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A Review on Highly Pathogenic Avian Influenza H5N1 Clade 2.3.4.4b in Mammals: Molecular Adaptation, Host Range Expansion, and One Health Implications

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Abstract

Influenza A virus H5N1 clade 2.3.4.4b has caused the largest highly pathogenic avian influenza (HPAI) panzootic on record, infecting over 175 million poultry globally and spilling over into more than 60 mammalian species (Table 2). The establishment of reassortant genotype B3.13 in US dairy cattle from March 2024, with 1,093 confirmed herds across 19 states by April 2026, is the first sustained HPAI transmission in a domestic ruminant and defines the pandemic risk landscape of the current era. Two mechanistically independent adaptive axes drive bovine fitness in B3.13 (Figure 1). The polymerase axis, encompassing PB2 M631L and PA K497R, remodels the viral replication complex to exploit host-specific ANP32A isoforms. The receptor axis, driven by hemagglutinin (HA) mutations D104G and V147M, enables efficient recognition of N-glycolylneuraminic acid, which comprises approximately half the sialic acid pool in bovine mammary tissue but is absent in humans. Crystallographic analysis has established that a single Q226L substitution in the HA receptor-binding site of a human clinical isolate of bovine H5N1 is sufficient to switch its receptor specificity from avian-type to human-type receptors, identifying this position as the highest-priority genomic surveillance target. Three pandemic barriers remain intact as of early 2026: B3.13 retains avian-type receptor preference, an elevated HA fusion pH, and full nucleoprotein (NP) sensitivity to human MxA restriction. Even so, partial ferret respiratory-droplet transmission, over 70 confirmed human cases, including the first US pediatric case, the emergence of a baloxavir-resistant clinical isolate, and ongoing convergent molecular evolution collectively define a pandemic risk level higher than any prior point in H5N1 history. Integrated One Health action combining mandatory bulk-milk genomics, wastewater surveillance, systematic wildlife serosurveillance, and accelerated ruminant mRNA vaccination is urgently required.

Keywords: *H5N1 clade 2.3.4.4b, PB2 M631L, ANP32 restriction, NeuGc receptor adaptation, B3.13 dairy cattle, mammal-to-mammal transmission, One Health, pandemic preparedness*

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 viruses have circulated in Eurasian poultry since A/goose/Guangdong/1/96 (Gs/GD) was identified in farmed geese in southern China in 1996 (Claas et al., 1998; Shortridge et al., 1998). The 1997 Hong Kong outbreak produced 18 human infections and six fatalities following direct poultry-to-human transmission, confirming H5N1 as a genuine zoonotic threat and prompting the first mass culling response in HPAI history (Claas et al., 1998; Matrosovich et al., 1999). For more than two decades, clade replacements within the Gs/GD lineage, including clades 2.1, 2.2, and 2.3.2.1, caused frequent epizootics, largely dead-end mammalian spillovers (Xie et al., 2023). Since 2020, the spread of clade 2.3.4.4b has significantly altered this epidemiological picture (Li et al., 2026). Clade 2.3.4.4b emerged in European wild birds in 2020 and drove the largest HPAI epizootic on that continent, subsequently persisted in seabirds including Northern Gannets (*Morus bassanus*) and Black-headed Gulls (*Chroicocephalus ridibundus*) from 2022 onward (EFSA, 2023; Fusaro et al., 2024). Unlike earlier clades, which mainly spread through dabbling-duck flyways, clade 2.3.4.4b outbreak globally via pelagic seabird migration routes, reaching North America in late 2021 and South America by late 2022 (Caliendo et al., 2022; Leguia et al., 2023). Studies confirmed the presence of H5N1 in Antarctica by December 2023 (Banyard et al., 2024). Reassortment with North American low-pathogenicity avian influenza (LPAI) strains generated more than 50 distinct genotypic constellations; of these, B3.13 and D1.1 proved epidemiologically dominant in North America (Nguyen et al., 2025; Peacock et al., 2025).

The October 2022 Spanish mink farm outbreak provided the first documented evidence of sustained mammal-to-mammal HPAI transmission, crossing a conceptual boundary between the era of sporadic avian-to-mammal spillover and the current era of active mammalian adaptation (Table 2; Agüero et al., 2023). Finnish fur farm outbreaks in July to October 2023 reinforced this conclusion (Kareinen et al., 2024). Phylogenetic analyses of South American pinniped isolates demonstrated sustained multinational horizontal transmission across Peru, Chile, Argentina, Uruguay, and sub-Antarctic territories, resulting in more than 52,000 confirmed animal deaths (Leguia et al., 2023; Uhart et al., 2024). The single most consequential development in the panzootic has been the establishment of B3.13 genotype H5N1 in US dairy cattle from March 2024, the first documented example of sustained HPAI transmission in a

domestic ruminant (Oguzie et al., 2024; Nguyen et al., 2025). From March 2024 to April 2026, HPAI was confirmed in 1,093 dairy herds across 19 US states, and 71 human cases were documented, constituting the most prolonged occupational HPAI exposure in US history (USDA-APHIS, 2026a; USDA-APHIS, 2026b).

This review deeply emphasizes on molecular basis of mammalian adaptation, the extended host range and species-specific pathogenesis, One Health implications, and important research gaps.

Molecular Adaptation Mechanisms in Mammalian Hosts

Genome Constellation and the ANP32 Restriction Bottleneck

The B3.13 genotype pairs a Eurasian clade 2.3.4.4b HA and N1 with North American LPAI-derived internal genes; this unique internal gene backbone proved significantly permissive for bovine-directed mutation selection (EFSA, 2025; Nguyen et al., 2025). The D1.1 genotype, a distinctive North American reassortant that preserved wild-bird dominance but independently generated cattle-infective variants in 2025, confirming the presence of multiple genotypic routes to bovine infection (Pekar et al., 2026). D1.1 cattle isolates carry PB2 D701N rather than M631L, which validates that different polymerase adaptation strategies can each arrive at productive bovine replication. A comprehensive panel of recombinant viruses representing more than 60 years of H5N1 evolutionary history confirmed that replicative fitness in bovine cells is highly variable across 2.3.4.4b genotypes. This is limited in viruses predating the clade global expansion, and is mainly determined by the internal gene cassette (Turnbull et al., 2025). Mutations in the PB2 polymerase subunit act as the key determinants of bovine adaptation, though their phenotypic effects are context dependent across different genetic backgrounds (Table 1).

Table 1. Key mammalian-adaptive mutations in HPAI H5N1 clade 2.3.4.4b

Mutation	*Protein / Principal Hosts*	*Functional Mechanism*	*Key Reference(s)*
PB2 E627K	PB2 residue 627; humans, mink, marine mammals, red foxes	Glu to Lys charge switch removes steric clash with the LCAR repeat of mammalian ANP32; strengthens contact with ANP32 E151; substantially enhances polymerase activity in human cells	Subbarao et al. (1993); Staller et al. (2024)
PB2 D701N	PB2 residue 701; South American pinnipeds; D1.1 cattle spillovers	Asp to Asn enhances RNP nuclear import through improved importin-alpha-1 binding; independent of ANP32 interaction-site remodelling; dominant adaptation in D1.1 Nevada cattle cluster	Gabriel et al. (2008); Steel et al. (2009); Uhart et al. (2024); Pekar et al. (2026)
PB2 M631L	PB2 residue 631; US dairy cattle (universal in B3.13 genotype)	Met to Leu restructures the PB2 627-640 loop; preferential binding to bovine ANP32A over ANP32B (validated by split-luciferase assay); substantially enhances polymerase activity alone and synergistically with PA K497R in bovine MAC-T cells; also complements swine and human ANP32 homologues; pre-existed at low frequency in clade 1.1.2 and 2.3.2.1c viruses in Cambodia and Vietnam 2013-2014	Dholakia et al. (2026); Capelastegui and Goldhill (2025); Zhang et al. (2025)
PA K497R	PA residue 497; approximately 95% of B3.13 clade iii cattle sequences	Located at the FluPol symmetric dimer interface; provides some polymerase enhancement alone; synergizes with PB2 M631L to restore wild-type polymerase activity in bovine mammary-gland explants	Dholakia et al. (2026)
PB2 T271A	PB2 residue 271; farmed mink (Spain, October 2022)	Distinct ANP32-adaptation pathway selected under high-density mammal-to-mammal transmission; enhances replication kinetics without altering the canonical 627 or 701 contact surfaces	Agüero et al. (2023); Peacock et al. (2023)
HA D104G	HA 130-loop; B3.13 cattle (increasing prevalence)	Repositions a polar residue beneath the 130-loop, relieving steric clash with the NeuGc N-glycolyl hydroxyl; broadens receptor usage to include SA-alpha-2,3-NeuGc and NeuAc; positive selection (dN/dS = 1.49)	Hassard et al. (2026)
HA V147M	HA 130-loop; B3.13 cattle (fixed by late 2025)	Enlarges the hydrophobic pocket surrounding the 130-loop, accommodating the NeuGc hydroxyl without compromising NeuAc binding; approximately doubles effective receptor density in bovine mammary epithelium; strongest positive selection in B3.13 HA dataset (dN/dS = 5.63; eight independent emergences)	Broszeit et al. (2019); Hassard et al. (2026)
HA Q226L	HA 220-loop; not yet at consensus frequency in circulating B3.13 (surveillance target only)	Single Gln to Leu change in the 220-loop switches receptor specificity of A/Texas/37/2024 from avian-type alpha-2,3-NeuAc to human-type alpha-2,6-NeuAc; structural basis resolved by crystal structure with LSTa/LSTc receptor analogues; effect enhanced by additional N224K mutation; Q226L alone may not achieve the sustained alpha-2,6 engagement required for full pandemic receptor phenotype	Lin et al. (2024)
M2 S31N	M2 ion channel; universal across clade 2.3.4.4b	Confers complete resistance to adamantane antivirals; retains full susceptibility to neuraminidase inhibitors and baloxavir marboxil. A baloxavir-associated PA I38M mutation was detected in one B3.13 human isolate from California in late 2024; treatment context unclear	Nguyen et al. (2025); Zhu et al. (2025)

Abbreviations: ANP32, acidic nuclear phosphoprotein 32; FluPol, influenza polymerase; LCAR, leucine-cysteine-alanine-arginine repeat; RNP, ribonucleoprotein; MAC-T, bovine mammary epithelial cell line; NeuGc, N-glycolylneuraminic acid; NeuAc, N-acetylneuraminic acid; dN/dS, ratio of non-synonymous to synonymous substitutions.

Table 2. Mammalian species affected by HPAI H5N1 clade 2.3.4.4b (2022 to early 2026)

Species	*Primary Transmission Route*	*Key Clinical Signs*	*Key Outcome / Scale*	*Reference(s)*
Dairy cattle (Bos taurus)	Contaminated milking equipment and fomites; single wild-bird spillover	Mastitis, sharply reduced milk yield, mild respiratory signs; high viral load in milk (10^8 to 10^9 TCID50/mL)	More than 1,093 herds in 19 US states (April 2026); CFR less than 5%; protective immunity develops but is non-sterilizing against heterologous D1.1	Nguyen et al. (2025); Caserta et al. (2024); Facciuolo et al. (2025); Tarbuck et al. (2026)
Domestic cat (Felis catus)	Ingestion of raw milk or colostrum; close contact with infected dairy farms	Severe neurological disease (ataxia, seizures), necrotising meningoencephalitis, interstitial pneumonia, myocarditis	CFR greater than 74%; infectious virus isolated from urine post raw milk ingestion	Burrough et al. (2024); Bonilla-Aldana et al. (2025); Frye et al. (2025); Naraharisetti et al. (2025)
American mink (Neovison vison)	Initial wild-bird introduction, then sustained farm-to-farm transmission	Respiratory distress and neurological signs	First documented sustained mammal-to-mammal HPAI transmission; PB2 T271A selected	Agüero et al. (2023); Kareinen et al. (2024)
South American sea lion (Otaria flavescens)	Pinniped-to-pinniped horizontal transmission	Neurological signs, respiratory distress, mass die-offs	More than 52,000 deaths across Peru, Chile, Argentina, Uruguay, and sub-Antarctic islands; pup CFR greater than 95%; PB2 D701N dominant	Leguia et al. (2023); Uhart et al. (2024)
Red fox (Vulpes vulpes)	Predation or scavenging of infected wild birds	Acute neurological deterioration, necrotising meningoencephalitis	Usually fatal; PB2 E627K frequently selected in brain tissue; respiratory samples often RT-PCR negative	Vreman et al. (2023); Elsmo et al. (2023)
Domestic swine (Sus scrofa)	Presumed wild-bird contact (route still under investigation)	Mild or subclinical infection in experimental conditions; replication in lower respiratory tract	First US detections late 2024; no transmission to sentinel pigs experimentally; HA S136N alpha-2,6-associated mutation selected at minority frequency in porcine respiratory tract	EFSA et al. (2025); Kwon et al. (2025); USDA-APHIS (2025)
Human (Homo sapiens)	Contaminated milk aerosols (B3.13); direct contact with infected poultry or wild birds (D1.1)	Conjunctivitis (approximately 89% of B3.13 cases); severe pneumonia and neurological disease (D1.1 cases)	More than 70 confirmed US cases (February 2024 to April 2026); 1 death (D1.1, Louisiana, January 2025); first pediatric US case (California, November 2024); no sustained human-to-human transmission	Garg et al. (2025); Rolfes et al. (2025); Zhu et al. (2025); CDC (2026)

Abbreviations: CFR, case fatality rate; TCID50, 50% tissue culture infectious dose; RT-PCR, reverse transcription polymerase chain reaction.

The bottleneck that limits avian IAV replication in mammals is mismatch between the viral polymerase complex and host ANP32 proteins. All birds except palaeognaths encode a long-form ANP32A containing 33 additional amino acids from alternative exon 4 splicing; this long-form isoform efficiently supports avian polymerase activity in comparison to short-form mammalian ANP32A or ANP32B (Long et al., 2016; Carrique et al., 2020). Adaptive polymerase mutations must therefore remodel the ANP32 interaction interface to maintain replication in mammals. Bovine cells are ANP32A-dominant, which is different from the ANP32B-dominant milieu of human, pig, and mustelid cells, that's why the B3.13 cattle lineage selectively fixed PB2 M631L rather than the classical PB2 E627K substitution (Peacock et al., 2023). Notably, recent ANP32 biology has demonstrated that M631L interacts not only with bovine ANP32A but also with swine and human ANP32 analogs, suggesting that it is not limited to bovine-specific adaptation but rather a broadly mammalian-permissive change that provides the greatest fitness gain specifically in the bovine cells (Capelastegui and Goldhill, 2025).

Polymerase Adaptations: PB2 and PA

The PB2 E627K (Glu to Lys at residue 627) solves the ANP32 problem by a charge switch that removes steric clash between PB2 and the negatively charged LCAR (leucine-cysteine-alanine-arginine) region of mammalian ANP32 proteins, while simultaneously firming interaction between the encapsidating polymerase subunit and ANP32 residue E151 (Subbarao et al., 1993; Camacho-Zarco et al., 2020; Staller et al., 2024). E627K predominates in human H5N1 isolates, farmed mink, marine mammals, and red foxes, and is recurrently selected in brain tissue of terrestrial carnivores such as red foxes, skunks, and cats, during acute mammalian infection (Vreman et al., 2023). PB2 D701N (Asp to Asn at residue 701) acts through a distinct mechanism by enhancing nuclear import of the viral ribonucleoprotein (RNP) complex through improved binding to importin-alpha-1 (Gabriel et al., 2008; Tarendeau et al., 2008); this substitution is dominant in South American pinniped isolates and in the D1.1 Nevada cattle cluster (Steel et al., 2009; Uhart et al., 2024; Pekar et al., 2026).

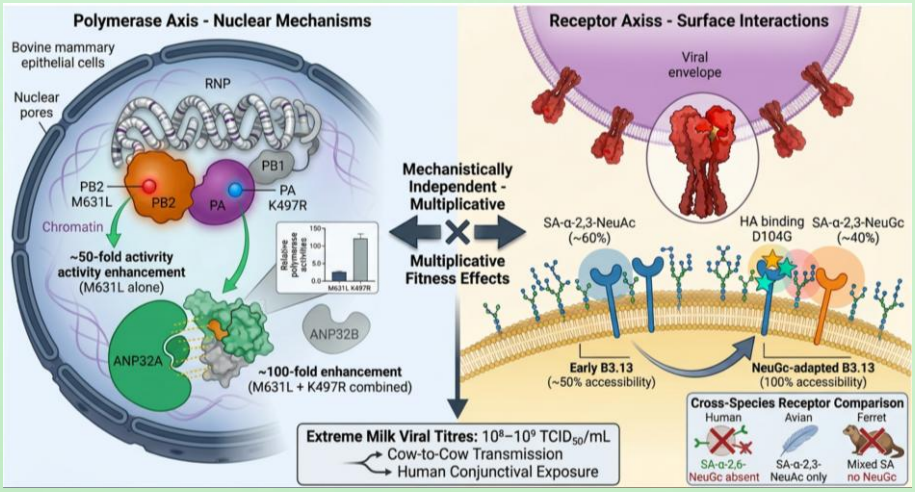

Figure 1. Two Independent Adaptive Axes in Bovine Mammary Epithelial Cells Driving H5N1 B3.13 Establishment and Pandemic Risk.

Left panel (Polymerase Axis, blue): PB2 M631L and PA K497R mutations remodel the viral polymerase complex to exploit the ANP32A-dominant bovine cellular environment. The mutations enhance polymerase activity ~50-fold (M631L alone) and ~100-fold in combination, enabling robust viral RNA synthesis in bovine mammary epithelium. RNP, ribonucleoprotein complex; ANP32A/B, acidic leucine-rich nuclear phosphoprotein family proteins. Adapted from Dholakia et al. (2026) and Sheppard et al. (2023).

Right panel (Receptor Axis, red/orange): HA D104G and V147M mutations in the 130-loop broaden viral tropism from alpha-2,3-linked sialic acids (SA-α2,3-NeuAc, ~60% of accessible receptors in early B3.13) to additional alpha-2,3-linked NeuGc species (~40% additional receptor pool). This dual-receptor usage effectively doubles accessible receptor density in bovine mammary tissue. Cross-species comparison shows NeuGc is synthesized only in cattle, horses, and pigs (via active CMAH enzyme) and is absent in humans (92 bp CMAH exon deletion), birds (CMAH genomic deletion), and ferrets (CMAH non-functional mutation). Adapted from Hassard et al. (2026).

Bottom panel: Combined polymerase and receptor adaptations produce exceptional milk viral titers (10^8-10^9 $TCID_{50}$/mL), enabling sustained cow-to-cow transmission via contaminated milking equipment and elevated human conjunctival exposure risk in dairy workers (Caserta et al., 2024; Baker et al., 2024).

$TCID_{50}$, tissue culture infectious dose 50; CMAH, cytidine monophospho-N-acetylneuraminic acid hydroxylase.

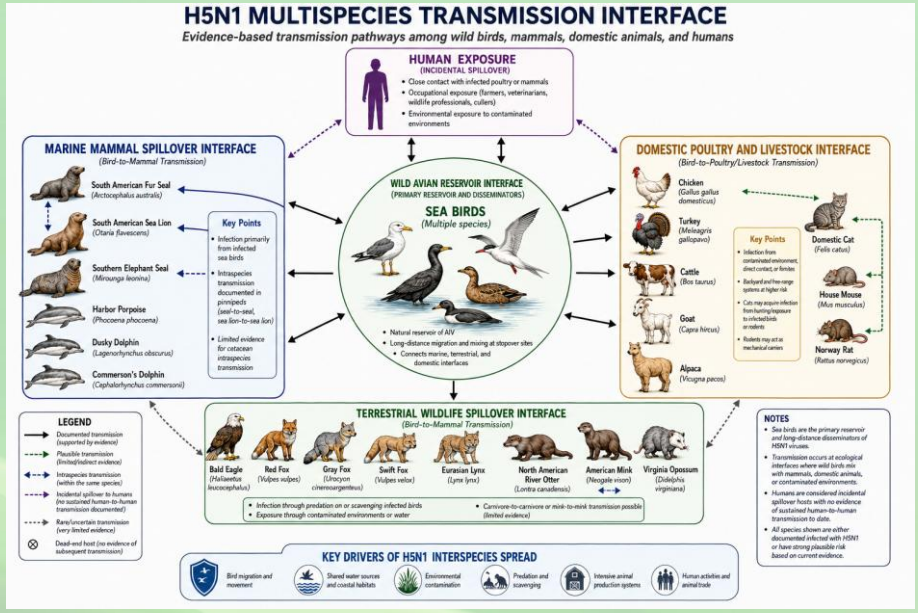

Figure 2. H5N1 Clade 2.3.4.4b Multispecies Transmission Interface

Central wild avian reservoir (sea birds) serves as the primary source of H5N1 dissemination globally. Four major spillover interfaces branch from this reservoir: (1) Marine mammal spillover to pinnipeds (seals, sea lions) and cetaceans (dolphins, porpoises) with documented sustained mammal-to-mammal transmission in pinniped colonies; (2) Domestic poultry and livestock spillover to chickens, turkeys, cattle, and companion animals (cats, dogs, rodents), with sustained infection in cattle representing the first domestic ruminant outbreak; (3) Terrestrial wildlife spillover to wild carnivores (foxes, eagles, otters, minks) primarily through predation on infected birds, with most spillovers representing dead-end infections except in high-density mink farm settings; (4) Human exposure through occupational and environmental contact with infected animals, with no sustained human-to-human transmission documented to date. Solid arrows indicate documented transmission routes with epidemiological or phylogenetic evidence; dashed arrows indicate plausible transmission with limited evidence. Six key ecological drivers facilitate spillover at these interfaces: bird migration, shared water sources, environmental contamination, predation and scavenging, intensive animal production systems, and human animal trade activities.

Data adapted from: Peacock et al. (2025), Kuiken et al. (2024), Uhart et al. (2024), Lair et al. (2024), Caserta et al. (2024), Burrough et al. (2024), Agüero et al. (2023), CDC (2024a), USDA-APHIS (2026a, 2026b), and related sources cited in References.

The most mechanistically distinctive adaptation of the 2.3.4.4b panzootic is PB2 M631L, which is fixed in all sequenced B3.13 cattle isolates. Unlike E627K, the Met-to-Leu substitution at residue 631 does not alter residue charge; instead, it remodels the PB2 residues 627 to 640 loop at the ANP32 interaction interface, promoting preferential physical binding to bovine ANP32A over ANP32B, confirmed by split-luciferase protein-protein interaction assay (Dholakia et al., 2026). Minireplicon assays using bovine-specific RNA polymerase I promoter demonstrated substantially enhanced polymerase activity in bovine MAC-T mammary epithelial cells for M631L alone, with further enhancement when combined with PA K497R (Dholakia et al., 2026). It is noteworthy that the Dholakia et al. (2026) study was conducted on bovine MAC-T cells; the extent of the M631L polymerase enhancement in primary human airway cells has not been directly measured within the same experimental context and drawing conclusions about the fitness in human airway settings from the bovine cells should be done with caution. The cryo-EM structure of H5N1 polymerase bound to ANP32B (PDB: 8R1J) shows that residue 631 is close to E627 and Q591 at the ANP32 bridging surface, supporting its role in this interaction (Staller et al., 2024).

Using retrospective genomic analysis by Zhang et al. (2025) identified that PB2 M631L is a common low-frequency variant in the H5N1 virus from Cambodia and Vietnam from 2013 to 2014. The two isolates of clade 1.1.2 reassortant (2013) and clade 2.3.2.1c (2014) are genetically different backgrounds where all B3.13 internal gene segments were lacking. This is particularly significant as M631L did not cause a bovine outbreak from 2013 to 2014 as it had been encapsulated in a genetic structure that did not have the extra permissive properties of the B3.13 internal cassette. Thus the mutation was already present in the world H5N1 gene pool, as a pre-existing variant, prior to its fixation following the panzootic 2.3.4.4b. Moreover, ongoing B3.13 evolution has generated additional convergent PB2 evolution, which has led to further emergence of PB2 D740N (three independent emergences, with a significant boost in activity in bovine cells) and to a double-mutant cluster with PB2 E627K and M631L occurring together that emerged in July 2025 and is the most active combination of mutations in polymerase activity tested (Dholakia et al., 2026).

PA K497R is found in ~95% of the B3.13 clade iii sequences, and is at the FluPol symmetric dimer interface near the M631L and ANP32 interaction region (Dholakia et al., 2026). It alone constitutes some polymerase enhancement as well, but most importantly, the M631L plus K497R combination is synergistic and fully recapitulates the activity of wild-type cattle/Texas polymerase in bovine mammary explants (Dholakia et al., 2026). The PB2 T271A, selected during the October 2022 Spanish mink farm outbreak, represents an independent adaptation pathway associated with enhanced ferret transmission efficiency in experimental models (Agüero et al., 2023; Peacock et al., 2023).

Accessory Protein Contributions: NP, NS1, M2, and PB1-F2

NP sequences of circulating B3.13 isolates retain full sensitivity to human MxA restriction at key residues 52, 100, 283, 313, and 357, as confirmed experimentally by Ankerhold et al. (2025). MxA-mediated innate immune restriction constitutes a major post-entry barrier to H5N1 replication in human airways, and the retention of MxA sensitivity in circulating B3.13 is therefore a critical current pandemic safeguard. The NS1 protein retains a PDZ-binding ESEV C-terminal motif associated with virulence in mammalian hosts through suppression of type I interferon signalling (Garcia-Sastre, 2011; Jagger et al., 2012). The M2 ion channel carries the S31N substitution, conferring universal adamantane resistance across clade 2.3.4.4b (Nguyen et al., 2025).

Consistent with NS1 interferon antagonism at the molecular level, human iPSC-derived respiratory organoid experiments demonstrated that the cattle-derived human isolate A/Texas/37/2024 suppresses STAT1-mediated transcriptional activity and induces minimal interferon and inflammatory cytokine responses despite efficient replication; bulk and single-cell RNA sequencing revealed reduced STAT1-mediated transcriptional activity compared to the historic A/Vietnam/1203/2004 strain, which triggers robust innate responses (Shichinohe et al., 2025). This intrinsic immune suppression could help explain why the B3.13 clade of H5N1 infections in humans have mostly been mild conjunctivitis, rather than the more severe cytokine-storm pathogenesis seen with previous H5N1 clades, but it should be interpreted with caution due to the different routes of exposure in natural human infection compared to organoid infection. The organoid model of the human respiratory system is itself a valuable alternative to cell lines, especially in recapitulative the stratified airway epithelial architecture; however, in vivo interactions of the airway with circulating leukocytes are not recapitulated. PB1-F2 accessory protein, which is derived from an overlapping reading frame of PB1, has been linked to mitochondrial membrane permeabilization, apoptosis in immune effector cells and increased inflammatory pathogenesis during mammalian H5N1 infection. The molecular surveillance gap of full characterisation of the integrity of the PB1-F2 reading frame and PA-X expression level of circulating B3.13 sequences in various host species still needs to be addressed.

Haemagglutinin: Receptor Binding, NeuGc Adaptation, and Fusion pH Barriers

The HA of B3.13 cattle viruses contains the polybasic cleavage site (RRRKKR) that is recognized by ubiquitous furin family proteases, which is responsible for the systemic pathogenicity in mammals (Horimoto & Kawaoka, 1994). Consistently, glycan microarray, bio-layer interferometry (BLI), and structural analysis all reveal that B3.13 HA has strong alpha-2,3-linked sialic acid (SA) preference with little or no binding to alpha-2,6-linked SA, which is characteristic of the avian receptor phenotype (Chopra et al., 2025; Santos et al., 2025; Song et al., 2025; Yang et al.,

2025). This absence of efficient alpha-2,6 binding is a critical barrier to pandemic emergence, as productive human upper respiratory tract attachment and aerosol droplet transmission require alpha-2,6-SA recognition.

A distinct and novel receptor adaptation axis has emerged in circulating B3.13 viruses through HA mutations D104G and V147M in the 130-loop. MALDI-TOF mass spectrometry glycomics demonstrated that bovine mammary gland, teat, trachea, and lung carry N-glycans capped with N-glycolylneuraminic acid (NeuGc) and N-acetylneuraminic acid (NeuAc) in approximately equal proportions (~50:50), with alpha-2,3 linkage predominating for both (Hassard et al., 2026). NeuGc is synthesized by cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH), which is permanently inactivated in humans by a 92 bp exon deletion (Chou et al., 2002), absent in birds by genomic deletion (Schauer et al., 2009), and non-functional in ferrets, mink, and pinnipeds (Ng et al., 2014; Nemanichvili et al., 2022). Cattle, horses, and pigs express high NeuGc via active CMAH (Suzuki et al., 1997; Nemanichvili et al., 2022). Since early B3.13 binds only alpha-2,3-NeuAc, it exploits only half the available mammary receptor pool. The mutations D104G and V147M functionally broaden rather than switch receptor specificity: D104G repositions a polar residue below the HA 130-loop to reduce steric clash with the NeuGc N-glycolyl hydroxyl group, while V147M enlarges the hydrophobic pocket in the 130-loop to accommodate NeuGc without loss of NeuAc binding (Broszeit et al., 2019; Hassard et al., 2026). This dual-receptor usage doubles the density of available virus receptors effectively, in bovine mammary tissue. The quantitative gain of utilizing the NeuGc and NeuAc pools is reflected in V147M having the strongest positive selection in the entire cattle B3.13 HA dataset ($dN/dS = 5.63$; $P(dN/dS>1) = 1.00$; with eight independent emergences prior to fixation by late 2025) (Hassard et al., 2026). The only known lineage of natural NeuGc adaptation is equine H7N7, which evolved strict NeuGc preference by the analogous Y161 residue (Gambaryan et al., 2012; Broszeit et al., 2019). Importantly, D104G and V147M are found to be neutral/mildly attenuating in primary human nasal epithelial cultures, reflecting that NeuGc absence in human tissues is an inherent fitness loss for bovine-adapted strains in humans (Hassard et al., 2026).

In a recent structural study highly relevant to pandemic risk, it was shown that a single HA Q226L substitution in the 220-loop (H3-equivalent numbering) could shift the receptor specificity of A/Texas/37/2024, a human clinical isolate of bovine H5N1, from avian-type alpha-2,3-NeuAc to human-type alpha-2,6-NeuAc receptors (Lin et al., 2024). In this study, surface plasmon resonance, ELISA using N-linked glycan sialosides, and glycan array analysis were used, and crystal structures of 2.7 angstrom were determined for both the wild-type HA bound to its avian receptor analogue LSTa and the Q226L HA bound to its human receptor analogue LSTc, providing a direct visualization of the structural basis for receptor switching. When Q226L is combined with the N224K mutation, it further enhances this switch (Lin et al., 2024). Structurally, the 130-loop (D104G and V147M are located here, broadening the NeuGc recognition) and the 220-loop (operates Q226L) are separate parts of the rim of the receptor-binding pocket and are involved in the receptor specificity through independent contacts (Hassard et al., 2026; Pekar et al., 2026). Historically, efficient human-type receptor binding has required the H2N2 emergence of 1957 in addition to the emergence of G228S, which was able to occur in the H3N2 strain of 1968. Historically, human-type receptor binding was efficient only if both H2N2 emergence from the 1957 strain and G228S occurred in the 1968 strain H3N2. The Lin et al. (2024) study shows that the mutation of Q226L suffices to change the specificity in binding assays, but binding assay affinity is not equivalent to the productive engagement of α2,6-NeuAc to trigger replication throughout the human upper respiratory tract. What is currently known is that Q226L has only one amino acid change (a relatively modest genetic cost), and that complete adaptation to the pandemic is probably a composite of these and other co-occurring mutations. Notably, none of the sequenced B3.13 genomes in the current analysis of Lin et al. (2024) contained the Q226L mutation, making HA position 226 the only most frequently targeted one in the genome through the context of genomic surveillance. Biochemical assay and cryo-EM analyses show that the HA membrane fusion pH at B 3.13 is around 5.9 (Song et al., 2025; Yang et al., 2025). This high fusion threshold is a second quantification of a pandemic barrier that is independent. Once triggered, HA-mediated membrane fusion is an irreversible conformational change and inactivates the virion. The aerosol-stable pandemic strains have a fusion pH of 5.0 to 5.5 (Herfst et al., 2012; Imai et al., 2012; Linster et al., 2014). Here, the lower fusion pH would ensure that HA is in the pre-fusion conformation upon entering target cells as a part of respiratory droplets (with a pH of 6.5 to 7.0) and in the slightly acidic environment of the upper respiratory tract, where fusion would not occur until the cell's endosomal pH dropped into the cell, promoting fusion. The fusion pH is about 5.9 and in B3.13 HA has the potential to trigger early during aerosol transit, resulting in a higher risk of rapid loss of infectivity before the virus reaches the susceptible airway epithelium. This raised pH for fusion is therefore an autonomous barrier that can be measured and is efficient for aerosol transmission in humans.

Neuraminidase: Stalk Integrity, Sialidase Efficiency, and Antiviral Susceptibility

The N1 neuraminidase (NA) isolates from mammalian hosts of subtype clade 2.3.4.4b show a distinctive structural and functional profile in which a long stalk of 18-20 amino acid residues (Hermann & Krammer, 2025) is consistently present. This structural feature is a major difference when compared to the short-stalk variants in which there are deletions from position 49-68 and which have been the most common in the more virulent H5N1 outbreaks affecting poultry (Zhou et al., 2009; Li et al., 2014). The extended stalk is essential for function, being directly linked to increased sialidase activity in mucus-rich tissues like the bovine mammary gland, and the human respiratory tract.

This improved activity allows efficient release of the virions from host cells by cleaving sialic acid receptors, thus inhibiting viral aggregation and favoring viral dissemination (Blumenkrantz et al., 2013). The prevalence of this long-stalk NA was confirmed by recent structural and biochemical analyses, which showed that this NA provides a significant fitness advantage in the unique microenvironment of bovine milk, where viral titers may reach extraordinary levels of 10^8-10^9 $TCID_{50}$/mL (Caserta et al., 2024; Halwe et al., 2025). The interplay between NA stalk length and HA affinity is crucial for viral fitness. The NA has to manifest a finely-tuned catalytic activity as the HA adjusts to novel host-specific receptors, such as NeuGc abundant in bovine tissues (Hassard et al., 2026). This balance promotes optimal exit of infected lactocytes with an intact receptor that is suitable for initial attachment. Any disturbances in this balance can significantly reduce the efficiency of replication and transmission of the virus.

In terms of therapeutic potential, neuraminidase inhibitors (NAIs) like oseltamivir and zanamivir are, in general, effective inhibitors of current 2.3.4.4B strains, but the occurrence of resistance markers is a growing concern. Significantly, a PA I38M mutation that leads to lower susceptibility to the cap-dependent endonuclease inhibitor baloxavir marboxil was found in a human isolate (A/California/150/2024) from the late 2024 outbreak in California (Zhu et al., 2025). The exact selective pressure that gave rise to this mutation is unknown, and the finding in a human clinical isolate highlights the importance of ongoing antiviral surveillance and resistance monitoring. Additionally, adamantane-class antivirals are no longer an option for all of these viruses, as the M2 S31N substitution is found in all members of clade 2.3.4.4b (Nguyen et al. 2025). These observations highlight the dynamic evolutionary capacity of H5N1 and the imperative for developing novel antiviral strategies and robust surveillance mechanisms to detect emerging resistance.

Host Range Expansion: Epidemiology and Pathogenesis

The global expansion of H5N1 into mammalian hosts has occurred through four major transmission interfaces (Figure 2): marine mammal spillover with sustained mammal-to-mammal transmission in pinnipeds (Table 2; Uhart et al., 2024; Lair et al., 2024); domestic poultry and livestock spillover leading to sustained infection in cattle (Caserta et al., 2024) and sporadic infection in cats (Burrough et al., 2024); terrestrial wildlife spillover primarily through predation with most infections being dead-end events except in confined mink settings (Agüero et al., 2023); and incidental human spillover through occupational exposure with no sustained human-to-human transmission documented (CDC, 2024a; Peacock et al., 2025).

Taxonomic Scope, Ecological Mechanisms, and Transmission Routes

Since 2021 there have been over 70 species of mammals infected by H5N1 clade 2.3.4.4 from seven taxonomic orders (Figure 2), the largest taxonomic range for any HPAI virus (Peacock et al., 2025; Kuiken et al., 2026; Li et al., 2026). The mechanisms of infection vary greatly along this spectrum. Infections in terrestrial species of carnivores and omnivores are mainly through predation and scavenging of infected wild birds, which results in high dose oral dosages that induce systemic infection (Elsmo et al, 2023). The infections are acquired from infected prey, or by direct contact with infected sea birds or through indirect contact via the environment (Mirolo et al., 2023; Lair et al., 2024). Farmed mustelids are likely to be infected through introductions into areas with high densities of birds and where mammal-to-mammal amplification of infection occurs (EFSA, 2023). The infection route of dairy cattle is distinct, tropism in the mammary gland, and the transmission is mostly from fomites and contaminated milking equipment, but not from respiratory exposure (Baker et al., 2024; Nguyen et al., 2025). Moreover, H5N1 remains viable for long periods after high-titre raw milk contamination in milking units, which greatly aids and facilitates the spread of the virus within the farm system (Figure 2; Le Sage et al., 2024).

Dairy Cattle: Mammary Tropism, Experimental Pathogenesis, and Immunity

For dairy cattle, the primary site of infection is the mammary gland epithelium, as evidenced by the large number of SA-alpha-2,3-linked receptor glycans on the luminal surface of alveolar lactocytes (Nelli et al., 2024; Kristensen et al., 2024). The concurrent expression of NeuGc-capped receptors (Hassard et al., 2026), approximately doubling accessible receptor density, together with the PB2 M631L and PA K497R polymerase adaptations tailored to bovine ANP32A, provide an extremely permissive replication environment (Nguyen et al., 2025; Dholakia et al., 2026). Thus, the concentration of viruses in raw milk reach 10^8-10^9 $TCID_{50}$/mL (Caserta et al., 2024; Halwe et al., 2025), far higher than concentrations in respiratory secretions of infected birds or other mammals. A rapid interstate spread followed a single wild-bird spillover into US dairy cattle, confirmed across 1,093 herds in 19 states by April 2026 (Nguyen et al., 2025; USDA-APHIS, 2026a).

Infection studies with bovine H5N1 have been conducted independently twice, both extensively characterizing the virus' ability to cause disease in cattle. Halwe et al (2025) intramammary inoculated lactating Holstein-Friesian cows of B3.13 genotype with moderate severity of necrotizing mastitis and high fever, but oronasal inoculated calves of B3.13 genotype severe nasal shedding with no transmission to sentinel calves. Baker et al. (2025) found severe mammary pathology with both routes of aerosol respiratory challenge in heifers and intramammary inoculation in lactating cows. One of the key results of the Halwe et al. study was that an isolate from a contemporary European

wild bird gave the same results as B3.13 in the same experimental set-up, thereby demonstrating that severe bovine mammary tropism is not unique to B3.13, but is also seen with genotypically different 2.3.4.4b isolates. The implication is that the bovine mammary niche might more generally be strongly permissive for the broader clade, and not just for B3.13-specific adaptations.

The H5N1 virus can be inactivated by pasteurization at 72°C for 15 seconds (Schafers et al., 2025). Guan et al. (2024) demonstrated that H5N1-positive raw cow milk inoculated into the nose of mice and ferrets resulted in infection, but not efficiently aerosolized from ferrets to ferrets, indicating that raw milk is an infection vehicle but is not an efficient aerosol source; and Kaiser et al. (2024) showed in independent experiments that H5N1-positive raw cow milk was also unable to infect mice or ferrets at room temperature, but was able to do so at both 63°C and 72°C, confirming that raw milk is an infection vehicle and is not an efficient aerosol source of infection at room temperature. However, raw milk, colostrum, and milk-contaminated equipment surfaces remain direct, high-titre zoonotic hazards (Burrough et al., 2024; Caserta et al., 2024). Retail amplicon whole-genome sequencing has since demonstrated that viral RNA persists through pasteurization and can be recovered from store-bought dairy products across multiple states (Lail et al., 2025). This finding has both surveillance utility and communication challenge: the detection of viral RNA in pasteurized milk does not indicate viable viruses, but it triggered substantial public concern about milk safety in 2024 to 2025. A review paper addressing both scientific and public health audiences should be explicit that RNA detection and infectious virus detection are not equivalent, and that FDA testing confirmed no infectious virus in commercially pasteurized products (FDA, 2025).

Facciuolo et al. (2025) demonstrated that dairy cows develop robust humoral immunity following primary H5N1 infection, which protects against homologous reinfection via the intramammary route. Primary infection induced HA-specific neutralizing antibody production, and experimentally rechallenged cows showed substantially attenuated clinical signs and reduced viral shedding, providing the most direct experimental evidence that bovine vaccination is immunologically viable. However, Tarbuck et al. (2026) found that natural immunity is non-sterilizing and strain-specific, while convalescent cows showed complete clinical protection against homologous B3.13 rechallenge one-year post-infection; heterologous rechallenge with the D1.1 genotype caused transient clinical disease and limited infectious viral shedding, with convalescent cows experiencing significantly milder disease than naive controls. These findings imply that the viral diversity accumulating in the US dairy cattle reservoir could progressively erode naturally acquired herd immunity, reinforcing the case for clade-matched vaccination before immune evasion becomes established.

Further experimental work has characterized the dry cow model as a methodologically useful alternative to lactating cow studies. Cool et al. (2026) demonstrated that cows 21 days into the dry-off period are highly susceptible to intramammary H5N1 inoculation and exhibit severe clinical and pathological outcomes similar to those observed in lactating cows. Since dry cows are not producing milk, the viral dynamics within individual quarters can be observed without the confounding influence of milk ejection and milking disruptions. This assists in a more accurate analysis of how the virus replicates and is spread within the mammary gland.

Other Mammalian Hosts: Pathogenesis and Transmission

Experimental models have played an important role in the evaluation of B3.13 isolates virulence and transmission capacity (Tarbuck et al., 2026). While mice of the C57BL/6J strain are more resistant to neuroinvasion, BALB/c mice are highly susceptible to B3.13, and typically exhibit severe neuroinvasive disease, which also depends on mouse strain (Tipih et al., 2025). This illustrates the host susceptibility importance of genetic background. Fabrizio et al. (2025) identified B3.13 viruses from cow milk and determined that both viruses are capable of causing 100% mortality at 101 TCID50 in mice, with neurologic symptoms such as ataxia and tremors, but they were not able to transmit to ferrets by air contact. Xiao et al. (2025) used reverse genetics to reconstruct the cattle isolate (A/dairy cattle/Texas/24-008749-003/2024) and compared it with A/Vietnam/1194/2004 in both non-lactating and lactating BALB/c mice. The cattle B3.13 construct was highly lethal with a mouse lethal dose (mLD_{50}) of 1.48 PFU by intranasal inoculation in BALB/c mice, with broader tissue tropism and higher titres in extrapulmonary organs than the Vietnam isolate. The reported mLD_{50} value applies specifically to this reverse-genetics B3.13 construct in BALB/c mice. Because values vary significantly across strains, routes, and viral histories, these results should not be directly extrapolated to other species or contexts.

Domestic cats (*Felis catus*) have emerged as a high-risk companion animal link between dairy farm infection chains and household human exposure. Fatal infections have been documented in indoor cats epidemiologically traced to dairy farm worker households, with ingestion of raw milk or colostrum from infected cows as the most plausible exposure route (Naraharisetti et al., 2025). A broad meta-analysis of all feline H5N1 cases documented a case fatality rate of 74% (Bonilla-Aldana et al., 2025), and more recent systematic reviews focusing specifically on RT-PCR-confirmed cases of clade 2.3.4.4b indicate a significantly higher mortality rate of approximately 89.6% (Coleman & Bemis, 2025), establishing cats as highly susceptible sentinel animals warranting active household surveillance. Extending the post-ingestion picture, Frye et al. (2025) isolated infectious H5N1 virus from the urine of domestic cats following ingestion of contaminated raw milk, the only study to demonstrate shedding of replication-competent virus in a non-respiratory compartment in any mammalian species during the current panzootic (Table 2).

This urinary shedding observing is related to household contamination pathways and highlights the case for treating domestic cats as sentinel animals that may passively distribute viruses via the home environment.

Marine Mammals and Multinational Pinniped Transmission

South American pinniped mass mortality occurrences totaled more than 52,000 confirmed individual elephant seals, fur seals and sea lions across Peru, Chile, Argentina, Uruguay, and sub-Antarctic territories (Leguia et al., 2023; Uhart et al., 2024; Kuiken et al., 2026). Phylogenetic analyses demonstrated sustained multinational pinniped-to-pinniped horizontal transmission, with PB2 D701N convergently selected as the dominant polymerase adaptation in isolates from geographically separated colonies (Pardo-Roa et al., 2025; Uhart et al., 2025), constituting the clearest genomic evidence of directional positive selection in any mammalian host outside the dairy cattle system (Leguia et al., 2023; Pardo-Roa et al., 2025). Elephant seal pups (*Mirounga leonina)* mortality exceeded 95% in some affected colonies (Campagna et al., 2024; Uhart et al., 2024). Confirmation of H5N1 on mainland Antarctica by December 2023, affecting both seabirds and marine mammals, represents the most extreme geographic extension of any HPAI panzootic (Banyard et al., 2024; Uhart et al., 2025). While phylogenetic data strongly supports pinniped-to-pinniped transmission, heavy surveillance reliance on coastal research stations suggests that mortality in remote, ice-dependent habitats is systematically underrepresented.

Domestic Swine: Susceptibility, Limited Transmission, and Reassortment Risk

H5N1 was confirmed in US domestic swine for the first time in late 2024, the first natural detection in pigs during the current panzootic (USDA-APHIS, 2025; EFSA, 2025). Kwon et al. (2025) studied pathogenesis and transmissibility, infecting pigs oro-respiratorially with the B3.13 virus A/Cattle/Texas/063224-24-1/2024, which was derived from the bovine virus receptor (BVR). They observed productive replication mainly in the lower respiratory tract with seroconversion in most of the principal-infected pigs and no self-limitation or spread to sentinel contact pigs. The HA gene from one pig contained a mutation, S136N, which was found at ~32% minority variant frequency in the bronchoalveolar lavage fluid. This mutation we wish to explain in particular is S136N, being an N-glycosylation sequon introduced at HA 136 which sterically obscures the receptor binding site with avian-type SAs leading to a reduction in the affinity of the avian-type SA and a shift in the equilibrium towards alpha-2,6-NeuAc engagement. The occurrence of this mutation at minority frequency in the pig's respiratory tract, even before consensus, is the first experimental evidence of selection pressure directing the mutation towards human type receptor binding where pigs have been exposed to bovine-derived B3.13. While this is reassuring in that no transmission to sentinel pigs, to date, is somewhat comforting, it should be considered a minimum, not maximum, estimate of swine risk due to the following reasons: The isolate used was from early 2024 prior to significant within-cattle evolution of the virus, experimental housing densities differ from commercial swine operations, and there are no published experimental challenge data from swine.

One of the key issues to consider is the possibility of reassortment from co-infection of swine with B3.13 and human seasonal IAV. The respiratory epithelium of pigs expresses all four major types of sialic acid receptors (SA-alpha-2,3 and SA-alpha-2,6, both with NeuAc and NeuGc), and the pig ANP32A has been shown to be an unusually effective cofactor for the activity of the hemi-annihilator polymerase of avian IAV, relative to the other ANP32A isoforms of mammals (Peacock et al., 2020; EFSA et al., 2025). Co-infection of contemporary human seasonal IAVs and B3.13, in the presence of simultaneous exposure to the virus, may lead to the emergence of chimeric viruses with human-adapted gene contents and 2.3.4.4b surface NA, which is a well-established route of emergence of pandemic IAVs (EFSA, 2025; Kwon et al., 2025).

Zoonotic Risk and Human Infections

There have been 71 confirmed cases of human infection with H5N1 in the US since February 2024, and nearly all cases have occurred due to occupational exposure on dairy farms (Rolfes et al., 2025; CDC, 2026). From March 2024 to May 2025, 41 cases were linked to dairy cattle, 24 to commercial poultry, 2 to backyard poultry and 3 were not associated with specific exposure sources. Most clinical presentations were mild, including 89% with conjunctivitis, 46% with fever, 41% with respiratory symptoms, 4 patients requiring hospitalisation and 1 death. Importantly, intensive investigations in all these cases did not reveal any person-to-person transmission (CDC, 2026). The infection cluster in California, the largest of any state, occurred between September and December 2024, with 37 dairy farm workers having confirmed exposure, from 29 dairy farms, and with a single patient being a pediatric patient who did not have a known animal exposure; this was the first pediatric H5N1 case identified in the United States (CDC, 2024a; Zhu et al., 2025). Among the 37 infected farm workers, 20 (54%) wore an eye protection, of which 13 wore goggles (Marshall et al., 2024). All were cases with no hospitalizations, which is consistent with the conjunctival route of exposure. A special scientific note about the pediatric case: The child was diagnosed with H5N1 infection via the real-time reverse transcription polymerase chain reaction (rRT-PCR) of nasal swab four weeks after testing negative for H5N1 antibody detection. Without the serological response, this is a very unusual occurrence among cases diagnosed by PCR as confirmed, but could represent very mild or limited infections or may be due to

the timing of when the antibody testing was done or, less likely, to a false PCR-positive diagnosis (Tobolowsky et al., 2025).

For case-based investigators relying on the number of confirmed cases in the denominator for calculating severity, this is a limitation. In Michigan and Colorado, H5N1 infection was identified via serology testing of 7% of dairy occupation samples (including cases that were asymptomatic), showing that surveillance of symptomatic cases underestimates the actual rate of infection (Mellis et al., 2024). According to pandemic risk modelling, it is likely the B3.13 has a higher effective reproductive number in humans than the data might suggest, but has not had enough secondary human cases to suggest a sustained human chain has developed. Another reason for the macaque route of exposure data, is that seroconversion occurred without clinical signs even with the oral-gastric inoculation of macaques at raw milk-relevant doses (Rosenke et al., 2025), implying subclinical infections in the general population may not be captured by the current surveillance framework. Biological and genotypic behaviours constitute the important barriers to mammalian potential risk, which is characteristic of the landscape of H5N1 mammalian risk. The first human fatal case of H5N1 in the USA was a patient in Louisiana with D1.1 genotype and suffered severe H5N1 human lower respiratory disease with neurological involvement, not B3.13, that was found in the USA (Rolfes et al., 2025). This is a type of D1.1 case, which is the classical presentation, however there have been studies using experimental models that have concentrated on the B3.13 dairy associated genotype. Experimental ferret transmissibility studies confirmed that A/Texas/37/2024, which is an isolate from a bovine case, was able to cause respiratory droplet transmission in 17%-33% of the ferret pairings and was fatal in the majority of experimental animals without the need for further adaptation (Eisfeld et al., 2024; Gu et al., 2024). Other studies, however, also confirmed the mode of transmission from cow milk to ferrets of B3.13 viruses: direct contact and not airborne contact (Fabrizio et al., 2025). To see how well the human isolate A/Texas/37/2024, B/Texas/2/2006, and B/Mexico/1/2024 could transmit through direct contact, a complementary hamster model study was conducted, which showed that transmission is more efficient when the human isolate is used, even though transmission via breathing is not efficient in all three strains (Nooruzzaman et al., 2026). Care needs to be taken in interpreting these transmission data; ferrets, like humans, are CMAH-null, and can only detect the fitness benefits of NeuGc adaptations in humans such as D104G and V147M (Ng et al., 2014; Tarbuck et al., 2026). The NeuGc-adapted strain in cattle has almost twice as many receptors as its NeuGc-naive previous strain, yet in ferrets does not possess such an advantage and thus has not been transmitted. Until CMAH-competent animal models are developed and validated, ferret data for NeuGc-adapted B3.13 strains provide a systematically incomplete picture of bovine-environment fitness (Nelli et al., 2024; Tarbuck et al., 2026). Despite these mammalian adaptations, three pandemic barriers nevertheless remain intact in all currently circulating isolates: absence of alpha-2,6-SA binding, a high HA fusion pH of approximately 5.9, and full NP MxA sensitivity (Eisfeld et al., 2024). Overcoming these constraints would require multiple co-occurring mutations, including the structurally significant Q226L shift, which has not yet been detected in a sustained human chain (Eisfeld et al., 2024; Tarbuck et al., 2026). This specific shift is structurally difficult for the H5 protein to achieve because the H5 receptor-binding pocket is narrower than that of H3 or H1 subtypes, meaning the Q226L substitution often results in a severe loss of overall binding avidity unless accompanied by compensatory mutations, such as the loss of the 158N glycosylation site, to stabilize the human-type receptor (Tharakaraman et al., 2013; Xiong et al., 2013).

One Health Surveillance and Countermeasures

Surveillance Innovations

The dairy cattle outbreak catalyzed the rapid deployment of several novel surveillance approaches with broad applicability to future HPAI events (USDA APHIS, 2024a; Tarbuck et al., 2026). Bulk tank raw milk PCR is now a federal interstate movement requirement, providing herd-level H5N1 screening that leverages the extraordinarily high viral titres in infected milk, which can exceed 10^6 to 10^8 $TCID_{50}$/mL in clinical cases (Caserta et al., 2024; USDA APHIS, 2024b; Halwe et al., 2025; Nguyen et al., 2025). An important operational limitation of this approach is that the assay detects viral RNA rather than replication-competent virus; RNA fragments from previously inactivated virus could, in principle, produce positive signals in bulk tank samples from herds where most cows have recently recovered (Eisfeld et al., 2024; FDA, 2024). This does not undermine bulk milk PCR as the most sensitive available herd-level screening tool, capable of detecting one infected cow in a herd of 500, but positive PCR results should prompt follow-up with viral isolation or individual animal sampling before concluding that active infection is ongoing, particularly when managing herd-movement decisions (Eisfeld et al., 2024; Nguyen et al., 2025).

Amplicon whole-genome sequencing of retail pasteurized dairy milk achieves greater than 90% H5N1 genome coverage from purchased products across 20 US states, enabling passive national-scale genomic surveillance without requiring direct farm access (Lail et al., 2025). This approach exploits the same biological fact that raises food safety concerns: viral RNA is heat-stable enough to survive pasteurization even when the infectious virus is not, with studies showing RNA persists even after exposure to 72°C for 15 seconds (FDA, 2025; Lail et al., 2025). FDA testing over 290 retail samples confirmed no infectious virus in commercially pasteurized products, and public communication should be unambiguous on this point (FDA, 2025).

The advent of wastewater-based epidemiology has ushered in a new era in H5N1 surveillance, and this approach has been used to detect viruses in municipal wastewater solids that were detected prior to the formal confirmation of H5 outbreaks in dairy herds in multiple catchments during the spring of 2024, using H5-specific droplet digital PCR (Wolfe et al., 2024; Louis et al., 2024). This helps public health control measures in time to contain outbreaks before they are diagnosed at a farm level, and can be used to ensure detection before a confirmed infections dairy herd has been reported (Louis et al., 2024; Kobayashi et al, 2025). Oxford Nanopore R10.4.1 chemistry is recommended for molecular characterization of HPAI vs R9.4.1 for whole genome sequencing of HPAI samples with 90% accuracy compared to ~60% at the critical boundary of low pathogenic/highly pathogenic discrimination (Ratcliff et al, 2024). Such improved sequencing accuracy is vital for assessing pathogenicity and monitoring the evolution of virus in environmental surveillance.

Medical Countermeasures

Vaccines

Vaccine development has proceeded rapidly in response to the emerging bovine H5N1 threat. A clade-matched replicating RNA (repRNA) vaccine encoding the 2.3.4.4b H5 HA conferred 100% single-dose protection against lethal bovine H5N1 challenge in mice, while a repRNA encoding the historical clade 1 HA provided only partial protection, directly establishing that a clade-matched antigen is required for full protection in contemporary challenge models (Hawman et al., 2025). The same repRNA platform subsequently achieved full protection against a lethal clade 2.3.4.4b challenge in cynomolgus macaques, resulting in robust neutralizing antibody titers and significantly reduced viral loads in the respiratory tract (Hawman et al., 2025).

Licensed adjuvanted human H5N1 vaccines, based on older clades, formulated with AS03 or MF59 adjuvants, generate cross-neutralizing antibodies against clade 2.3.4.4b at seroconversion rates of 60% to 95% with two to three doses, providing bridging immunity while clade-matched vaccines are produced (Khurana et al., 2024). Fabrizio et al. (2025) confirmed that sera from ferrets vaccinated with WHO-recommended candidate vaccine viruses (IDCDC-RG78A and NIID-002) (Miyakawa et al., 2024; WHO, 2024) for H5N1 clade 2.3.4.4b effectively neutralize B3.13 viruses, validating the current stockpile strategy. Furthermore, a novel mRNA-LNP bovine vaccine provided complete protection against a high-dose H5N1 challenge in lactating dairy cows by the 19th week post-vaccination with no adverse effects on health or milk production (Kong et al., 2026). These findings, along with the development of natural immunity after initial exposure in dairy cows of Facciuolo et al. (2025), support the notion that vaccination would be highly effective at inducing protective antibody responses in cattle.

5.2.2. Antivirals

Oseltamivir, zanamivir, and peramivir retain full activity against all characterized H5N1 (clade 2.3.4.4b, genotype B3.13) isolates, as phenotypic assays show no significant elevation in half-maximal inhibitory concentration (IC_{50}) values for these neuraminidase inhibitors (Hussain et al., 2025; Fabrizio et al., 2025; WHO, 2026). Also, Pascua et al. (2026) confirmed the efficacy of these neuraminidase inhibitors against the current strain despite the presence of PA-I38M mutations in specific isolates. However, the NA-H275Y substitution (N1 numbering) remains the most significant threat to this class. This mutation, which disrupts the binding pocket for oseltamivir and peramivir while sparing zanamivir, has been detected at low frequencies in animal-derived clade 2.3.4.4b viruses (Pascua et al., 2026). Notably, recombinant NA protein assays have confirmed that NA-H275Y confers highly reduced inhibition, increasing IC_{50} values up to 184-fold for oseltamivir and 1724-fold for peramivir, and NA-H275Y is one of the few resistance-conferring mutations that does not significantly impair enzyme function or viral fitness in the B3.13 background, facilitating its potential for spontaneous emergence and persistence (Fabrizio et al., 2025; Pascua et al., 2026). Other markers of reduced NAI susceptibility, such as NA-I223V and NA-S247N, have been identified in avian isolates but have not yet become fixed in the bovine-associated lineages (Nguyen et al., 2023).

Cap-dependent endonuclease inhibitors, such as baloxavir marboxil, target the endonuclease activity of the PA protein. The principal marker of resistance is the PA-I38X substitution (where X is T, M, or F). In late 2024, a human isolate (A/California/150/2024) was found to harbor the PA-I38M mutation, which conferred a 17-fold reduction in baloxavir susceptibility (Fabrizio et al., 2025; Pascua et al., 2026). Mechanistically, the substitution of isoleucine with methionine at position 38 sterically hinders the binding of baloxavir within the cap-snatching site. In murine models, this mutation significantly reduced the efficacy of oral baloxavir treatment across multiple dosage levels, resulting in higher viral titers and lower survival rates compared to wild-type controls (Fabrizio et al., 2025; Pascua et al., 2026). While PA-I38T typically confers a more dramatic reduction in susceptibility (up to 614-fold depending on the genetic background), PA-I38M is more frequently observed in recent H5N1 clusters, possibly due to a lower fitness cost in the bovine-adapted polymerase complex (Pascua et al., 2026).

Adamantane resistance is primarily mediated by the M2-S31N substitution. While this mutation is nearly universal in seasonal H3N2 and H1N1pdm09 viruses, its prevalence in clade 2.3.4.4b is genotype dependent. It is also a defining feature of the D1.1 genotype but is notably absent in the predominant B3.13 genotype, which retains in vitro susceptibility to amantadine and rimantadine (Andreev et al., 2026; Fabrizio et al., 2025). However, in vivo studies have shown that amantadine fails to protect mice against lethal B3.13 infection, likely because the highly

efficient polymerase complex and rapid replication kinetics of this virus can overwhelm the inhibitory effects of M2 blockers (Andreev et al., 2026). In addition, the efficacy of the PB1-targeting favipiravir has been demonstrated only as marginal in human respiratory organoid models with very high effective concentration (EC50) indicating it is not the right candidate for use in monotherapy for the currently circulating H5N1 strains (Shichinohe et al., 2025). The collective results indicate the precarious state of the existing therapeutic arsenal, meaning that if mutations are spread, like I38M, and one drug class is lost, the clinical management of the influenza virus will be virtually left solely with neuraminidase inhibitors (Fabrizio et al., 2025; WHO, 2026).

Governance Gaps and Policy Priorities

Several important governance gaps were identified in the course of the response to the HPAI H5N1 outbreak in the United States, which may provide some lessons for future response efforts.Several critical governance gaps were identified during the HPAI H5N1 investigation in the U.S., which may serve as lessons for future investigations to contain HPAI H5N1 outbreaks (Owusu & Sanad, 2025; Lowen et al., 2025). One of the difficulties was the lack of sufficient financial compensation for voluntary testing of infected herds before movement restrictions were put in place, allowing the virus to spread undetected within affected farms (USDA, 2024; Owusu & Sanad, 2025). This lack of early diagnosis enabled the spread of the virus more widely, making containment more complex (Owusu & Sanad, 2025). Moreover, there was significant delay during the early phase of the outbreak in releasing key genomic sequence information. This precluded independent analysis of the phylogenies as well as hindered international scientific transparency, limiting the ability of the global scientific community to quickly understand and respond to the changing nature of the virus (Otto & Edgerton, 2025; Owusu & Sanad, 2025). Sub-optimal personal protective equipment (PPE) compliance was also a significant finding among the workers on the farms. An eye protection was not worn by a significant number of farm workers during early case ascertainment in Colorado, and during an outbreak of the disease later in California, this also seemed to be directly linked to the clinical presentation of conjunctivitis seen in humans (Marshall et al, 2024; Bagdasarian & Wineland, 2024; CDC, 2024b).

Lessons learned from these indicate three integrated policy response are necessary to aid efforts in future HPAI containment. Firstly, the current movement based-testing program needs to be replaced by continuous bulk milk surveillance of the herd, along with mandatory live public sequence reporting (Lail et al., 2025; USDA, 2024). This would help in getting ahead of an outbreak and prevent the critical delay that exists between detection of outbreak and genome sequencing, allowing a more agile reaction. Secondly, automated alerts for priority surveillance mutations (PSMs) should be incorporated into national bioinformatics workflows (Pinsky & Bradley, 2024; CDC, 2024c). These include specific NP residues (52, 100, 283, 313, and 357) associated with MxA resistance (Mänz et al., 2013; Götz et al., 2016), HA positions (110, 154, 216, and 318) critical for fusion pH reduction, HA residues (Q226 and G228 equivalent) for alpha-2,6 binding, and deep mutational scanning (DMS) NeuGc hotspots (H141, E142, T143, L145, K168, and Y173) in circulating bovine isolates (Dadonaite et al., 2024). Such alerts would help to proactively inform of the possibility of viral adaptation and zoonosis risk (Pinsky & Bradley, 2024; CDC, 2024c). Third, the speed at which clade-matched H5N1 mRNA vaccines are being fielded to dairy cows is an important intervention (Chiba et al., 2024; Kong et al., 2026; Santos et al., 2026). The approach to minimize evolutionary selective pressure within the primary mammalian reservoir while protecting the agricultural workforce from occupational zoonotic exposure is currently the most viable option to reduce evolutionary selective pressure.

Other key policy changes are needed across the dairy industry, including in areas of high risk (USDA-APHIS, 2024; Owusu & Sanad, 2025). These include stringent entry/exit, equipment disinfection and animal movement control procedures on the farm (USDA-APHIS, 2024). Vaccination strategies for dairy cattle need to be explored further and protocols for international trade need to be harmonised in order to create a coordinated response worldwide (EFSA AHAW Panel, 2025; Kamel et al., 2025). Building public health infrastructure, particularly at the local level, is also crucial in order to improve the ability to respond quickly (CDC, 2024d). Moreover, continued investment and research into genetic resistance to avian influenza in cattle and development of robust vaccines for use in cattle is critical (Kamel et al., 2025; Owusu & Sanad, 2025). Finally, science-based communication strategies are needed to accurately inform consumers of the safety of dairy products and thus, ensuring consumer confidence (FDA, 2025). To conclude, targeted financial support mechanisms should also be developed that will help the producers who are affected by outbreaks of the disease to cope with the severe economic losses.

Discussion

The U.S. dairy cattle H5N1 clade 2.3.4.4b is a significant change in the ecology of influenza viruses, as a bird virus has spilled over into a new mammal host. Since 2021, Clade 2.3.4.4b viruses have been spreading in the world and infecting millions of birds and leading to spillovers into various mammals, including sea lions, cats, mink and cattle (Capelastegui and Goldhill, 2025). The two predominant genotypes in 2024 in the USA were B3.13 in dairy cattle and D1.1 in poultry (Hatta et al., 2025).

The H5N1 B3.13 has been detected in the U.S. dairy herd and is an important epidemiological change because of the converging yet independent adaptive axes. The polymerase axis with mutated PB2 M631L and PA K497R is

responsible for efficient replication in bovine mammary epithelial cells and allows the virus to exploit the ANP32A-dominant bovine cellular environment, leading to dramatic increased viral titres in milk, which can drive fomite (via contaminated equipment) bovine-to-cow transmission (Sheppard et al., 2023; Fabrizio et al., 2025; Dholakia et al., 2026; Baker et al., 2024; Caserta et al., 2024). Additionally, these polymerase modifications promote coronavirus multiplication in primary human airway epithelial cells, which greatly increases the potential for occupational exposure to this virus (Dholakia et al., 2026; USDA-APHIS, 2026b).

NeuGc is a prevalent form of SA at the alveolar lactocyte surface, which represents about 50% of the sialic acid pool, and the receptor axis (HA D104G and V147M in the 130-loop) increases the initial attachment efficiency in bovine mammary tissue, with a broader use of the receptors (Caserta et al., 2024; Good et al., 2024; Hassard et al., 2026). These two adaptation axes helped increase the availability of receptors and boosted the efficiency of the polymerases, and that is why these mutations spread so fast and why so many herds spread the virus across jurisdictions by April 2026, and why the US outbreak of the disease in cattle was unprecedented in its scale.

A critical point of ongoing scientific discourse revolves around the capacity of bovine B3.13 HA to bind alpha-2,6-NeuAc, the canonical human upper respiratory tract receptor. So, several scientific controversies require direct acknowledgement. First, the question of whether bovine H5N1 strains can engage alpha-2,6-NeuAc to any measurable degree remains debated. Santos et al. (2025), Chopra et al. (2025), and Yang et al. (2025) consistently demonstrate poor alpha-2,6-NeuAc binding in B3.13 isolates by glycan microarray and BLI, while Good et al. (2024) reported expanded receptor breadth for a D104G mutant. The NeuGc broadening mechanism characterised by Hassard et al. (2026) may explain some earlier ambiguous alpha-2,6 signals if NeuGc-containing alpha-2,6 glycans were not separated from NeuAc-containing alpha-2,6 glycans in earlier array designs. Second, the strength of the evidence for mammal-to-mammal transmission in South American pinnipeds is phylogenetically compelling (Uhart et al., 2024). However, surveillance bias toward coastal research stations may inflate apparent transmission scope. Third, the partial ferret airborne transmissibility of A/Texas/37/2024 (17-33% droplet transmission pairs; Gu et al., 2024) is directly relevant but does not translate linearly to human pandemic potential, as the three additional barriers (alpha-2,6-SA binding, reduced HA fusion pH, and NP MxA resistance) remain intact.

The structural insights reported by Lin et al. (2024) simultaneously clarify and sharpen the understanding of the pandemic barrier. The confirmation that a single Q226L substitution in the 220-loop can switch HA specificity to human-type receptors, distinct from the 130-loop adaptions, resolves the structural basis for the receptor-switching step more precisely than was previously understood. This finding implies that the current observation of limited alpha-2,6 binding in circulating B3.13, while epidemiologically significant, is mechanistically tenuous, being merely one mutation removed from a potentially pandemic-enabling phenotype (Lin et al., 2024; Nguyen et al., 2025; Dholakia et al., 2026). While the partial ferret airborne transmissibility of A/Texas/37/2024 (17 to 33% droplet transmission pairs) does not translate linearly to human pandemic potential (Gu et al., 2024), as three independent barriers remain simultaneously intact. The historical pandemic precedent, where H2N2 and H3N2 emergence required receptor-binding changes at exactly Q226 and G228 in the context of broader reassortment, means that any detection of Q226L variants in routine surveillance should be treated as an immediate escalation trigger of public response.

At the end, six research priorities emerge from this synthesis. Experimental assessment of whether NeuGc-adapted B3.13 strains (D104G plus V147M) show altered transmissibility in ferrets, which lack NeuGc, is urgently needed, as current ferret models cannot detect NeuGc-specific fitness changes. Longitudinal whole-genome sequencing of chronically infected cattle herds is needed to characterise fixation dynamics of emerging mutations. Full animal-model characterisation of D1.1 bovine isolates is outstanding. Experimental determination of whether swine serves as a reassortment vessel for B3.13 and human seasonal IAV is critical following the 2024 US swine detection. Multi-host organoid models enabling direct replication comparisons across bovine mammary, porcine respiratory, and human bronchial tissues under control conditions would advance mechanistic understanding substantially. Finally, formal cost-effectiveness analyses of bovine H5N1 mRNA vaccination programmes are needed to inform policy deployment.

Conclusions

Clade 2.3.4.4b H5N1 has redrawn the host-range rules for HPAI. For the first time in the 30-year history of the Gs/GD lineage, sustained HPAI transmission has been established in a domestic ruminant species, and for the first time, two mechanistically distinct and orthogonal adaptive axes are operating simultaneously in the same host, each independently increasing bovine fitness and zoonotic exposure risk. Structural crystallography has now resolved the minimal mutational distance separating circulating B3.13 HA from human-type receptor specificity: a single Q226L substitution in the 220-loop. The pandemic barriers that remain intact as of early 2026 are real and consequential, specifically the absence of alpha-2,6-linked sialic acid binding, the high HA fusion pH (~5.9), and full NP sensitivity to human MxA restriction. Nevertheless, more than 1,000 infected dairy herds, over 70 human cases including the first US pediatric case, the emergence of a baloxavir-resistant clinical isolate, viral RNA detected in bovine semen, ongoing emergence of convergent mutations including PB2 E627K plus M631L double-mutant variants, and partial ferret aerosol transmissibility collectively define a pandemic risk level higher than at any prior point in H5N1 history.

The path to early detection and prevention of pandemic emergence requires integrated One Health surveillance combining mandatory bulk-milk genomics with real-time public sequence reporting, wastewater surveillance for early geographic warning, systematic wildlife and occupational health serosurveillance, and accelerated deployment of ruminant mRNA vaccines to reduce evolutionary pressure in the primary mammalian reservoir, represents the only realistic pathway to early detection of the adaptive mutations identified in this review whose emergence in circulating sequences would require immediate escalation of pandemic response.

Declarations

Ethical Approval Certificate

Not applicable. This study is a review article based on published literature and does not involve any experimental procedures with animals or humans.

Author Contribution Statement

Muhammad Ahsan Yaseen: Conceptualization, investigation, formal analysis, writing the original draft, review, and editing.

Muhammad Tahir Sarfraz Khan: Manuscript review and editing.

Faiza Abbas: Literature search and screening

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Conflict of Interest

The authors declare no conflict of interest.

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Abbreviations

ANP32A	Acidic leucine-rich nuclear phosphoprotein 32A
ANP32B	Acidic leucine-rich nuclear phosphoprotein 32B
HA	Hemagglutinin
RNP	Ribonucleoprotein complex
PB2	Polymerase basic protein 2
PA	Polymerase acidic protein
CMAH	Cytidine monophospho-N-acetylneuraminic acid hydroxylase
NeuAc	N-acetylneuraminic acid (sialic acid)
NeuGc	N-glycolylneuraminic acid (sialic acid)
SA	Sialic acid
α2,3 / α2,6	Alpha-2,3 or alpha-2,6 glycosidic linkage
$TCID_{50}$	Tissue culture infectious dose 50
mL	Milliliter
B3.13	H5N1 clade 2.3.4.4b genotype B3.13
H5N1	Highly pathogenic avian influenza H5N1
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
dN/dS	Ratio of nonsynonymous to synonymous substitutions

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