/2024 strain (Fig. 4 A). This substitution was associated with increasing polymerase activity in humans (JS Long et al., 2016). A severe case in a teenager also showed this E627K mutation (55% frequency) in the D1.1 genotype by deep sequencing (AN Jassem et al., 2024). This suggests that the adaptability of the 2.3.4.4b virus to humans remains relatively weak in both genotypes. However, the emergence of the PB2-E627K mutation does highlight the need for careful monitoring of adaptive mutations.

By analyzing the genome of the B3.13 genotype (Fig. 4 A and Fig. 4 C), we found 2 more unique mutations associated with adaptation in mammalian cells, including PB2-M631L, and PA-K497R. PB2-M631L (Fig. 4 A) showed dominant regulation on viral polymerase activity in mammalian cells (X Zhang et al., 2017). PA-K497R (Fig. 4 C) is associated with increased polymerase activity in mammalian cell lines (S Yamayoshi et al., 2018). The PA-142E mutation identified in B3.13 A/Texas/37/2024 has been shown to increase virulence in mice, resulting in its classification as mammalian-adapted mutations, as well (Fig. 4 C) (JH Kim et al., 2010). Meanwhile, PB1–207R and PA-400P mutations in the D1.1 genotype have been identified as avian-adapted mutations owing to their role in reducing polymerase activity in mammalian cells or virulence in mice (DJ Hulse-Post et al., 2007; BL DesRochers et al., 2016). Previous research also suggested that M1-S85N (D Elton et al., 2013) may be related to avian-like adaptation of influenza virus, and this mutation was also found in H5N1 genotype D1.1. Our analysis also revealed that shared mutations between B3.13 and D1.1, including M1–30D, M1–43M, and M1–215A mutations of matrix protein 1 (M1) in H5N1, can lead to increased virulence in mammals (Fig. 4 C) (A Suttie et al., 2019), which may explain the emergence of the D1.1 genotype in cases feline and human infection. In summary, B3.13 contains unique mutations that reflect adaptations to mammals, which are absent in D1.1, whereas D1.1 exhibits distinct mutations associated with avian adaptation. These findings highlight the differences in host adaptability between the two genotypes of the influenza virus.

3.4. Mutations and resistance to clinically used antivirals

Despite differences in host adaptability between B3.13 and D1.1, both genotypes of H5N1 2.3.4.4b currently circulating across North America have demonstrated the ability to infect humans. Notably, both genotypes have been associated with similar conjunctivitis symptoms following human infection. To control the spread of the H5N1 clade 2.3.4.4b outbreak in the United States, the CDC currently recommends four antiviral drugs that remain sensitive to the H5N1 B3.13 genotype, containing three NA inhibitors (NAIs) and one PA endonuclease inhibitor, Baloxavir (U.S. CDC, 2024d). Previous studies have shown that spatial conformation of the hydrophobic pocket in the NA active site is determined by interactions among its constituent amino acids. For example, the H274Y (Fig. 5 A) mutation affects the spatial orientation of E276, bringing the residues closer to the binding site and making the hydrophobic pocket smaller, resulting in decreased NA binding affinity (PJ Collins et al., 2008).

Similar to the sequence analysis of HA, we also conducted signature analysis on the targets of the noted drugs (Fig. 5 A and Fig. 5 B). In B3.13, we identified a unique mutation, NA-N70S, which was detected in human, cows, and poultry, and a 46-fold increase in the IC50 of zanamivir was observed in H1N1 with this mutation (JL McKimm-Breschkin, 2013). Additionally, a shared mutation, NA-S247N (0.04% in the B3.13 genotype and 1.67% in the D1.1 genotype), in the NA active site (Fig. 5 A and Fig. 5 C), which was identified in both B3.13 and D1.1 genotypes, could confer a moderate reduction in oseltamivir affinity (J Pokorná et al., 2018). Other CDC recommended antivirals, such as the nucleic acid endonuclease inhibitor Baloxavir marboxil (BXM), inhibit viral RNA synthesis and block viral replication. The shared PA-I38M mutation in both B3.13 (0.04%) and the human-infected H5NX 2.3.4.4b (1.5%) could reduce the activity of Baloxavir acid (BXA) (SV Svyatchenko et al., 2021) by 4- to 10-fold, while the PA-F105S (0.07%) mutation in B3.13 only exhibited resistance to another PA inhibitor, L-742,001 (M-S Song et al., 2016).

Other clinically used drugs, such as Amantadine, inhibit influenza virus infection as a proton channel inhibitor by displacing water molecules in the channel close to the inner side of the virus via hydrophobic groups. Mutations on M2-V27I/A (0.56%) (R Musharrafieh et al., 2018) and M2-A30S (0.07%) (J He et al., 2024) observed in B3.13 (Fig. 5 C) could confer drug resistance to an M2 inhibitor like Amantadine. Inhibitors on other targets, such as the drug Arbidol, which targets HA, bind to HA2 and inhibit membrane fusion between virus and receptor (Fig. 5 B). Whereas in A/Texas/37/2024, similar to H1N1, an extra segment of alpha-helix on HA-D57-N60 may cause spatial blockage, hindering Arbidol from entering the binding site (RU Kadam et al., 2017). Overall, the B3.13 genotype displayed various mutations, both inside and outside the active sites of antiviral targets, which could potentially result in unpredictable effects on drug resistance. This reinforces the need for strategies to prevent the spread of resistance mutations.

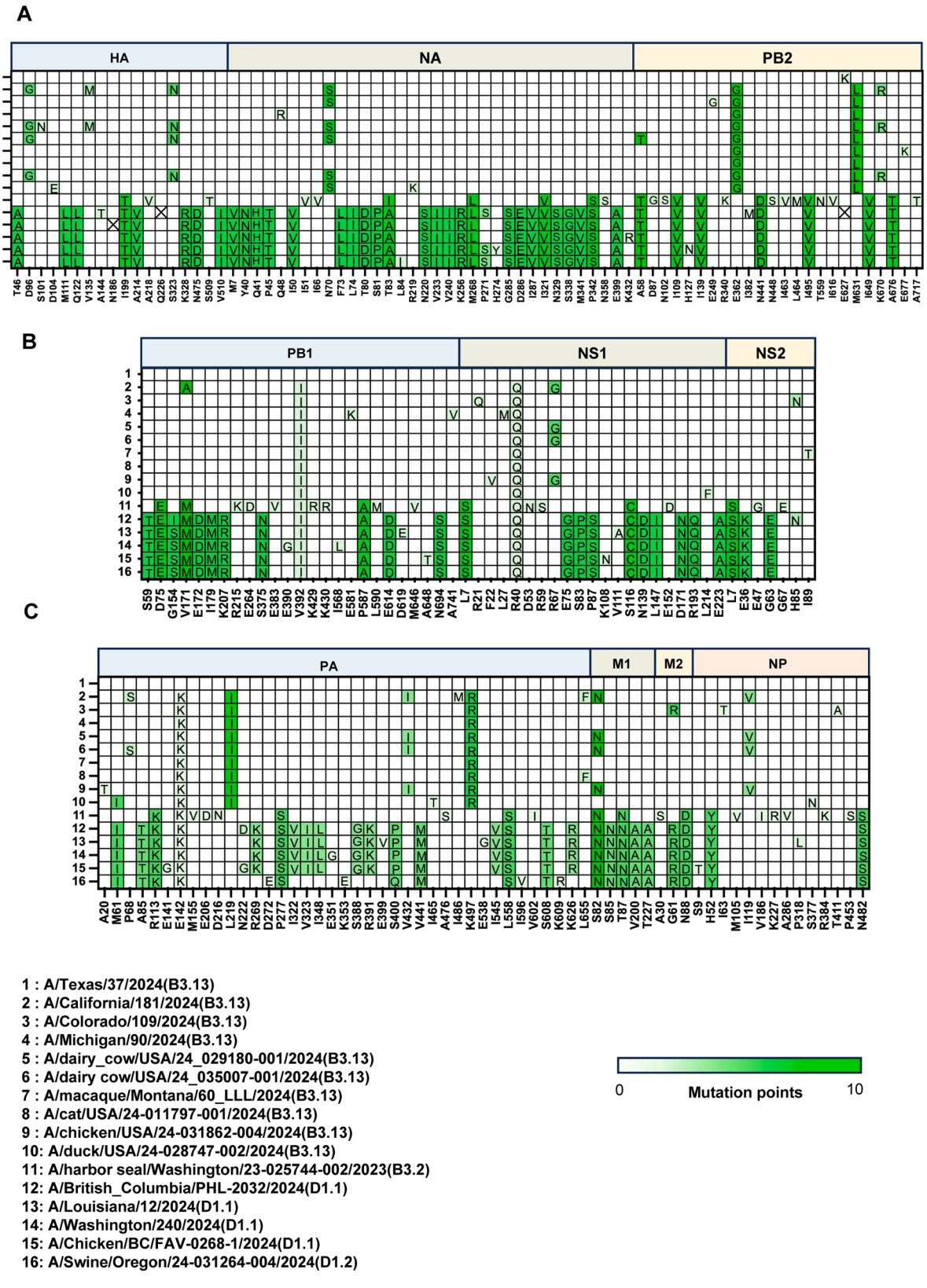
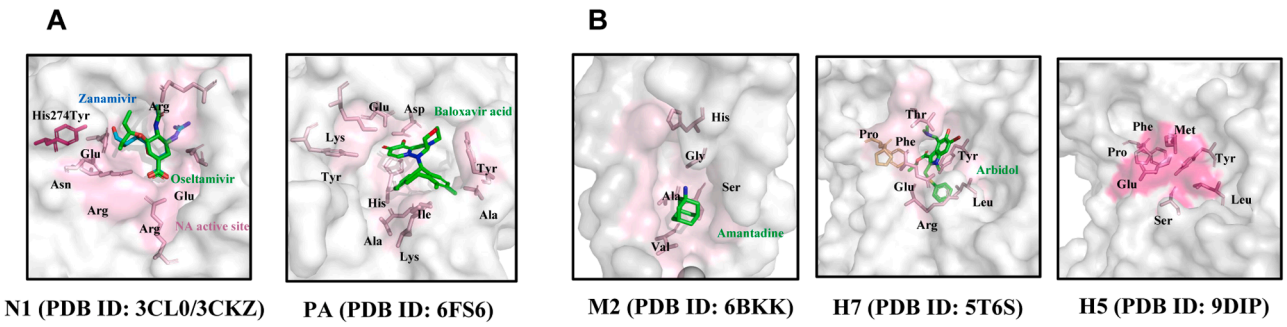


Fig. 4. Comparative genomic heatmap in the genome of the circulating H5N1 in North America. (A) HA, NA and PB2 amino acid substitutions of H5N1 viruses isolated from human, avian and other mammalian cases in North America. Heatmap depicts the frequency of each amino acid substitution, with green gradient indicating prevalence of the mutation across analyzed sequences. Specific mutations on each site (with H3/N2 numbering for HA and NA based on A/Aichi/2/1968 (H3N2) and PB2 numbering according to alignments with A/Goose/Guangdong/1/1996 (H5N1)) are labeled directly on the heatmap grid. (B) and (C) heatmaps demonstrated the substitution of PB1, NS1, NS2, PA, M1, M2, and NP across the analyzed H5N1 clade 2.3.4.4b sequences.



N1 (PDB ID: 3CL0/3CKZ) PA (PDB ID: 6FS6) M2 (PDB ID: 6BKK) H7 (PDB ID: 5T6S) H5 (PDB ID: 9DIP)

C

Protein	Mutation	B3.13(2,839)	D1.1(300)	H5NX 2.3.4.4b (Human, 65)
Neuraminidase (NA) active site	NA-70	N70S (80.5%)	/	N70S (33.8%)
	NA-110	S110Y (0.11%)	/	S110E (33.8%)
	NA-136	/	/	Q136H (3.1%)
	NA-197		P197S (0.33%)	
	NA-198	/	/	D198N (32.3%)
	NA-222	I222S (0.04%), I222T (0.04%)	/	/
	NA-247	S247N (0.04%)	S247N (1.67%)	/
	NA-436	I436S (0.07%)	/	I436L (33.8%)
Polymerase acidic (PA)	PA-24	Y24H (0.14%)	/	/
	PA-38	I38V (0.11%), I38M (0.04%)	/	I38M (1.5%)
	PA-105	F105S (0.07%)	/	/
	PA-122	V122I (0.04%)	/	/
	PA-137	K137R (0.07%)	/	/
Matrix 2 (M2)	M2-13	N13S (3.87%)	N13T (2%), N13S (0.33%)	N13S (16.9%)
	M2-16	E16G (0.39%)	E16G (0.33%), E16D (1%)	E16G (1.5%))
	M2-27	V27I (0.53%), V27A (0.04%)	V27A (0.66%)	V27T (1.5%)
	M2-28	I28T (2.9%), I28N (0.04%)	I28V (1%)	/
	M2-30	A30S (0.07%)	/	/
	M2-31	/	S31N (5%)	S31N (3.1%)

Fig. 5. Catalytic pockets and mutations of resistance to antivirals against AIVs. (A) Catalytic site of N1 neuraminidase in complex (PDB ID: 3CL0, 3CKZ) with oseltamivir and zanamivir. Baloxavir acid binding to PB2 protein (PDB ID: 6FS6). All interacting amino acid side chains are highlighted in light pink. (B) Structural basis of M2 proton channel – Amantadine complexes (PDB ID: 6BKK) binding pattern and comparison between the H7N9 HA2-Arbidol binding sites (PDB ID: 5T6S) and H5N1 HA2-Arbidol binding sites (PDB ID:9DIP). (C) Antiviral resistance mutation frequencies of all detected B3.13, D1.1 and human-infected clade 2.3.4.4b H5N1 viruses targeting Neuraminidase (NA), Polymerase acidic (PA), and Matrix 2 (M2).

4. Discussion

Multiple outbreaks of human infections caused by H5-subtype influenza viruses (G Graziosi et al., 2024), such as H5N2 (V Apostolopoulos et al., 2024), H5N6 (P Chen et al., 2019), H5N8 (Y Moatasim et al., 2024), and the recently detected H5N1, have been widely reported in North America (Fig. 1 A), presenting a significant threat to human health. Fortunately, no confirmed cases of human-to-human transmission of H5N1clade 2.3.4.4b have been reported in North America. By analyzing the genomic signatures of emerging strains, we can reasonably predict the cross-species transmission potential of influenza viruses and their adaptation to hosts. In this study, we conducted a phylogenetic analysis of two major emerging genotypes of H5N1 clade 2.3.4.4b involved in the current outbreak: B3.13 and D1.1. While the B3.13 genotype gains mammal adaptation, the D1.1 genotype is likely linked to poultry as a transmission source (O Dyer, 2024). Our research compared host adaptability and pathogenicity of B3.13 and D1.1 and provided guidance for the use of clinical drugs.

Through phylogenetic analysis, we identified potential intermediate host sources for the B3.13 and D1.1 strains infecting humans. Notably, the HA and NA sequences of A/Texas/37/2024 share a high degree of similarity with strain A/Cat/USA/24–011797–001/2024 isolated from

cats. Additionally, over 40 cat-origin viral sequences (94 feline-origin viral sequences) of the B3.13 genotype from the North America region have been uploaded to GISAID. As companion animals, cats are more likely to have direct contact with humans compared to dairy farm animals (E Burrough et al., 2024). These findings underscore the need to strengthen public health surveillance of influenza viruses in animals, in particular those aimed at preventing spillover from cats to humans. Furthermore, cat-derived sequences dominate the D1.1 genotype among mammalian isolates, raising concerns about the potential for recombination between the B3.13 and D1.1 genotypes and intermediate hosts like house cats. Notably, a strain isolated from a human case in Washington (A/Washington/240/2024) exhibited extremely high amino acid sequence similarity to a D1.2 genotype strain isolated from pigs in Oregon (A/swine/Oregon/24–031264–004/2024) with 100%, 99.3%, and 100% identity in HA, NA, and PB2 proteins, respectively (Fig. 2 and Fig. 4 A). This finding suggests a potential zoonotic link between swine and humans since previous studies have demonstrated that swine can serve as important intermediate hosts for influenza viruses, particularly those with adaptive mutations in the α-2,6-sialic acid receptor, which increase the virus's ability to infect human via upper respiratory tract (G Neumann et al., 2009). Given the potential for zoonotic spillover and the severe outcomes in humans, we suggest bolstering the surveillance of D1.1 genotype H5N1 viruses in both humans and animals, particularly in farmed species, such as swine, or companion animals, such as house cats, which may facilitate cross-species transmission and speed its evolutionary trajectory.

Additionally, as anticipated, receptor-adaptive mutations, such as HA-137A and HA-160A, have emerged in the HA of the B3.13 genotype, potentially boosting its ability to recognize human receptors. However, compared to the RBS region of A/Indonesia/5/2005, recent studies suggest that the HA of A/Texas/37/2024 features a broader binding groove (H Song et al., 2025). This structural characteristic enables only weak binding to human-preferring receptors, while retaining a strong preference for avian-preferring receptor binding. Recent studies have also highlighted critical mutations associated with changes in receptor-binding specificity. For example, the Q226L (T-H Lin et al., 2024) mutation was artificially introduced in the HA of the B3.13 genotype A/Texas/37/2024 strain increasing the virus's ability to bind efficiently to the α-2,6-linked sialic acid receptor, thereby supporting its adaptation to infect human hosts. Currently, no experimental research has verified whether the HA of D1.1 still relies on recognizing α-2, 3-linked sialic acid as its receptor. Our research observed that, with respect to host distribution, D1.1 isolates remain predominantly avian-derived (283 strains), with only 11 strains detected in mammals. Clinically, human infections with D1.1 present with symptoms of conjunctivitis as well. Unlike the upper respiratory tract, the conjunctiva is characterized with α-2,3-linked sialic acid. This tissue-specific difference in receptor expression suggests that D1.1 may continue to utilize α-2,3-linked sialic acid as its receptor during infection.

An unresolved question remains. Since B3.13 exhibits mammalian adaptive mutations, but still tends to utilize avian receptors, we asked why the D1.1 genotype virus (Fig. S1), which has not been observed to possess sufficient mammalian adaptive mutations, could lead to severe cases and fatalities in the recent two human cases. We explain the two outliers noted above as follows. The fatal patient in Louisiana was over 65 years old and had underlying health conditions, lowering tolerance to HPAIVs infection. For the Canadian adolescent, physicians used deep sequencing to identify low-frequency mixed nucleotides in PB2-E627K (52% allele frequency), HA-E190D (28% allele frequency) and HA-Q226H (35% allele frequency), all of which were identified as human adaptive mutations (AN Jassem et al., 2024), thus promoting virulence and pathogenicity in this case. In addition, upon admission, the cycle threshold value for the affected adolescent was 27.1, indicative of a high viral load. The C-reactive protein (CRP) level was significantly elevated at 199 mg/L, suggesting a severe inflammatory response. However, no specific cytokine profile was identified in this case. By 20 days post-admission, a throat swab from the upper respiratory tract tested negative, while the tracheal aspirate remained positive with a Ct value of 39.9. The human lower respiratory tract harbors α-2,3-linked sialic acid receptors as well, which may predispose individuals to severe symptoms and pneumonia.

Effective vaccination remains a critical strategy for controlling influenza outbreaks within human populations. Currently, the recommended vaccine components for the seasonal influenza strains in the Northern Hemisphere include A/Victoria/4897/2022 (H1N1) pdm09, A/Thailand/8/2022 (H3N2), and B/Austria/1,359,417/2021 (B/Victoria lineage). H1N1 and H5N1 are both classified as Group 1 influenza viruses, and the neutralizing antibodies induced by the vaccine are most likely to cross-neutralize H5N1. However, due to the relatively low sequence similarity between these strains, traditional seasonal influenza vaccines typically elicit broadly neutralizing antibodies only after multiple rounds of repeated exposures (A Roos et al., 2015). Surender et al. conducted serological experiments using sera from individuals vaccinated with stockpiled, U.S.-licensed vaccines targeting early H5N1 strains. Their findings indicate that vaccination with early H5N1 viruses (A/Vietnam, clade 1 and A/Indonesia clade 2.1) can induce cross-reactivity against the H5N1 2.3.4.4b clade. Moreover, the use of MF59 or AS03 adjuvants significantly increased seroconversion rates, demonstrating that stockpiled U.S.-licensed adjuvanted H5N1 vaccines may function as effective bridging vaccines (S Khurana et al., 2024). Building on our experience in combating the highly variable SARS-CoV-2, we have demonstrated that boosted immunization with adjuvanted antigen, such as CF501/SARS-CoV-2 RBD-Fc (Z Liu et al., 2023), can effectivelinduce broadly neutralizing antibodies and robust T cell responses against sarbecoviruses, thereby enhancing the breadth of neutralization (Z Liu et al., 2024). These findings underscore the essential role of adjuvants as a universal, broad-spectrum strategy in the development of vaccines against viruses with pandemic potential.

Although emerging genotypes of H5N1 clade 2.3.4.4b in North America have only caused sporadic human infection cases so far, only a failure of imagination could lead to a denial of the possibility that the virus, or its recombinant strains, could cause outbreaks in wide swathes of human populations. Therefore, in order to prepare a proper response to a potential pandemic, the CDC has already recommended neuraminidase inhibitor (NAI)-based antivirals (L Gubareva et al., 2022) and Baloxavir, which targets the PA protein, for the current epidemic of H5N1 2.3.4.4b infection. However, the emergence of mutations, such as NA-S247N, has raised concerns regarding drug resistance. To account for this problem, the synergistic use of drugs targeting different viral proteins has proven to be an effective strategy for inhibiting resistance development (T Bobrowski et al., 2021). Accordingly, other antivirals, such as Amantadine, which inhibits the M2 proton channel, was identified as a strong synergistic option to complement NAIs. In the severe case of D1.1 influenza A (H5N1) infection in a Canadian adolescent, physicians employed a combination antiviral therapy consisting of oseltamivir (NAI), Amantadine, and Baloxavir (AN Jassem et al., 2024). This approach aimed to improve antiviral efficacy, while minimizing the risk of resistance. Notably, our study indicated the rising rate of resistance mutations in the B3.13 strain, calling for the use of combination antiviral therapy in managing severe influenza infections and mitigating the emergence of drug resistance. By targeting multiple viral mechanisms, such strategies can improve treatment outcomes and preserve the efficacy of existing antiviral drugs. While this investigation provides insights into the molecular signatures of circulating H5N1 clade 2.3.4.4b, several limitations should be acknowledged. This research is grounded in publicly available databases and previously published references. Also, the viral isolates analyzed in this study were obtained from GISAID and may exhibit certain biases. For instance, farm animals such as cattle and chickens are the most common sources of viral isolates in B3.13 and D1.1, respectively. This prevalence could be attributed to the relative ease of sample collection from farm animals compared to wild animals. Despite these limitations, this study offers valuable

surveillance guidance for the prevention of H5N1 pandemics in populations, while also advocating for the synergy of multi-target antivirals strategy.

In summary, our findings offer insights into why D1.1 has not triggered a human pandemic and provide a molecular basis for understanding its limited cross-species transmission. Moreover, our study is the first to report the frequency of antiviral resistance mutations in both B3.13 and D1.1, suggesting that these mutations could facilitate the emergence of reassorted avian influenza virus strains in intermediate hosts, such as domestic cats and pigs, thus calling for global surveillance and implementing multi-target antiviral strategies to mitigate the risk of future pandemics.

CRediT authorship contribution statement

S.J., L.L., and Q.W. conceived the idea and supervised the project; G. Z., Y.S., H.G., and Y.W. performed the experiments; G.Z., and Y.S. drafted the manuscript; S.J., L.L., and Q.W. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors claimed no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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