

1 **Highly Pathogenic Avian Influenza A (H5N1) clade 2.3.4.4b Virus detected in dairy cattle**

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15 **Abstract**

16 The global emergence of highly pathogenic avian influenza (HPAI) A (H5N1) clade 2.3.4.4b
17 viruses poses a significant global public health threat. Until March 2024, no outbreaks of this
18 virus clade had occurred in domestic cattle. We genetically characterize HPAI viruses from dairy
19 cattle showing an abrupt drop in milk production. They share nearly identical genome sequences,
20 forming a new genotype B3.13 within the 2.3.4.4b clade. B3.13 viruses underwent two
21 reassortment events since 2023 and exhibit critical mutations in HA, M1, and NS genes but lack
22 critical mutations in PB2 and PB1 genes, which enhance virulence or adaptation to mammals.
23 The PB2 E627K mutation in a human case underscores the potential for rapid evolution post-
24 infection, highlighting the need for continued surveillance to monitor public health threats.

25 **Introduction**

26 Highly pathogenic avian influenza (HPAI) A (H5N1) virus belonging to clade 2.3.4.4b
27 represents a significant global concern due to its severe impact on poultry populations, wildlife,
28 and potential risks to human health. The origin of clade 2.3.4.4b can be traced back to 2020
29 when the H5N1 virus first emerged in domestic poultry in countries in East and Southeast Asia
30 (<https://wahis.woah.org#/home>). Initial outbreaks were primarily confined to avian species,
31 causing significant mortality among infected birds and posing substantial economic losses to the
32 poultry industry (1-3). However, clade 2.3.4.4b exhibited a remarkable capability for geographic
33 spread and host adaptation, leading to its dissemination across multiple continents through
34 migratory bird pathways and global trade networks since 2020 (3-8). In late 2021, clade 2.3.4.4b
35 H5N1 virus was introduced to North America from Eurasia and disseminated throughout the
36 continent via wild birds, subsequently infecting numerous wild terrestrial mammals, such as
37 foxes, skunks, bears, bobcats, and raccoons, posing a significant concern to public health (5, 9,
38 10).

39 Migratory birds play a significant role in transmitting HPAI viruses due to their ability to carry
40 the virus over long distances (11-14). Texas lies within the Central Flyway, a major migratory
41 flyway stretching from Canada to Mexico in North America
42 (<https://tpwd.texas.gov/huntwild/wild/birding/migration/flyways/>). Additionally, Texas
43 experiences some overlap in bird migration with neighboring states that belong to the Mississippi
44 Flyway. This convergence of flyways heightens the risk of HPAI viral transmission, as migratory
45 birds traverse diverse landscapes and habitats, including dairy cattle operations. In February and
46 March 2024, a syndrome occurred in dairy cattle in the Texas panhandle region where affected
47 animals developed a nonspecific illness and abrupt drop in milk production. Similar clinical

48 cases were subsequently reported in dairy cattle in southwestern Kansas and northeastern New
49 Mexico and mortalities in wild birds and domestic cats were observed within and around the
50 affected sites in the Texas Panhandle. Here we present our findings on the detection, genomic
51 characterization, phylogenetic analysis, and mutation adaptations of HPAI viruses, clade 2.3.4.4b
52 H5N1, identified in dairy cattle, domestic cats, and wild birds in Texas. As this manuscript is
53 being prepared for submission, the USDA has also confirmed the detection of this HPAI virus
54 strain in dairy herds in Idaho, Michigan, Ohio, North Carolina, and South Dakota. Furthermore,
55 the first human case of this virus in Texas, after contact with infected dairy cattle, has also been
56 reported ([https://www.dshs.texas.gov/news-alerts/dshs-reports-first-human-case-avian-influenza-](https://www.dshs.texas.gov/news-alerts/dshs-reports-first-human-case-avian-influenza-texas)
57 [texas](https://www.dshs.texas.gov/news-alerts/dshs-reports-first-human-case-avian-influenza-texas)).

58

59 **Results**

60 **Detection of clade 2.3.4.4b HPAI viruses in domestic dairy cattle and cats in Texas in** 61 **March, 2024**

62 In February 2024, veterinarians in the Texas panhandle region observed lactating dairy cattle
63 showing reduced feed intake, decreased milk production, and thickened yellow milk resembling
64 colostrum. The syndrome peaked 4-6 days after onset and subsided within 10-14 days, mainly
65 affecting older cows in mid to late lactation. By early March 2024, similar cases were reported in
66 southwestern Kansas and northeastern New Mexico, with mortalities observed in wild birds and
67 domestic cats near the affected areas. On March 21, 2024 milk samples from dairy cattle and
68 fresh tissues from cats in Texas were received at the Iowa State University Veterinary Diagnostic
69 Laboratory (ISU VDL). RT-PCR testing yielded positive results for influenza A virus (IAV) H5
70 clade 2.3.4.4b in the milk samples from the affected dairy cows along with brain and lung tissue

71 from two domestic cats that reportedly consumed raw colostrum and milk at a dairy in Texas.
72 The presence of highly pathogenic avian influenza (HPAI) H5N1 clade 2.3.4.4b was confirmed
73 by National Veterinary Service Laboratories (NVSL) in Ames, IA, USA.
74 The two milk samples from two cows and brain and lung samples from two cats, which tested
75 positive for IAV, underwent next-generation sequencing (NGS) and full genome sequences were
76 successfully obtained on March 23, 2024 for subtyping and other further analyses. NGS analyses
77 confirmed that all four individual samples were positive for HPAI A (H5N1). These sequences
78 have been deposited in GenBank with the Bioproject number PRJNA1092030 (Supplementary
79 Table 1). Several days later, the NVSL determined six HPAIV genome sequences from six wild
80 birds, one sequence from a skunk, one from a human case, and an additional four from dairy
81 cattle in Texas. These sequences are available in the GISAID database (<https://gisaid.org>) and
82 were included in this study for analysis (Supplementary Table 2).

83 **Phylogenetic and reassortment analysis**

84 To track the genetically most closely related strains, we conducted a comprehensive search
85 within the Global Initiative on Sharing Avian Influenza Data (GISAID) database. Supplementary
86 Tables 3-10 present the results, indicating that the viruses isolated from wild birds, cows, cats,
87 and humans in Texas during March 2024 shared a common ancestor with nearly 100 percent
88 homology. To elucidate the phylogeny of HPAI A (H5N1) viruses in our study, we conducted
89 individual phylogenetic analyses for each genome segment including a subset of HPAI A
90 (H5N1) reference sequences obtained from avian and mammalian sources in America submitted
91 to GISAID since January 1, 2021. Our analysis revealed that the genomes of viruses from two
92 cows and two cats closely aligned and formed a cluster within the HPAI A (H5N1) subclade
93 2.3.4.4b shown in Figure 1 (for HA gene) and Supplementary Figures S1-S8 (for HA, NA, PB2,

94 PB1, PA, NP, M, and NS genes). Specifically, we evaluated the time to the most recent common
95 ancestor (tMRCA) of H5N1 viruses in Texas in 2024 by constructing the maximum clade
96 credibility (MCC) tree of the HA gene using BEAST v1.10.4 (Figure 1). Referring to the lineage
97 classification of clade 2.3.4.4b H5N1 viruses in the United States by GenoFlu
98 (<https://github.com/USDA-VS/GenoFlu>) (3), the HA genes of the clade 2.3.4.4b H5N1 in
99 America since 2021 were divided into three lineages: ea1, ea2, and ea3. The HA genes of our
100 four HPAI H5N1 viruses, along with others from dairy cattle, wild birds, a skunk, and a human
101 during this outbreak period in 2024, were grouped under lineage ea1. In addition, the NA genes
102 were clustered within ea1, PB2 in am2.2, PB1 in am4, PA in ea1, NP in am8, M in ea1, and NS
103 in am1.1 lineages (Figures S1-S8).

104 The automated data pipeline available at <https://github.com/USDA-VS/GenoFlu> was further
105 applied to define their genotype (3). As illustrated in Figure 2, our four HPAI H5N1 viruses,
106 along with others from dairy cattle, wild birds, a skunk, and a human during this outbreak period
107 in 2024 belonged to genotype B3.13, resulting from a reassortment event involving genotype
108 B3.7 and a low pathogenic avian influenza (LPAI) virus. The B3.7 genotype, which emerged in
109 2023, contributed seven gene segments, including PB2, PB1, PA, HA, NA, M, and NS, while the
110 NP gene of B3.13 was originating from an LPAI virus resembling A/mallard/Alberta/567/2021
111 (11N9)-like strains. According to our GenoFlu analysis, the B3.7 genotype represents a 4+4
112 reassortant strain, with the HA, NA, PA, and MP genes originating from the H5N1 virus strain
113 A1 in 2020, while the remaining segments (PB2, PB1, NP, and NS) are closely related to LPAI
114 viruses. Our findings provide compelling evidence that the HPAI H5N1 viruses during this
115 outbreak period in 2024 underwent reassortment events involving both HPAI and LPAI viruses.

116 **Critical amino acid mutation analysis**

117 We conducted a comprehensive analysis of amino acid mutations, closely scrutinizing them to
118 identify any changes potentially associated with increased affinity to human-type receptor
119 heightened virulence, transmission or adaptation to mammalian hosts, and the mutants for
120 antiviral resistance. We focused on comparing critical sites among eight HPAI virus isolates
121 originating from dairy cattle and two from cats with those from terrestrial and marine mammals
122 in the public source. This included an extensive dataset comprising 173 strains from Canidae, 39
123 strains from Felidae, 53 isolates from Mustelidae, six strains from Ursidae, three strains from
124 other species of Bovidae, two strains from Procyonidae, as well as 68 marine mammal isolates,
125 comprising 38 strains from Phocidae, 16 strains from Otariidae, and 14 strains from Delphinidae
126 (Table 1). All 8 HPAI H5N1 isolates derived from dairy cattle and two cats demonstrated the
127 presence of residues 137A, 158N, and 160A within their HA segments, which may increase
128 binding affinity to the human-type receptor, while none contained residues 192I, 225D, or 228S
129 (15, 16). This consistent pattern mirrors that observed in the majority of HPAI isolates from both
130 terrestrial and marine mammals. Furthermore, all eight HPAI H5N1 isolates originating from
131 dairy cattle and two cats exhibited residues 30D, 43M, and 215A in M1 (17-19), as well as 42S,
132 103F, and 106M in NS1 (20). Once again, this pattern is aligned with the prevalent composition
133 observed across HPAI isolates from terrestrial and marine mammals, these mutants may increase
134 the viral virulence in mammals. It is noteworthy that mutations 591K, 627K/V/A, or 701N in
135 PB2, previously associated with mammalian host adaptation and enhanced transmission (18, 21,
136 22), were absent in all eight HPAI H5N1 isolates originating from dairy cattle and two cats,
137 while the HPAI virus from the human case exhibited E627K mutation in PB2. Conversely, these
138 mutations displayed a high frequency of occurrence in strains from Felidae and a lower
139 frequency in strains from Canidae, Mustelidae, Phocidae, Otariidae, and Delphinidae.

140 Additionally, no critical site mutations associated with increased influenza antiviral resistance
141 have been identified in the virus.

142 **Discussion**

143 The widespread outbreaks of HPAI A (H5N1) clade 2.3.4.4b virus, since October 2020, have
144 raised significant concerns regarding its impact on various mammalian species globally. Recent
145 data reveal that, as of the latest assessment, 37 new mammal species have been afflicted since
146 2021. The majority of these cases involve wild terrestrial mammals such as foxes, skunks, bears,
147 bobcats, and raccoons (9, 23, 24). Intriguingly, there have been sporadic infections among
148 domestic pets like domestic cats and dogs (25), as well as marine mammals, including dolphins
149 and sea lions (26). Moreover, from January 2022 to April 2023, eight documented human cases
150 of H5N1 influenza from clade 2.3.4.4b have been recorded, several of which were severe or fatal
151 (<https://www.cdc.gov/flu/>), underlining the gravity of this situation. Adding to this growing list
152 of affected species, we now characterize an H5N1 influenza virus strain from clade 2.3.4.4b
153 infecting dairy cattle associated with a sudden drop in milk production. The detection of this
154 virus in bovine milk raises a potential public health concern related to zoonotic transmission
155 through unpasteurized milk. This underscores the need for public awareness, pasteurization of
156 milk to maintain adequate food safety, outbreak management, and a holistic approach to human
157 health management.

158 In addition to being the first documented occurrence of HPAI A (H5N1) clade 2.3.4.4b virus
159 infection in domestic dairy cattle, early pathology observations in this outbreak revealed an
160 apparent tissue tropism for mammary gland in lactating domestic dairy cattle (personnel
161 communication). Prior to this incident, the clade 2.3.4.4b IAV has typically caused systemic and
162 respiratory diseases in wild mammals (9). Gross and microscopic lesions in wild mammals were

163 frequently observed in organs such as the lung, heart, liver, spleen, and kidney, with some cases
164 resulting in lesions in the brain leading to neurological signs. Furthermore, while it is widely
165 recognized that certain strains of HPAI H5N1 clade 2.3.4.4b virus can breach the blood-brain
166 barrier (9, 23, 25, 27), this is the first instance where the virus may penetrate the blood-milk
167 barrier and be present in milk, raising potential public health concerns.

168 During this outbreak, HPAI virus strains from various sources such as wild birds, dairy cattle,
169 cats, and a skunk, along with a human, displayed remarkably high nucleotide identities in their
170 genome sequences, forming a distinct phylogenetic subcluster. These findings suggest an
171 introduction of the 2.3.4.4b strain into Texas and neighboring regions by wild birds. The
172 widespread detection of this HPAI virus strain across diverse regions and species underscores the
173 complexity of its transmission pathways. Given the established role of migratory birds as
174 reservoirs for avian influenza viruses (11-14, 26, 28, 29), it is important to highlight Texas's
175 location within the Central flyway. Texas also has overlap in bird migratory patterns with
176 neighboring states that are part of the Mississippi Flyway. Furthermore, the outbreak's
177 occurrence in March coincides with the onset of the spring migration season, enhancing the
178 likelihood of viral dissemination through migratory bird populations. Considering these factors, a
179 highly plausible transmission route is hypothesized: wild birds may spread the virus through
180 direct contact or contamination of water sources or feed stuffs utilized by dairy cattle or other
181 animals such as skunks. Consequently, other cattle in the herd, workers and domestic felids on
182 dairy farms may contract the virus through direct contact with infected cattle or after consuming
183 raw colostrum and milk from infected cattle. The detection of the same strain of HPAI viruses in
184 various wild bird species, such as blackbirds and common grackles in Texas and Canada geese in
185 Wyoming (Central Flyway), provides further support for this hypothesis. Another potential

186 transmission scenario involves bovine-to-bovine spread. Recently, the USDA has verified the
187 presence of this HPAI virus strain in dairy herds located in Idaho, Michigan, Ohio, North
188 Carolina, and South Dakota (<https://www.aphis.usda.gov/news/agency-announcements/usda->
189 [confirms-highly-pathogenic-avian-influenza-dairy-herd-idaho](https://www.aphis.usda.gov/news/agency-announcements/usda-confirms-highly-pathogenic-avian-influenza-dairy-herd-idaho)). In these cases, a documented
190 history exists of cattle introduction from farms in the initial outbreak area, further supporting the
191 hypothesis that lateral transmission can occur among cattle.

192 Our thorough examination of mutation adaptations, particularly those linked to human receptor
193 binding affinity, increased virulence, transmission, or adaptation to mammalian hosts, offers
194 critical insights into the risks posed by this specific strain of HPAI viruses. Notably, all HPAI
195 viruses originating from dairy cattle and cats exhibit consistent amino acid residues in the HA
196 gene, including 137A, 158N, and 160A, which have been documented to enhance the affinity of
197 avian influenza viruses for human-type receptors (*15, 16*). Additionally, these dairy cattle-
198 derived and cat-derived HPAI viruses harbor key virulence-increasing amino acid residues, such
199 as 30D, 43M, and 215A in M1 (*17-19*), as well as 42S, 103F, and 106M in NS1(*20*). The
200 presence of these amino acid mutations raises legitimate concerns regarding the potential for
201 cross-species transmission to humans and other mammalian species. It is noteworthy that crucial
202 mutations associated with mammalian host adaptation and enhanced transmission, specifically
203 residues 591K, 627K/V/A, 701N, in PB2 (*18, 21, 22*), and 228S, along with the virulence-
204 increasing residue 66S in PB1-F2(*30*), were conspicuously absent in all HPAI virus strains
205 derived from dairy cattle and cats. This observation suggests that the current overall risk to
206 human health is relatively low. However, it is imperative to recognize that influenza viruses have
207 the capacity for rapid evolution within their host environments post-infection. A recent human
208 case with direct contact with infected dairy cattle revealed a genetic change (PB2 E627K)

209 (<https://www.cdc.gov/flu/avianflu/spotlights/2023-2024/h5n1-analysis-texas.htm>), indicating the
210 potential for adaptation or transmission events. This underscores the dynamic nature of influenza
211 viruses and the importance of continued surveillance and vigilance in monitoring potential
212 threats to human health.

213

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- 274 25. F. S. François-Xavier BriandComments to Author , Isabelle Pierre, Véronique Beven,
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294

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297 this manuscript. Additionally, we extend our gratitude to the faculty and staff at the ISU VDL
298 who contributed to the processing and analysis of clinical samples in this investigation.

299

300 **List of Supplementary Materials**

301 Materials and Methods

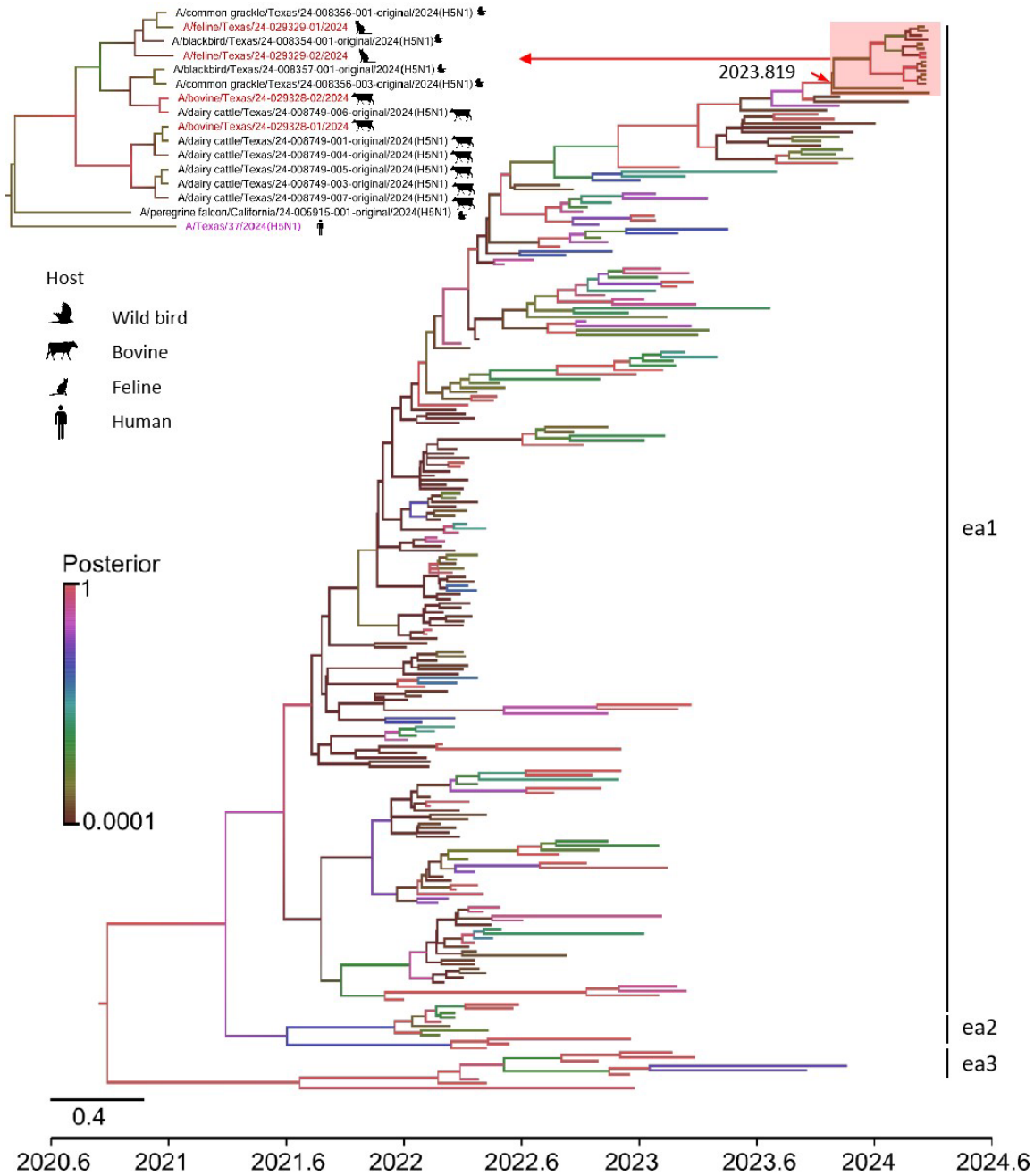
302 Figures S1-S8

303 Table S1-S10

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Figure 1.



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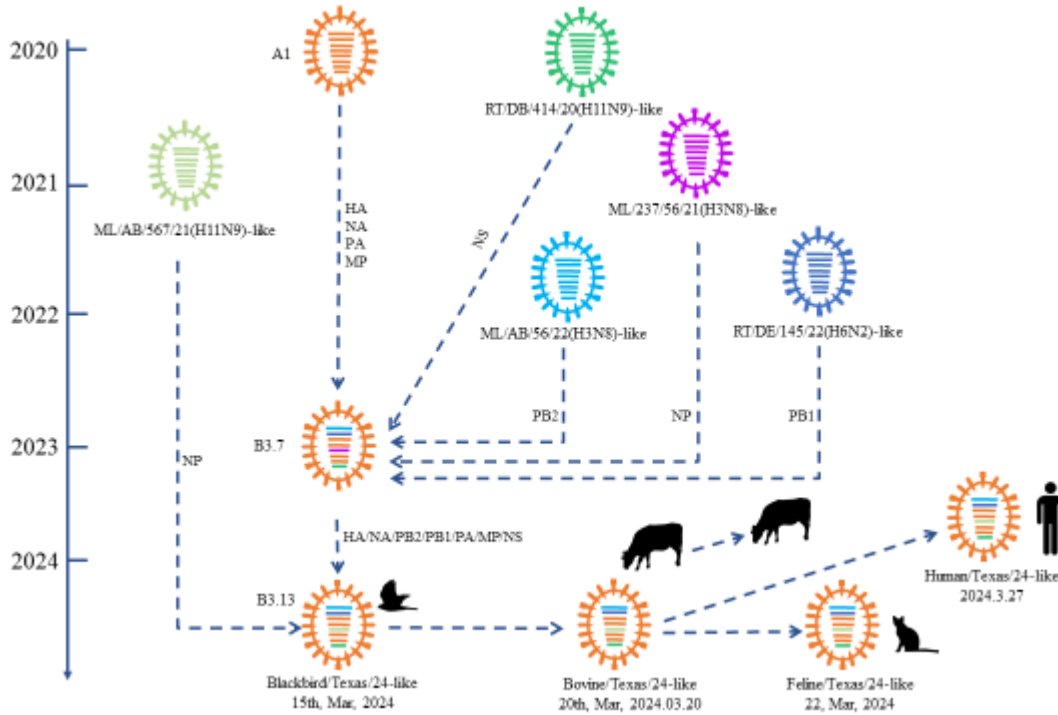
310 **Figure 1. Maximum clade credibility (MCC) tree of the HA genes of clade 2.3.4.4b H5N1**
311 **viruses in the United States since 2021.**

312 The MCC tree is constructed by using BEAST v1.10.4 software package. Each branch is colored
313 using posterior probability. The red frame represents H5N1 of Texas in 2024. The H5N1 viruses
314 isolated in this study are shown in red, human isolate is shown in blue.

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349 **Figure 2**

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353 **Figure 2. Schematic representation of genomic composition and reassortment time of HPAI**
354 **H5N1 viruses from dairy cattle and other animals and human in March 2024**

355 Viral particles are represented by colored ovals containing horizontal bars representing the eight
356 gene segments (from top to bottom: PB2, PB1, PA, HA, NP, NA, M, and NS). Each color
357 represents a separate virus background. The illustration is based on GenoFLU
358 (<https://github.com/USDA-VS/GenoFLU>) and phylogenetic analysis. ML: mallard, RT: ruddy
359 turnstone, AB: Alberta, DB: Delaware Bay.

360

361 **Table 1 Mutations detected in the clade 2.3.4.4b H5N1 viruses have contributed to**
 362 **increased binding to human-type receptors and virulence in mammals.**

Anim al Cate gory	Host (No. of strains)	Amino acids in HA that may increase the affinity to human-type receptor (H3 number)						Mutations in different genes that may increase virulence in mice									
								PB2			PB 1- F1	M1			NS1		
		13 7A	15 8N	16 0A	19 2I	22 5D	22 8S	591 K	627K/ V/A	70 1N	66 S	30 D	43 M	21 5A	42 S	10 3F	106 M
Terres trial	Canidae (173)	17 3	17 3	17 3	/ ^a	/	/	1	20	2	16 8	17 3	17 3	17 3	17 2	17 3	173
	Felidae (39)	37	35	37	/	/	/	/	32	34	38	38	38	38	38	38	38
	Musteli dae (53)	53	53	53	/	/	/	/	13	6	47	53	53	53	53	52	53
	Ursidae (6)	6	6	6	/	/	/	/	3	2	6	6	6	6	6	6	6
	Dairy cattle (8)	8	8	8	/	/	/	/	/	/	/	8	8	8	8	8	8
	Bovidae (3)	3	3	3	/	/	/	/	/	/	/	3	3	3	3	3	3
	Procyon idae (2)	2	2	2	/	/	/	/	2	/	2	2	2	2	2	2	2
Marin e	Phocida e (38)	38	38	38	/	/	/	1	6	3	38	39	39	39	39	39	39
	Otariida e (16)	16	16	16	/	/	/	10	/	2	16	16	16	16	16	16	16
	Delphin idae (14)	14	14	14	3	/	/	2	/	5	14	14	14	14	14	14	14

363 a, No such mutant.

364