Review Article

Molecular Determinants and Epidemiological Patterns of H5N1 Avian Influenza within the Zoonotic Context

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Abstract | The continued evolution of highly pathogenic avian influenza (HPAI) strain H5N1 represents a mounting global threat that transcends species boundaries. Initially confined to avian populations, the virus has undergone molecular adaptations, most notably within clade 2.3.4.4b that now enable cross-species transmission to marine mammals, domestic cats, cattle, and humans. This review examines the molecular epidemiology of H5N1, highlighting key mutations, including PB2 E627K and HA Q226L, that facilitate mammalian replication and host receptor binding. Molecular diagnostics including conventional PCR, real-time PCR, clade-typing assays, short PCR protocols, and advanced platforms like next-generation sequencing (NGS) and whole-genome sequencing (WGS) have revolutionized detection and surveillance strategies. To visualize the virus's global trajectory, we conducted a temporal and spatial data analysis from 2004 to 2025 using bar graphs and global heat maps, leveraging outbreak records from WAHIS, CDC, and the Global Animal Disease Information System. These data reveal emerging reservoirs, persistent hotspots, and concerning patterns of host adaptation. Within a One Health framework, this review emphasizes the urgency of synchronized surveillance, cross-sectoral data sharing, and real-time analytics to preempt zoonotic spillovers. Looking ahead, the future of H5N1 containment lies in the integration of AI/ML tools for spillover risk forecasting, the development of mRNA-based or universal influenza vaccines, and the expansion of global genomic databases to enable real-time tracking of viral evolution. Strengthening diagnostic access in wildlife and marine mammal sectors and developing portable field-level detection systems remain high-priority goals.

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Introduction

Avian influenza viruses (AIVs) are a remarkable health issue at a global scale due to their high levels of circulation and the high rates of mortality (Kim *et al.*, 2023). The segmented genome of influenza A viruses including avian subtypes has at least 11 viral encoded proteins as encoded by 8 single-stranded RNA segments, including the surface antigens hemagglutinin (HA) and neuraminidase (NA). A total of 16 HA and 9 NA subtypes have been found in avian species which made them to be diverse in their antigenicity (Nakhaie *et al.*, 2018; Shi *et al.*, 2023). Diversity in the genes of these glycoproteins results in the cleaving of these proteins and acts as the basis of distinguishing the serotypes of AIV. On the basis of their virulence in chicken (measured as intravenous pathogenicity index [IVPI]), avian influenza viruses (AIVs) are classified into two types: highly pathogenic avian influenza viruses (HPAIV) and low pathogenic avian influenza viruses (LPAIV) (Liu *et al.*, 2023; Scheibner *et al.*, 2023). The development and spread of certain subtypes of HPAIV, H5N1, H5N8, H7N9 has recently caused significant threats to the health of the population. Of these, H5N1 has been found to be the most virulent as it has observed high rates of case fatality in both fowls and man (Kim *et al.*, 2023). HPAI H5N1 virus was initially identified in 1959 in poultry in Scotland, there are first positive cases of the disease in humans Hong Kong in 1997 (Claas *et al.*, 1998; Kim *et al.*, 2023; Shi *et al.*, 2023).

Such recent outbreaks of HPAI H5N1 infection in other parts of the world have contributed to increased health alert in the world. The H5N1 strain that is currently circulating belongs to the prototype A/goose/Guangdong/1/96 lineage (Krammer and Schultz-Cherry, 2023). The impact of this strain has resulted in one of the greatest outbreaks observed among wild avian populations and has also spread into the United States and several other countries (Adlhoch *et al.*, 2023). The epidemiological monitoring of 2022 and March 1, 2023, showed extensive detections of HPAI H5N1 in domestic poultry, captive birds, and wild birds in around 28 countries within Europe (Gelb Jr *et al.*, 2005). Notably, the effects of this outbreak did not limit itself to avian species, in Peru, 3,487 sea lions were reported dead directly as a result of the infection. Such significantly widened interspecies transmission capacity between pelicans and sea lions emphasizes complicated ecology of spreading viral infections among non-related hosts (Adlhoch C and 2023). Memorable contagion of avifauna can increase the probability of cross-species spread to economically relevant animals or even people, *yet also* allow maintenance of the virus in bird reservoirs (https://www.cdc.gov/flu/avianflu/data-map-wild-birds.html).

Global HPAI H5N1 animal outbreaks remind us of the nature of such urgent need to receive a complete cognition of the virus and its consequences to the community (Figure 1). An in-depth study on the virology as well as epidemiology of HPAI H5N1 becomes critically important so as to effectively and timely respond to the current outbreak of animals, limiting its effects as well as avoiding any further zoonotic transmissions, as there is an ever-increasing incidence of human infections and the possibility of their severity.

In March 2024, Texas circumstances were reported involving HPAI H5N1 infections in dairy cows (Caserta *et al.*, 2024). The result was surprising because influenza A viruses do not seem to generally cause productive infections in cattle, in contrast with influenza C and D viruses. The spillover event (probably in Texas, late in 2023) carried the H5N1 virus, recognized to belong to genotype B3. 13, into the dairy cattle population (Nguyen *et al.*, 2025). There was first noticed unexplained sickness and milk loss among dairy producers, however, it was only in March 2024 that the presence of H5N1 was confirmed. Since its first spillover, the virus was transmitted rather quickly among dairy cattle in the U.S., which is most likely facilitated by the interstate movement of infected animals. Up to February 22, 2025, 973 dairy herds in 17 states had been affected with this strain of H5N1 (https://www.aphis.usda.gov/livestock-poultry-disease/avian/avian-influenza/hpai-detections/hpai-confirmed-cas and es-livestock).

As the USDA did not apply mandatory nationwide milk surveillance until December 2024, infected herds were detected to a large extent through voluntary reporting, clinical manifestation or other production losses. It seems that transmission between lactating cow occur mainly through contaminated milk and milking equipment as opposed to living through the respiratory system. The milk of infected cows is rich in infectious virus-7 to 9 log 10 units per milliliter and the causative agent may be viable on milking equipment several days with the appropriate

environmental conditions (Le Sage *et al.*, 2024). The various infection pathways cause varied clinical presentations in cattle. Cows with infection via the intramammary route frequently develop overt disease, massive losses in milk yield, and mainly lose virus via milk when acute infection has cleared (Krammer *et al.*, 2025). On the contrary, animals infected through the respiratory route shed less virus and are usually not heavily affected by the disease (Campbell *et al.*, 2025; Halwe *et al.*, 2025).

The B3.13 genotype of the H5N1 that circulated in dairy cows has thwarted attempts to curtail the spread so far and its spread remains unabated. The detection of the D1.1 genotype in the dairy herds within Arizona and Nevada was also confirmed by the National Milk Testing Strategy in February 2025. Many HPAI H5N1 genotypes exist in the wild bird population and currently, there is no data to show that they have slowed in their spread (Youk *et al.*, 2023). B3.13 is mostly transmitted among avian hosts and has a unique lineage N1 neuraminidase, but in 2024 North America reported an increase in human infections with genotype D1.1. Out of this, one person died of the infection and another two experienced serious respiratory disease that forced them into the hospital (Hermann and Krammer, 2025). Even milder diseases linked with D1.1 genotypes were identified in the group of the poultry industries workers. During 2024, the proportion of both B3.13 and D1.1 genotypes -m about to the detecting growing clade 2.3.4.4b- enhanced the relative number of human infections attributed in North America. By February 22, 2025, 70 human cases were confirmed in the United States.

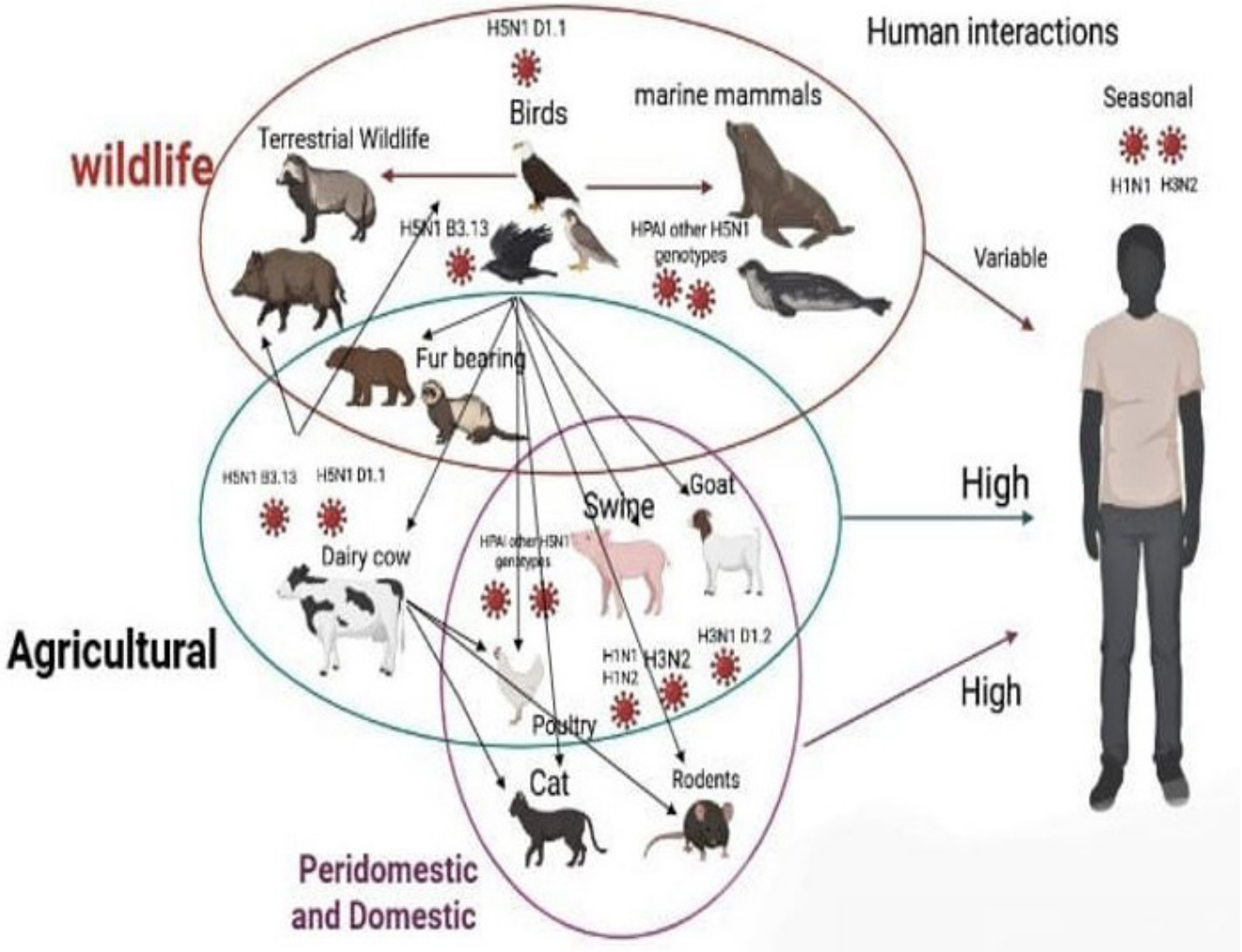

Figure 1: *Species of animals infected with H5N1 avian influenza. The species that are wild are defined in green and those that are livestock in blue as well as the species that are domestic or peridomestic in red. Orange arrows indicate transmission routes caused by an avian reservoir whereas transmission routes caused by a bovine host are illustrated using purple arrows. Influenza A virus icons show strains circulating in hosts with high potential of genetic reassortment.*

This review aims to bridge molecular virology with analytical epidemiology to illuminate the evolving threat of H5N1 avian influenza not just as a disease of birds, but as an increasingly unpredictable zoonotic challenge. By integrating insights from viral genomics, diagnostic advancements, and data-driven visualization, we strive to construct a clearer, more actionable understanding of how this virus mutates, spreads, and potentially crosses species barriers. Our goal is to equip scientists, veterinarians, public health officials, and policymakers with the tools and knowledge to anticipate spillover events before they occur, not merely react to them. In an era where viruses don't respect species or borders, this review underscores the urgent need for a unified One Health strategy that is proactive, interdisciplinary, and globally coordinated.

Molecular epidemiology of HPAI H5N1

Heat-sensitive glycoprotein that is the main protein component on the surface of the H5N1 virus, hemagglutinin (HA) is one of the key components in the early manifests of a disease. It assists attachment of viruses by attaching to certain receptors present on the host cell surface. Moreover, HA also mediates membrane fusion, which is a critical step in release of viral genome into the cytoplasm of the infected host cell (Kosik and Yewdell, 2019) (Figure 2). Conversely, neuraminidase (NA) plays its roles in the later phases of the viral replication cycle in cleaving glycoproteins and receptors on the surface of newly formed viral particles which consist of sialic acid. The enzymatic action of otherwise NA stimulates the progeny virions release by the infected cells and thus increases the viral diffusion into other cells or even different target host (McAuley *et al*., 2019).

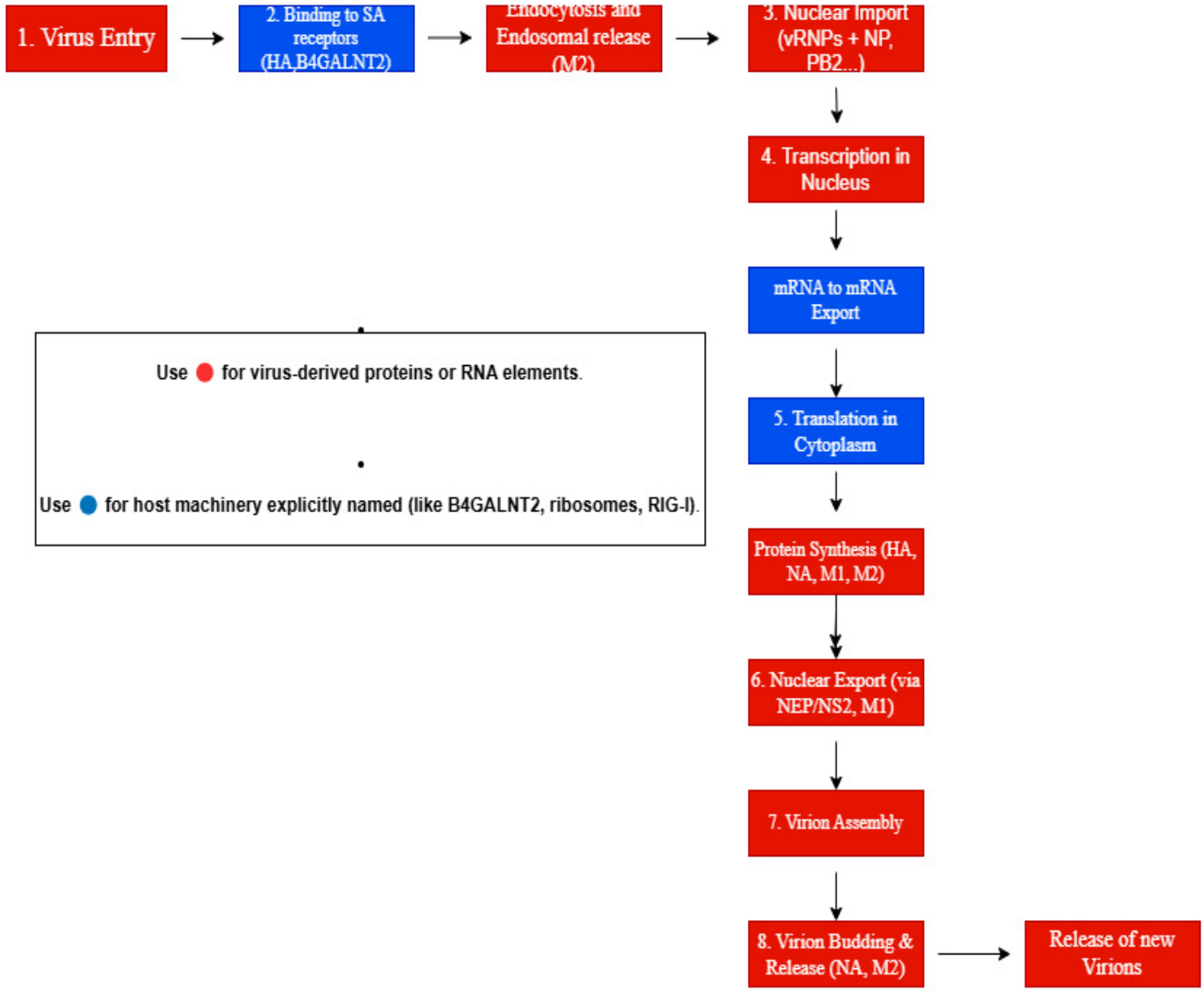

Figure 2: *Schematic representation of H5N1's infection mechanism: HA facilitates attachment and fusion, while NA enables progeny virion release from the host cell.*

HPAIH5N1 virus is a subtype of a virus called influenza A virus which is a virus known to belong to Orthomyxoviridae family. It contains a single-stranded, negative-sense RNA genome of about 13.5 kilobases in length. This genome is divided into eight separate pieces of RNA all of which are translated to generate a specific protein needed to replicate and cause pathogenesis. Its main viral proteins are hemagglutinin (HA, 568 amino acids), nucleoprotein (NP, 498 amino acids), neuraminidase (NA, 499 amino acids) matrix protein 1 (M1, 252 amino acids), matrix protein 2 (M2, 97 amino acids), polymerase basic 1 (PB1, 757 amino acids), polymerase basic 2 (PB2, 759 amino acids (Sangsiriwut *et al.*, 2018; Noor *et al.*, 2022) (Figure 3).

Synergistic action of hemagglutinin (HA) with neuraminidase (NA) is essential in the effective spread and replication of the H5N1 virus. Viral genome of HPAI H5N1 is bound by a nucleoprotein (NP) which forms a viral ribonucleoprotein complex (vRNP) by binding to the three subunits of the polymerase which are PA, PB1 and PB2. This complex of vRNP is central to important points of the viral life cycle such as the RNA transcription, replication and packaging (Te Velthuis and Fodor, 2016).

Clade dynamics and molecular evolution of H5N1

The evolution of HPAI H5N1 in the last 60 years has been substantial (Figure 4). The virus major division has been divided into ten big clades (0-9) along with some small and subclades which depict the genetic diversification of the virus, 2014. Other transcontinental outbreaks of note are the H5N1 clade 2.2 (2005-2006), 2.3.2.1c (2009-2010), H5N8 2.3.4.4a and H5N1 2.3.2.1c (2014-2015), and H5Ny 2.3.4.4b (2016-2017) (Bi *et al.*, 2016; Samantha *et al.*, 2020; Weifeng and George, 2021; Xiao-Ning *et al.*, 2021).

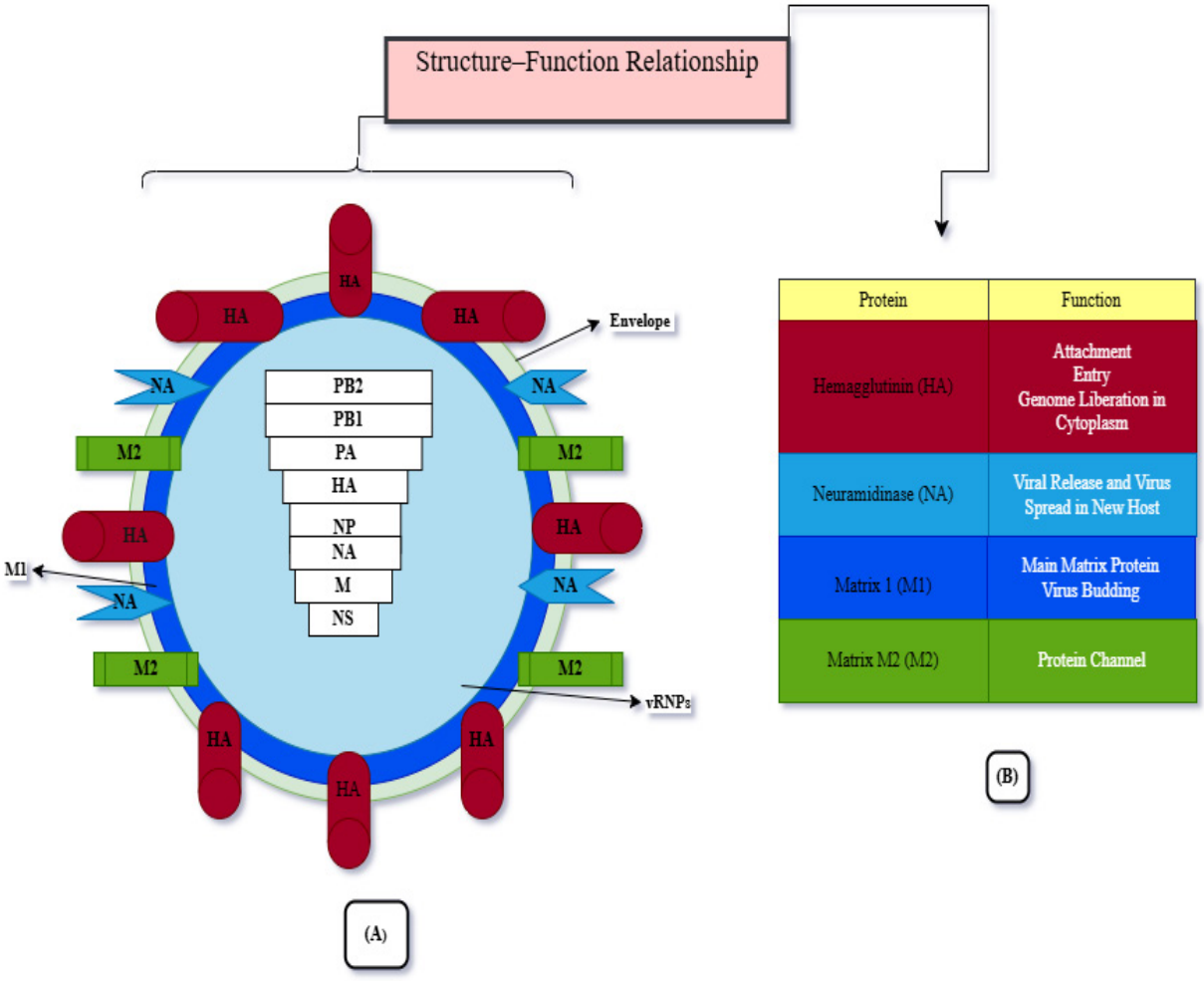

Figure 3: *(A) schematic representation of the segmented genome of highly pathogenic avian influenza virus H5N1, highlighting the eight RNA segments and corresponding encoded proteins. (B) Functional summary table of H5N1 viral proteins, outlining their roles in viral entry, replication, immune evasion, and virion assembly.*

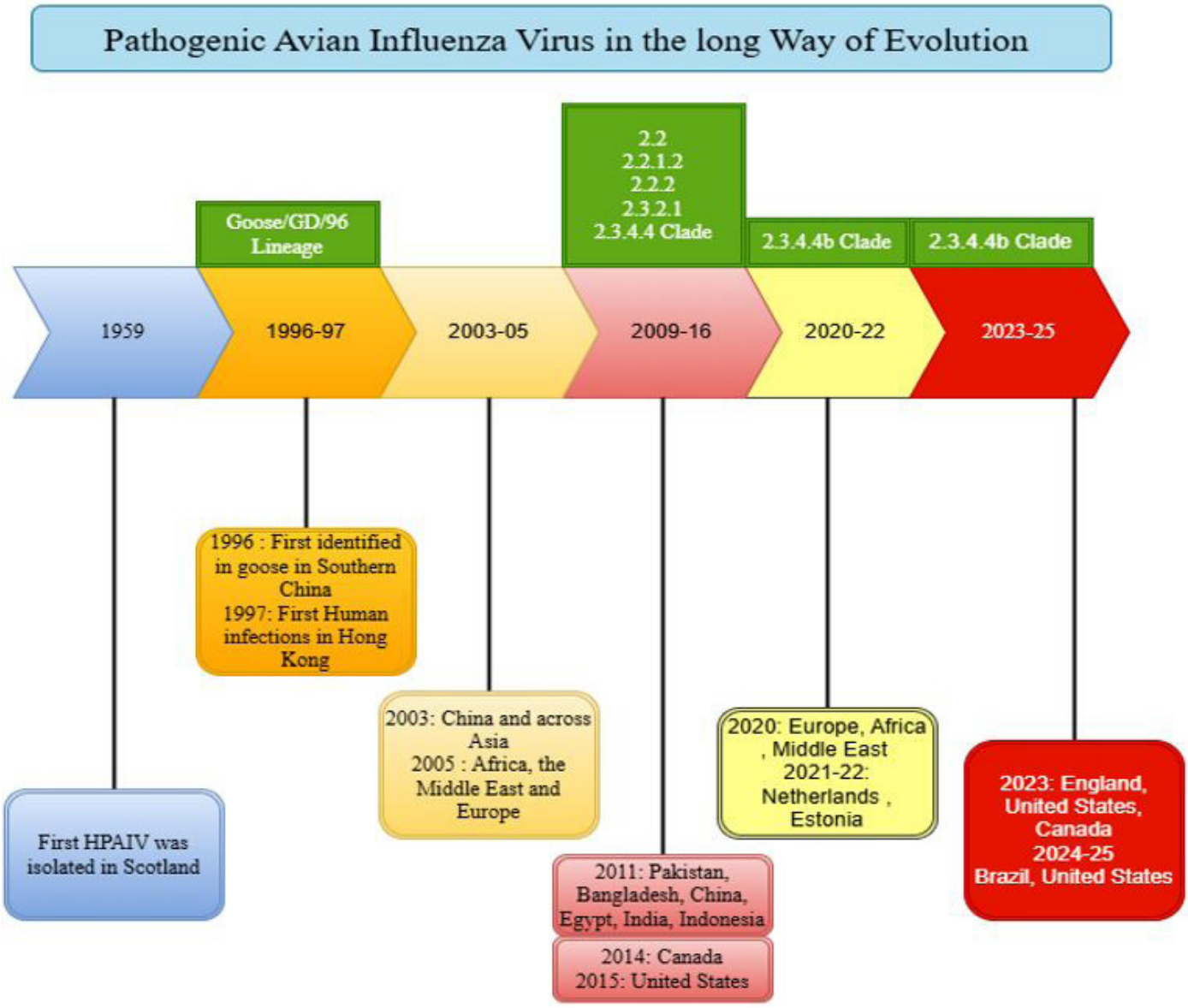

Figure 4: *Chronological flowchart illustrating the evolutionary trajectory of highly pathogenic avian influenza (HPAI) viruses from their first major outbreak in 1959 to current H5N1 clade dynamics in 2025. Each box highlights key epidemiological, genetic, or zoonotic milestones contributing to the virus's global persistence and pandemic potential.*

Through evolutionary analysis of Sanger sequencing of both bird and seal viral genomes, scientists deduced that there were at least two spillover events into the seal population. Least adaptation was observed in some strains of seals suggesting that human beings could also act as a possible intermediate host. Such results provide evidence that a continued monitoring of both coastal birds and marine mammals is necessary to compare the pandemic potential of H5N1 more accurately (Puryear *et al.*, 2023). Additional testing proved events of reassortment between some of the clades 2.3.2.1c, 2.3.2.1b, and 2.3.2.1a and the introduction of H9 viruses genetic material into clade 2.3.2 AIVs (Marinova-Petkova *et al.*, 2016; Nguyen *et al.*, 2016). The Ecuadorian isolates of the H5N1 viruses were identified to have been part of this highly pathogenic clade (Bruno *et al.*, 2023). Genetic similarities between these Ecuadorian strains with viruses isolated earlier in a Mexican falcon and Canadian red fox indicate the possibility of a route of transmission between the continents of North and South America through various host species. Also, the Ecuadorian strains are similar to those identified in the wild chicken in Chile, in Peruvian pelicans and in Venezuelan pelicans, highlighting the distribution of HPAIV clade 2.3.4.4b in different countries of South America. Domestic poultry is wide in the rural and low-income locations of Ecuador where a shortage of veterinary oversight and inferior public health infrastructure could predispose these people to issue of zoonotic transmission, which is common due to the proximal interaction between people and

polluted creatures (Bruno *et al*., 2023). A phylogenetic relationship study made by Noisumdaeng *et al*. (2022) has characterized the genetic evolution of HA and NA genes in HPAI H5N1 by using maximum likelihood (ML) applied to the phylogenetic reconstruction and to delineate two distinct HA clades (clade 1 and clade 2. 3. 4) and two lineages of NA within a corresponding H5 clade 1 viruses. Based on Bayesian molecular clock reconstruction, HA and NA genes of Thai H5N1 originated during the end of 2001 (HA: 2001.87, 95% HPD: 2001.342002.49; NA: 2002.38, 95 % HPD: 2001.992002.82), which points to the fact that the virus may have been circulating before its official isolation in 2004 (Noisumdaeng *et al*., 2022). Nucleotide similarity between Thai H5N1 HA clade 2.3.4 and interclade H5Nx 2.3.4.4 were 92.4196.8, with subclade classifications containing 2.3.4.1, 2.3.4.2 and 2.3.4.3 (Auewarakul *et al*., 2007; Noisumdaeng *et al*., 2022).

The SARS outbreak was moderated by mutation in the subunit of polymerase, PB1, PB2, and PA as well as H5N1 infection has important roles in the infectiousness and transmissibility coupled with resistance to neutralizing drugs (Danzy *et al*., 2014; Chauhan and Gordon, 2022). The catalytic center of the viral RNA polymerase complex, the PB1 subunit, can modulate replication fidelity, as well as host adaptation. H99Y in particular, has been implicated in the polymerase activity and replication efficiency in mammalian cells which are increased egregiously (Liang, 2023). Likewise, alterations in the PB2 subunit that is required in Cap binding and host adaptation have been found to enhance the frequency of replication dramatically and enhance adaptation to mammalian hosts.

The PB2 subunit substitution E627K involving the replacement of glutamic acid by lysine at amino acid position 627, has been proven to be one of the most well documented mutations that increase the adaptation of H5N1 to mammalian hosts. The given mutation is essential to effective reproduction within human cells, and it was firmly linked with the rise of pathogenicity in mammals. The other PB2 mutation is D701N that entails the substitution of aspartic acid at position 701 with asparagine. Similarly, to E627K, this substitution intensifies the activity of polymerase and was reported to correlate with increased virulence in mammalian system (Czudai-Matwich *et al*., 2014; Danzy *et al*., 2014; Liang, 2023). The PA subunit is the third component of the viral RNA polymerase complex, which becomes an important endonuclease, which is required to conduct the process of cap-snatching of RNA during the input of viral mRNA. The mutational studies have shown fine-grained amino acid residue-specific effects that have defined the PA through key residues that mediate replication ability as well as pathogenicity. Remarkably, mutations PA-44, PA-101, PA-127, PA-185, PA-224, PA-237, PA-241, PA-343, PA-347, PA-353, PA-383, and PA-573 were all reported as causing the changes in the replication kinetics and the virulence phenotype of H5N1 viruses (Zhong *et al*., 2018). Such mutations probably affect either contact between PA and host proteins or change the thermodynamics of the polymerase complex at various temperatures or host cellular conditions.

Molecular diagnostic techniques

Detection of avian influenza viruses using molecular diagnostic methods has been multiply used because of its highly sensitive diagnosis, fast turnaround time, scale ability and moderate pricing. Considering that outbreaks occur all over the world, these assays have been very useful in the response to outbreaks, surveillance and control of the outbreaks. Such molecular platforms as standard reverse transcription polymerase chain reaction (RT-PCR), real-time (qRT-PCR), and nucleic acid sequence-based amplification (NASBA) are commonly used to detect avian flu in poultry and have variations in terms of primers, probes, enzymes and frequencies in genes sequences (mostly in hemagglutinin (HA) and neuraminidase (NA) genes) that categorizes the avian flu under specific subtypes e.g., H5N1 (Wang *et al*., 2017; Fu *et al*., 2023). Real-time RT-PCR (qRT-PCR) is a gold standard of them. It provides once again high sensitivity and specificity, enables the use of quantitative viral RNA analyses and is routinely used in both diagnostic laboratories, as well as national surveillance schemes also in areas at high risk of infections (Slomka *et al*., 2023). This fast and precise detection of H5N1 is a prerequisite to early intervention, control and minimisation of economic losses accompanying outbreaks. Given their critical role, the following molecular diagnostic techniques are commonly used for the detection of H5N1 avian influenza virus.

Reverse-transcription polymerase chain reaction (RT-PCR)

Polymerase chain reaction (PCR) is a method

frequently used in the molecular sciences that is based on two oligonucleotide primers expedited enzymatically in neuropsychology, which increases a distinctive sequence of DNA in an *in vitro* specimen. Within the setting of avian influenza virus (AIV), reverse transcription PCR (RT-PCR) aims to use reverse transcriptase to produce complementary DNA (cDNA) made by viral RNA the template and is then multiplied using particular primers which pick up known sequences of the gene organisms (Doak and Zaïr, 2011). Payungporn *et al*. (2004) designed a one-step multiplex RT-PCR assay to simultaneously detect H5N1 by targeting the matrix (M), hemagglutinin (H5), and neuraminidase (N1) genes simultaneously. The single-tube, one-step format enhances specificity, decreases the turnaround time and greatly lessens the potential of cross-contamination. Notably there was no cross-reactivity with other subtypes reported (Payungporn *et al*., 2004). The methods of real-time RT-PCR (qRT-PCR) have since advanced and been more specific and wider adopted. The assays are fluorescence based (e.g. SYBR green, Taq Man probes) and can be used to measure viral load in real time. A qRT-PCR is much more sensitive than the traditional RT-PCR and virus isolation techniques, as well as much faster and ideal to provide high-throughput diagnosis. Nevertheless, it does not supersede the fact that its sensitivity would still be affected by PCR inhibitors, lack of proper RNA extraction, or RNA degradation, before amplification (Chen *et al*., 2007). Primers and probes against the H5, N1 and N8 genes were used to develop a multiplex qRT-PCR assay to enable detection of H5N1 and H5N8 simultaneously. Although being slightly less sensitive than the SYBR Green-based method, it had the same sensitivity as single plex RT-PCRs, and it was able to discriminate between subtypes using only a single reaction (Park *et al*., 2017). The RT-PCR with fluorescent real-time detection is already considered the most promising method of detecting the mRNA, especially in the diagnosis of the avian influenza viruses such as H5N1 (Wu *et al*., 2013). Nevertheless, despite the effectiveness, the procedure cannot be considered the standard method of detecting H5N1, probably because of the regional validation of the method or its non-uniformity internationally (Kis *et al*., 2013). Fluorescent-based real-time RT-PCR has been a major improvement in terms of enhancing the speed, accuracy and sensitivity in detecting the H5N1 avian influenza virus (AIV). Fluorescent-based real-time RT-PCR has been a major improvement in terms of enhancing the speed, accuracy and sensitivity in detecting the H5N1 avian influenza virus (AIV) (Ellis *et al*., 2007). Following this, Lu *et al*. (2008) brought TaqMan Minor Groove Binder (MGB) probes into real-time RT-PCRs. The probes provide more robust thermal stability and greater binding specificity to enable them to be used in direct fluorescence detection instead of needing the usage of a gel electrophoresis. This greatly reduces the time taken to detect it with a higher accuracy. Conversely, SYBR Green I, a non-specific dye that reversibly binds to double-stranded DNA has been widely used in most applications on account of relative simplicity and decreased cost. Contrary to the TaqMan-based systems, the use of SYBR Green allows using any set of primers and, therefore, is so versatile when it is related to rapid diagnosis development (Yin *et al*., 2001; Lu *et al*., 2008). In 2008, it was Naguib *et al*. (2015) who also implemented a SYBR Green powered real time fluorescent RT-PCR model that was specifically designed to identify H5N1 AIV. Owing to its rapidness and suitability to clinical screening, the method was appropriate in providing the full correspondence with the sequence analysis of viral RNA isolates (Naguib *et al*., 2015). Although RT-PCR in real-time is a key component of avian flu monitoring as it has high sensitivity, high specificity and a rapid turnaround, performance is very sensitive to the quality of the lab and operator skill. Poor technique, inadequate RNA extraction or contamination may result in either false positives or negatives and it becomes imperative that a strict control and standardization of each step of the diagnosis is followed.

Quantitative real-time PCR for quantification and clade tracking

The detection and subtyping of viruses, such as influenza A viruses (IAVs), are widely performed using quantitative real-time PCR (qRT-PCR) because of the high sensitivity and specificity coupled with quantitative ability of the method. In this method, real time observation is achieved by the use of fluorescent molecular dyes or chemically marked oligonucleotide probes to observe the course of amplification. The TaqMan probe (Applied Biosystems, CA, USA) is one of the most popular probe systems, which consists of a fluorophore at the 5 end and a quencher at the 3 end of oligonucleotide (Maru *et al*., 2025). In amplification, a probe anneals to a single-target site of the PCR product. When Taq polymerase extends the primers its inherent 5-3 exonuclease activity cleaves

the probe to separate the fluorophore and quencher. This leads to a measurable rise in fluorescence which is linearly related to the virus derivative of the PCR output allowing it to be assessed to quantify viral RNA. SYBR Green (Applied Biosystems, CA, USA) is another commonly used qRT-PCR method; its dye specifically binds to double-stranded DNA therefore it has bright fluorescence when intercalated. In spite of poor RNA/single-stranded DNA affinity with SYBR Green, bound to the double-stranded amplicon it emits bright fluorescent signal, which makes it possible to assess the success of the reaction. The sensitivity, specificity and wide range of quantification of both probe-based methods (e.g. TaqMan) as well as intercalator-based methods (e.g. SYBR Green) allow qRT-PCR to be among the best relevant tools used in diagnosing and monitoring IAVs (Dovas *et al.*, 2010; Yang *et al.*, 2020). Specifically, the TaqMan technique is preferred because of its high specificity and sensitivity, particularly avian IAVs. To detect the influenza A virus, qRT-PCR targeting the highly conserved gene, called the matrix (M) gene, is very common because it is highly preserved in all the subtypes of IAV. The strategy also increases the possibility of the assay to identify a diverse range of IAV strains (Habib-Bein *et al.*, 2003). TaqMan qRT-PCR system is very sensitive in detection of influenza A virus (IAV) in human and avian host. This type of probe-based system provides specific and very sensitive identification though it demands the design of target specific probes, and this may add to the complexity and price of assay. Comparatively, however, SYBR Green does not impose a particular probe, so it is preferable to use in terms of screening and diagnosis and is easier to work with. But since SYBR Green binds non-specifically to all double-stranded DNA, optimal reaction conditions are required to reduce non-specific amplification. In spite of this shortcoming, the procedure enables the analysis of melting curves, which are able to distinguish between IAV subtypes and slight variations in the sequence of matrix genes (Giglio *et al.*, 2003; Krafft *et al.*, 2005). This aspect renders SYBR Green especially appropriate to high-throughput surveillance and diagnostics systems where velocity and low cost are vital. The latest technology deployed in TaqMan qRT-PCR has made it possible to the specific identification of highly pathogenic avian influenza (HPAI) H5N1 virus (HA and NA genes). TaqMan assays have also accurately quantified the HPAI H5N1 in human and wild bird samples and quantificational data indicate that the technique is both robust and clinically useful (Agüero *et al.*, 2007; Ellis *et al.*, 2007). TaqMan method as well has superior analytical sensitivity. As an example, minor groove binder (MGB)-conjugated-based have been reported to monitor as low as 0.001 TCID 50/reaction or 0. 08 EID 50/reaction (Di Trani *et al.*, 2006). In an attempt to improve the performance of probes, high-affinity analogs of DNA (locked nucleic acids; LNAs) have been embedded into probe design. Compared with native probes, the LNA-modified probes enhance the stability of thermal and enzymatic degradation, and it also increases the assay sensitivity and stability of the assay (Wahlestedt *et al.*, 2000; Braasch and Corey, 2001). A qRT-PCR assay using a locked nucleic acid (LNA)-TaqMan was developed to distinguish between two lineages (clades 1 and 2) of the highly pathogenic avian influenza (HPAI) H5N1 virus amidst the disease infected samples in humans (Tran-Tan *et al.*, 2010). In this approach, the sensitivity of the diagnostic testing was found to be 97 percent and effectively identified 56 of 58 H5N1-positive clinical specimens to have an analytical sensitivity of 10 to 100 viral copies per reaction.

HPAI H5N1 viral RNA was also detected in chicken tissues such as cardiac tissue and skeletal muscle after intranasal inoculation, although this study was performed in the lab (Das *et al.*, 2008). In addition, qRT-PCR with SYBR Green allowed the effective subtyping of all HA and NA genes of avian IAVs that could be considered a versatile and affordable one used in wide-ranging surveillance (Tsukamoto *et al.*, 2012). There was also another development when Hoffmann *et al.* (2007) developed a qRT-PCR assay against the HA cleavage site using subtype-specific probes. FliH5-CSFAM was one of such probes; it had the ability to be used to specifically identify HPAI Qinghai-lineage H5N1 viruses or relatively associated Asian viruses (Hoffmann *et al.*, 2007). All these studies indicate significance of qRT-PCR probe based as well as intercalator based in clinical diagnosis, molecular epidemiology and active surveillance of HPAI H5N1 infection.

Super high-speed qRT-PCR (SHRT-PCR)

Super high-speed qRT-PCR (SHRT-PCR) has been described as an experimental adaptation of conventional real-time RT-PCR designed to shorten amplification time. Initial studies showed that, using specialized thermocycling systems, amplification could be completed in less than 20 minutes while

maintaining analytical performance comparable to standard RT-PCR assays (Sakurai *et al.*, 2011; Sakurai and Shibasaki, 2012). However, despite these early demonstrations, SHRT-PCR has not been widely adopted in routine diagnostic or surveillance settings. Its use is constrained by limited sample throughput, the need for dedicated instrumentation, and reduced suitability for fully quantitative analyses. As a result, conventional qRT-PCR and, more recently, digital PCR continue to represent the primary molecular diagnostic methods for influenza detection, while SHRT-PCR remains a niche approach with mainly experimental or proof-of-concept relevance.

Next-generation sequencing (NGS) and whole genome sequencing (WGS)

The molecular diagnosis and surveillance of highly pathogenic avian influenza (HPAI) H5N1 viruses are now rapidly evolving with next-generation sequencing (NGS) and whole-genome sequencing (WGS). In contrast to traditional approaches, including RT-PCR-based methods, which are directed at identifying specific segments of a gene, NGS can deliver an unbiased, high-throughput examination of all parts of the viral genome, using an unprecedented resolution to discover viral evolution, genetic variability, and transmission (Liu *et al.*, 2020; Marchenko *et al.*, 2024). The full genome sequencing of the eight gene regions of the influenza A virus (hemagglutinin (HA), neuraminidase (NA) and internal genes PB1, PB2, PA, NP, M, and NS) is possible using modern NGS platforms such as Illumina, Oxford Nanopore and Ion Torrent. This whole-genome strategy is vital in detecting and analyzing genetic reassortment event-another significant evolutionary strategy through which influenza viruses gain new characteristics such as host range and zoonotic generations. In H5N1 scenario, WGS has played a crucial role in expensive reassortments between the avian and mammalian influenza viruses and this act can increase the danger of an emerging pandemic (Kim *et al.*, 2025).

In addition to tracking viral evolution, NGS offers powerful mutation surveillance capabilities. Mutations in the HA gene such as Q226L and G228S are associated with increased binding to human-type sialic acid receptors, which could enhance human-to-human transmission. Similarly, mutations in the PB2 gene, such as E627K and D701N, have been linked to increased replication efficiency in mammalian hosts. The early identification of such mutations through WGS allows public health authorities to assess potential risks and implement control measures. Furthermore, WGS can detect changes in the neuraminidase gene (NA) that are known to confer resistance to antiviral drugs like oseltamivir (e.g., the H275Y mutation), thus supporting clinical decision-making and antiviral stewardship (Pawestri *et al.*, 2020; Alkie *et al.*, 2023).

Current gaps and limitations

Inadequate diagnostic access in wildlife/marine mammal surveillance

There remains a significant diagnostic gap in surveillance of HPAI in wildlife and marine mammals, especially with recent spillovers observed in seals and sea lions. Limited field-accessible tools and logistical barriers hinder early detection in these non-traditional hosts (Puryear and Runstadler, 2024).

Lack of rapid tools for field-level detection in non-avian hosts

Although RT-PCR and NGS are powerful, they are often lab-bound and unsuitable for remote field settings. The development of portable, species-agnostic diagnostics remains limited, affecting timely intervention during zoonotic outbreaks in mammals.

Data analytics and visualization

In the age of big data and cross-species pandemics, visual analytics has become indispensable for decoding the complex epidemiological behavior of H5N1 (Chretien *et al.*, 2014). Beyond molecular insights, data visualization allows us to observe trends over time, pinpoint geographic hotspots, and unravel the multi-host ecology of the virus. By translating raw outbreak records into intuitive graphs and heat maps, we can detect not just where and when the virus persists, but also how it adapts and circulates across hosts and ecosystems (Health, n.d; Nations n.d; Organization, n.d).

Data sources and synthesis

Influenza surveillance data summarized in this review were obtained from publicly available databases maintained by WAHIS, the U.S. Centers for Disease Control and Prevention (CDC), and the Global Animal Disease Information System (GADIS). Data were compiled from published surveillance reports and datasets for all available years; no primary data collection or automated web scraping was performed.

Only laboratory-confirmed influenza reports with clearly defined country-level and temporal information were included, while records lacking these details were excluded. To minimize duplication across sources, data were harmonized and summarized at the country–year level, ensuring that overlapping reports contributed only once to aggregated estimates. Data were available through 2025, the most recent reporting year at the time of manuscript preparation, and years with incomplete reporting were interpreted cautiously.

Figure 5 presents the global time-series of influenza prevalence aggregated across all reporting countries from 1995 to 2025 (Health, n.d; Nations n.d; Organization, n.d). During the early period (1995–2007), global prevalence remained relatively low and stable. This pattern likely reflects a combination of lower reported burden and more limited global surveillance coverage during this time.

A pronounced global peak is observed in 2009, corresponding to the H1N1 influenza pandemic, characterized by a rapid increase followed by an abrupt decline, consistent with a pandemic-driven event rather than gradual epidemiological change. Following this period, influenza prevalence did not return to pre-2009 levels. Instead, a sustained upward trend is evident from approximately 2011 through 2019, suggesting long-term changes in detected influenza burden, surveillance intensity, diagnostic capacity, or a combination thereof.

A sharp decline is observed during 2020–2021, which is unlikely to represent a true disappearance of influenza circulation. Rather, this reduction coincides with the COVID-19 pandemic period and likely reflects the combined effects of non-pharmaceutical interventions, altered healthcare-seeking behavior, and disruptions in routine influenza surveillance and reporting. Subsequently, influenza prevalence rebounded rapidly after 2021, reaching the highest levels observed in the time series, consistent with resumed surveillance and renewed circulation in the post-pandemic period.

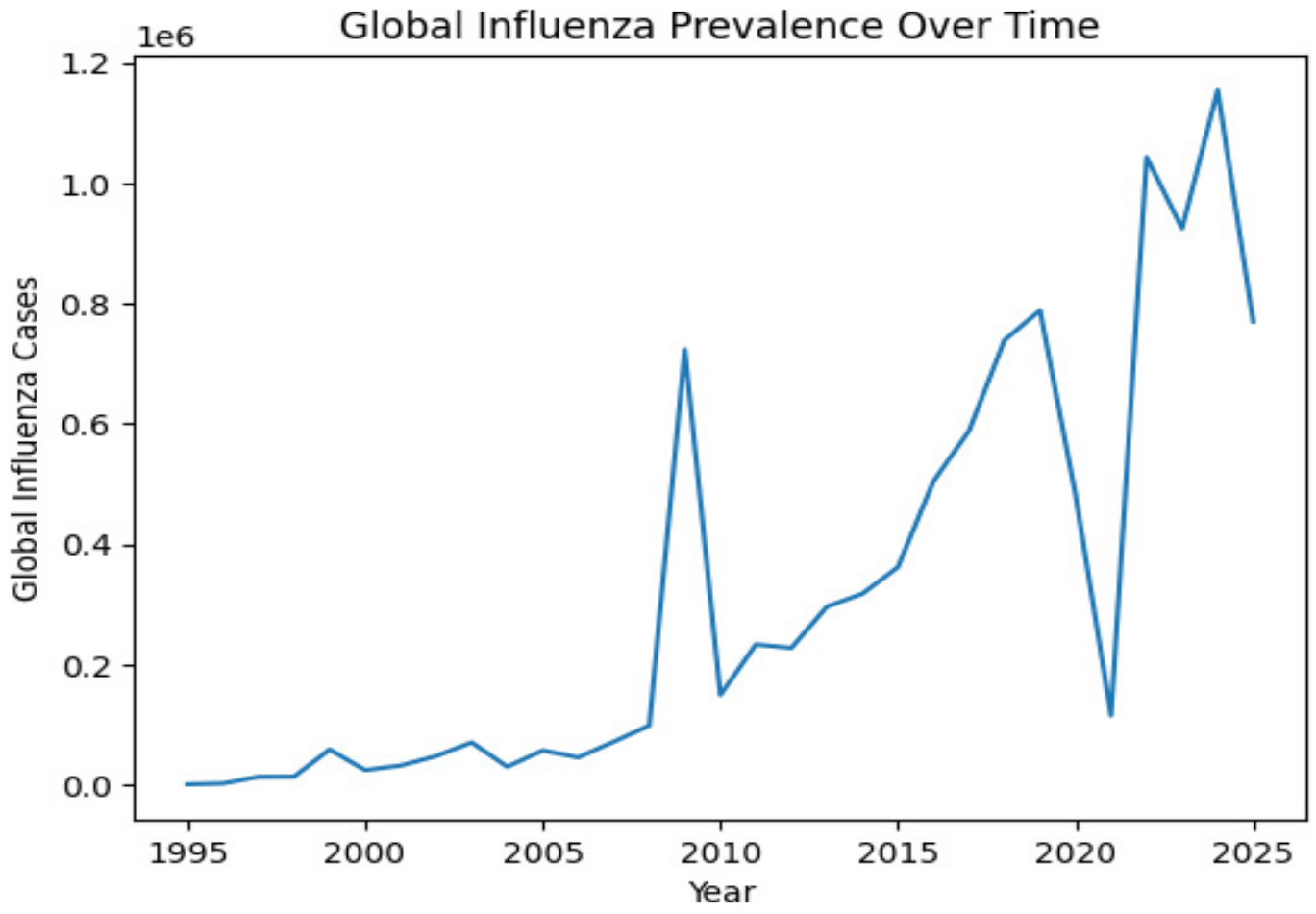

Figure 5: *Global influenza prevalence over time (1995–2025).*

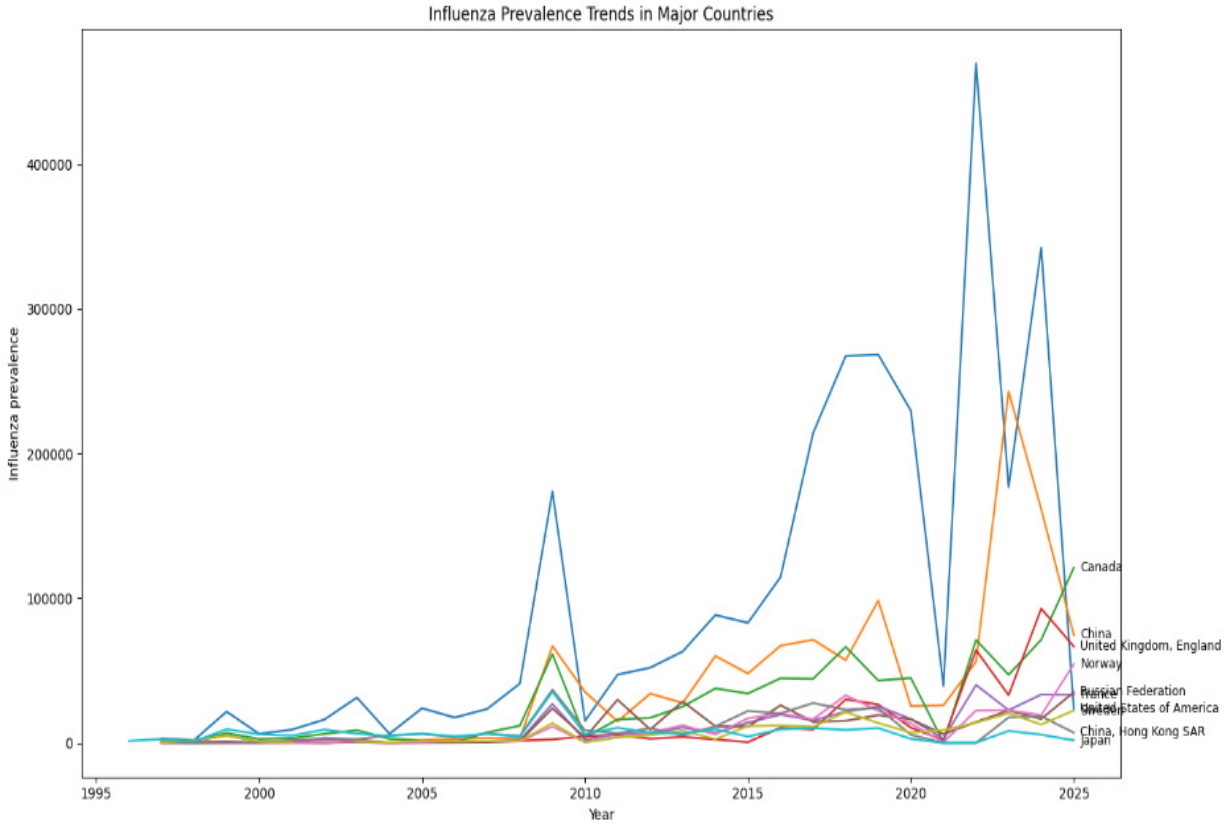

Figure 6: *Influenza prevalence trends in major countries.*

Annual influenza prevalence trends for the same set of major contributing countries shown in Figure 6. Country names are annotated directly on the curves to facilitate comparison of temporal dynamics, illustrating both synchronized pandemic-related changes and substantial differences in the magnitude and timing of national influenza burden.

Transmission to mammals and humans

Highly pathogenic influenza A (H5N1) viruses have been the cause of severe respiratory disease and high mortality in humans; nonetheless, continued human-to- human transmission is not a common occurrence (Herfst *et al.*, 2014). Nevertheless, genetic mutations or reassortments especially when the influenza strain is acquired by humans is of serious concern because they may promote the potential to be transmitted through air droplets and thus increase the possibility of another pandemic (Imai *et al.*, 2013). Recent outbreaks show rapid global spread, frequent spillover into mammals, and emerging mammal to mammal transmission, raising concerns about new evolutionary pathways and potential zoonotic threats (Peacock *et al.*, 2025) in Figure 7.

H5N1 avian influenza, first identified in domestic geese in China in 1996, has evolved into a highly diverse and globally widespread virus (Sacristán *et al.*, 2024). The H5 2.3.4.4 clade, emerging in 2004, gave rise to multiple subclades, notably 2.3.4.4b, which rapidly spread across Asia, Europe, Africa, and the Americas. Migratory birds played a central role in its unprecedented expansion into Central and South America, the Caribbean, and Antarctica by 2023. Recent outbreaks reveal an alarming pattern of cross-species transmission. In European fur farms, H5N1-infected mink, foxes, and raccoon dogs, with mammal-to-mammal transmission driven by viral mutations. In South America, sea lions and dolphins demonstrated sustained transmission marked by distinct adaptations (Sacristán *et al.*, 2024; Galli *et al.*, 2025). In March 2024, H5N1 clade 2.3.4.4b (B3.13) was detected for the first time in US domestic ruminants, infecting dairy cattle and goats. Infected cattle exhibited fever, gastrointestinal symptoms, neurological signs, and drastic drops in milk production, with high viral loads in raw milk. Viral presence in alpacas, blowflies, and farm environments suggests multiple transmission routes. Limited human, cat, and poultry infections have also been documented. While human transmission remains rare, these expanding zoonotic events raise significant concerns about H5N1's pandemic potential as it continues to adapt across diverse hosts.

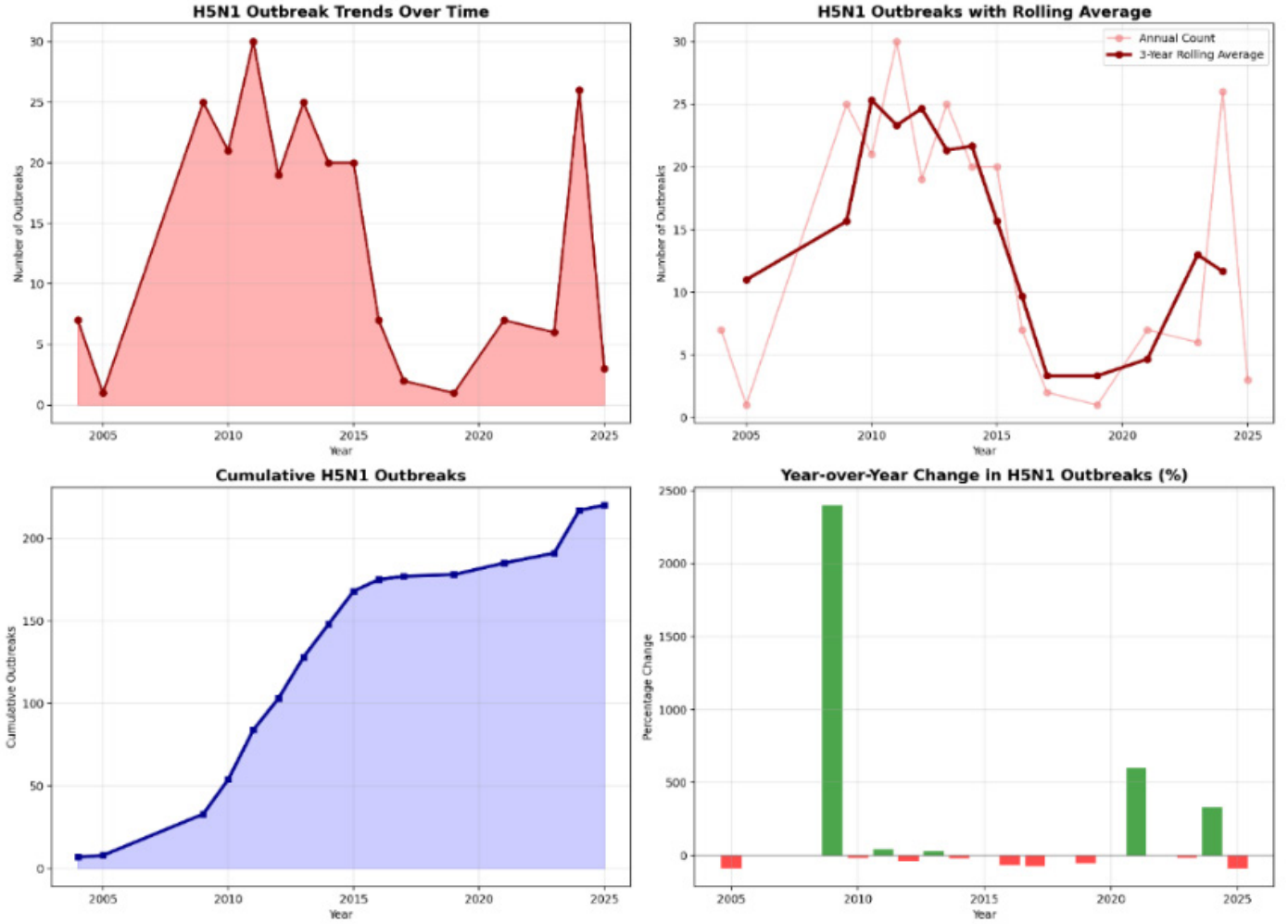

Figure 7: *Temporal analysis of H5N1 outbreaks from 2005 to 2025 using multiple time series visualizations.* Figure 7 *presents four complementary views of H5N1 outbreak dynamics over two decades: (A) annual outbreak counts showing raw temporal trends; (B) outbreak counts with a rolling average to highlight smoothed patterns; (C) cumulative outbreak totals indicating long-term disease burden; and (D) year-over-year percentage change in outbreak frequency, capturing shifts in epidemic intensity. Together, these panels reveal both short-term volatility and long-term persistence of H5N1 activity across time.*

Major die-offs among sea lions in Peru, Chile, Argentina, and Brazil have resulted in over 15,000 deaths, attributed to clade 2.3.4.4b strains carrying mutations associated with mammalian affinity (Leguia *et al.*, 2023; Tomás *et al.*, 2024). Evidence from Brazil and Uruguay supports sea lion-to-sea lion transmission, indicating localized mammalian transmission cycles (Tomás *et al.*, 2024). Similar patterns have been observed in seals and minks, particularly in fur farms, where sustained mammal-to-mammal transmission was linked to viral evolution.

In the U.S. and Europe, cats have been infected through exposure to raw dairy or infected birds, often exhibiting neurological symptoms and systemic disease (Ching and Ching, 2018). In 2024, clade 2.3.4.4b B3.13 was detected in U.S. dairy cattle, spreading between animals likely via contaminated milking equipment and resulting in fever, neurological symptoms, gastrointestinal distress, and significant milk production losses (Bassanese, 2025). Viral detection in associated species such as alpacas and sheep, as well as in environmental samples and blowflies, suggests diverse and indirect transmission routes. The virus's presence in farm environments emphasizes the complexity of its spread.

Influenza A viruses, especially H5N1, originate from wild aquatic birds, which act as natural reservoirs. Zoonotic transmission occurs through inhalation, ingestion of contaminated food or water, or direct contact with infected birds, secretions, or contaminated surfaces (Kalthoff *et al.*, 2010). Intermediate hosts like pigs and other mammals

(Figure 8) play a crucial role by allowing avian and human influenza viruses to co-infect and reassort, leading to new viral variants with potential human infectivity (Alexander and Brown, 2000). Backyard poultry farming, especially in developing countries, increases human exposure. Genetic mutations during replication or reassortment may enhance the virus's ability to infect human respiratory cells, increasing zoonotic and pandemic risk.

Forecasting spillover and strengthening preparedness: Future prospects in H5N1 surveillance and control

The strain H1N1 evolution into a severe pathogen is one of the major health concerns, the future of the surveillance system and response towards the Influezna control lies on the conjunction of genomic studies, AI Artificial intelligence, computational analysis and real time data analysis (Charostad *et al*., 2023). With the aim of enhancing genomics data bases to research and develop mRNA-based vaccines and to forecast the zoonotic spillover and mutation through computational tools (Dabla *et al*., 2021).

Modifying global genomic databases for artificial intelligence driven surveillance

Genomic databases mainly GISAID, NCBI GenBank, influenza research database severe are the pain pillars for influenza genomics, phylogenetic analysis and to track an outbreak by continuously monitoring. But it's time to change for repositories to evolve from manually achieving and analyzing data to have AI based systems that could results in high output data analysis and AI readable and machinable metadata structures (Daniels and McCauley, 2023; Musa *et al*., 2024). There are other key features that should be kept in mind to innovate databases. Good quality and high-resolution whole genome sequencing should be routine practice for suspected H5N1 across birds, poultry industry and clinical samples of humans should be a standard practice. Also, clinical status along with host species, location and date of sample collection should be accessible and annotated for maximum downstream usability (Azeem and Yoon, 2025). Moreover, genomic repositories should also be built with the scalability and modularity for AI to speed up the quality control, sequence and the metadata validation. These improvement in the structures in the databases will enable the prediction model tools to access tasks like tracking of mutation, antigenic drift and shift in influenza prediction of clade with better accuracy. These enhanced and modified system would be the backbone of the surveillance system that would ultimately identify the transmission dynamics of virus and risk associated with it.

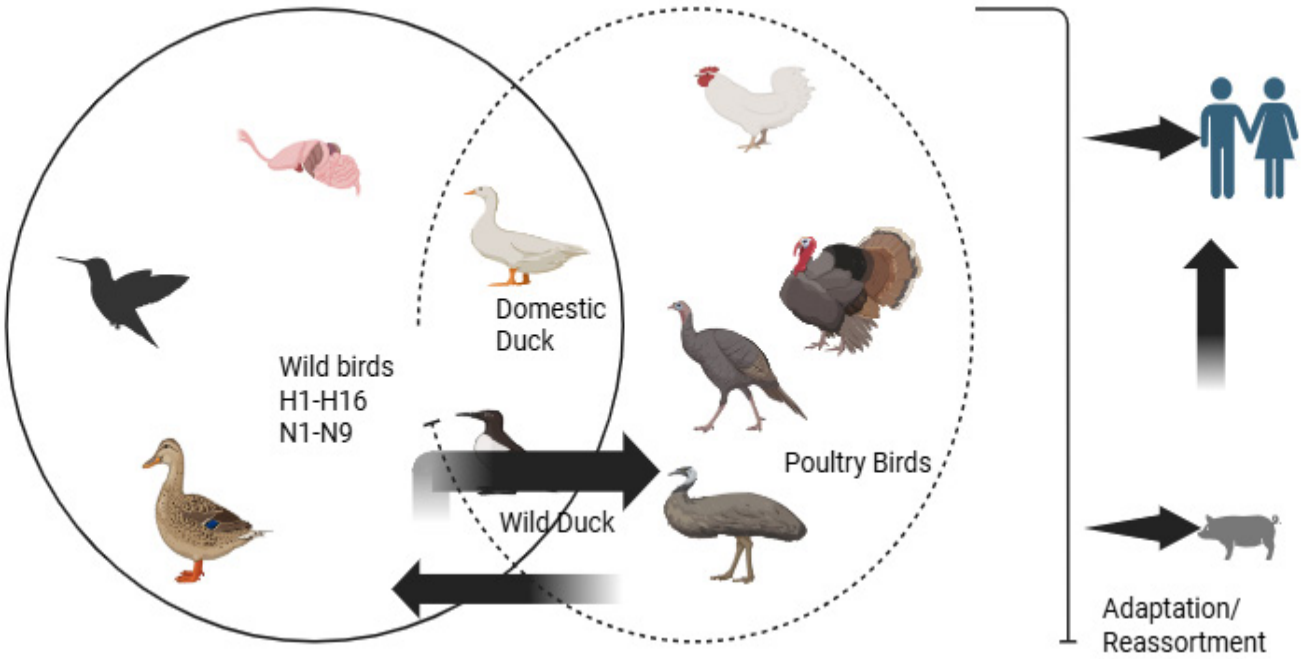

Figure 8: *The Transmission dynamics and host range of avian influenza viruses (AIVs), illustrating the interspecies spread of H5N1. Wild aquatic birds serve as the natural reservoir for all AIV subtypes (H1–H16, N1–N9), with viral exchange occurring between wild ducks and domestic waterfowl. This facilitates spillover into poultry species, where further viral adaptation and reassortment can occur. Mammalian hosts, including pigs, may act as intermediate mixing vessels, ultimately enabling zoonotic transmission to humans.*

Development of mRNA-based and universal vaccine development

As conventionally vaccines are laborious to develop and came with unwanted immune response. The development of new generation of vaccine for viruses with genetic drift and shift like H5N1 influenza could be gamechanger. As this was observed in COVID-19 pandemic, the success of the mRNA-based had revolutionized the vaccine development approaches across globe (Lim *et al*., 2024). Utilizing the updated genomics databases, circulating H5N1 strains could be analyzed and mRNA-based platform could be tailed to rapid prototyping and development of strain specific viruses (Shafi *et al*., 2025). Also, long terms efforts must be puts towards the development of vaccines that could be used globally and protect against multiple strains of Influenza. This could be done by targeting the conserved immunogenic epitopes like hemagglutinin (HA), internal viral protein like NP, M1 that would provide immunity across different clades and subtype of influenza (Olukitibi, 2024; Sidney *et al*., 2025). To provide better targeted immunity modern approaches, reverse vaccinology and computational vaccinology along with ML based prediction of epitope can improve antigen selection and immunogenic predictions of an epitope. To provide better accessibility, nasal sprays vaccines providing mucosal immunity are good options for low resource setting.

Artificial intelligence (AI) and machine learning (ML) for predicting zoonotic spillover

The most fascinating prospect lies in utilizing AI models and machine learning to predict the zoonotic spillover events of H5N1. Traditional methods are time consuming and are inflexible to adapt to dynamic environment. but modern advancement in AI could resolve this issue. Because they can handle large data set and can uncover the interaction between specie-specie interaction, host ecology and impact of environmental changes.

To effectively predict zoonotic spillover occurrences using AI and machine learning (ML) models, a wide range of multidimensional data sources including those that reflect the intricacy of emergence of pathogens is required. These models depend on combined data sets containing genomic data (e.g. codon usage bias, mutation rates, receptor-binding affinities, and reassortment signals), host ecology (e.g. species density, migration patterns, host-receptor compatibility, and phylogenetic relatedness), environmental and climatic factors (such as temperature variation) and climatic patterns, habitat degradation, and change of land use), and anthropogenic factors (e.g. urbanization, wildlife trade, livestock rearing, and biosecurity breaches (Choudhury *et al*., 2024; Bowyer *et al*., 2025). These parameters are converging, which allows AI models to identify the non-obvious patterns and create predictive signatures that can be characterized by spillover potential to be high.

Such rich datasets are processed with the assistance of various AI/ML approaches. To determine the classes of viruses into zoonotic and non-zoonotic classes, a supervised learning model is performed using random trees and XGBoost models to predict the possibility of host switching or acquiring an adaptable nature to humans (Murthy, 2023; Choudhury *et al*., 2024). On the other hand, the unsupervised machine learning techniques (e.g. clustering algorithms and principal component analysis (PCA) have been used to detect previously undocumented host-pathogen associations as well as ecological groupings of potential transmission concern. Also, sophisticated deep learning models have been utilized across the board on several applications, e.g., time-series outbreak predictions, and phylogenetic reconstruction, as well as the prediction of the host-pathogen protein-protein interaction (PPIs) (Wang *et al*., 2024). The spatiotemporal modeling algorithms can also dynamically map the changing risk areas, raising the indicators of early warnings since they depend on the time-resolved ecological or epidemiological developments.

A number of real-life platforms can be attributed to the operationalization of these approaches. An example of a project that uses AI models to better understand the ecology of viruses and their spillover is PREDICT, which was an initiative of USAID under the One Health program (Gwakisa *et al*., 2023). It allowed finding possible sources of zoonotic viruses in wildlife and mapping transmission hotspots, shedding light on how viruses work in different ecological systems and their ways to jump hosts. Spillover, produced by the EcoHealth Alliance, incorporates over 30 virological and ecological factors into open ML models in order to prioritise surveillance across potential novel viruses, through assigning risk scores to the unseen pathogen (Holmes, 2022; Nandi,

2023). DeepViral Deep learning platform enables more host-virus relationship prediction based on sequence and functional data, and thus contributes to the detection of such spillover risks that have previously been unknown. Taken together, these tools indicate that AI/ML has a proactive potential to predict high-risk pathogen and ecosystems and have them intervened upon before human transmission takes place, a critical ability in an age of quickening zoonotic dangers (Madan *et al*., 2022; Nandi, 2023).

Integration with the one health framework

The emerging technologies, including AI-based modeling, genomic surveillance, vaccine development, and high-quality data visualization, are perfectly aligned with the concept of the One Health approach that highlights the connection of human, animal, and environmental health and underlines interdependence. In addition to promoting interdisciplinary cooperation, this paradigm also improves the surveillance and response systems to the diseases. The use of artificial intelligence has a transformative presence in One Health because it allows combining a variety of complex and heterogeneous datasets across veterinary, clinical, and ecological disciplines that have traditionally been relatively siloed. The use of AI tools in regard to investigating zoonotic sources is exemplified in the context of the COVID-19 pandemic during which the integration of human health records with wildlife surveillance data was used to explore which species might be the source. AI makes it easy to predict possible reservoir hosts and intermediate species likely to cause zoonotic spillover- including when the triumph of civets and camels in the zoonotic spillover of SARS-CoV and MERS-CoV, respectively were successfully predicted by using predictive modeling (Innes *et al*., 2022; Zhang *et al*., 2024).

Furthermore, environmental monitoring technologies through AI can monitor land-use transformation, habitat encroachment, and climate abnormalities in real-time environments, providing early indicators of environmental disturbance, which can increase the spread of pathogens. An example is the satellite AI system that has been applied to foretell the Rift Valley Fever outbreaks in Africa using rainfall, vegetation indices, and livestock motion. The integration of genomic intelligence and AI products and services in One Health systems enables policy makers to integrate evidence-based biosecurity policies. The most effective example is the Global Avian Influenza Data Sharing (GAINS) platform that combines surveillance and genomics data and uses that information to direct policy-related decisions in both human and animal health areas. The cross-ministry coordination between the ministry of health, the ministry of agricultural and the ministry of environment has provided a harmonized and proactive perspective in outbreak preparedness in line with the global health security objectives and has made the country resilient in the face of possible upcoming zoonotic risks (Gupta *et al*., 2022; Subedi *et al*., 2024).

Problems and next requirements

Although the combination of AI and genomics in the context of zoonotic disease monitoring has become a game-changer, there are multiple severe challenges that still need to be resolved to achieve the equally accessible and effective deployment (Srivastava *et al*., 2025). There are still data-scarce areas of large geographic extents and animal taxa, especially under low-resource conditions. Numerous viral genomes lack high-quality metadata that include host species, ecological context or clinical outcomes, there is a constraint on how well predictive models can perform. One more virulent strain is named computational inequity- most of the low- and middle-income countries do not have the infrastructure to perform high-throughput sequencing, analyze data through the cloud, or access AI tools fueling disparities in the implementation of these and other outbreak detection and response interventions (Ciecierski-Holmes *et al*., 2022; López *et al*., 2022).

As well, the topic of model interpretability is also being brought to the forefront due to deep learning algorithms, which tend to be a black box. The absence of the explainable AI also makes it hard to verify that the scientists and policymakers can take confident steps based on the model-produced forecasts. There also exist integration obstacles: surveillance data are frequently saved in closed or non-interoperable platforms, limiting their functionality in swarming AI systems. In addition, ethical and privacy issues, notably in human health surveillance, require data governance structures that can safeguard personal data and enable fast sharing of data in a time of outbreaks (Mello and Wang, 2020; Igwama *et al*., 2024).

These obstacles can only be addressed through a coordinated international effort. This encompasses

the ongoing investment in capacity-building, including training local researchers and creating regional sequencing hubs and extending internet infrastructure, and in open-access technologies and common guidelines on data sharing and validation of models. Such collaborative frameworks are the only way through which the benefits of AI and genomics can be shared fairly so that every region can contribute to, and benefit, the global pandemic preparedness agenda.

Conclusion

H5N1 is no longer localized and just purely avian. It is a chameleon, trans-nation viral menace that has gradually metamorphosed beyond a poultry scourge into a serious inter-species infiltrator with actual zoonotic implications. The H5N1 virus history since its initial outbreak in Southeast Asia and its recent invasion of marine mammals, domestic cats and even bovine reveal a grim image of the flexibility and survivability of the virus. Such trends are forcing the scientific community to disconnect with the old concept of species-specific confinement and instead adopt a higher degree of integration, foresight, and technology-outfitted thinking into the equation.

The evolution of H5N1 has not been randomized at the molecular level but horribly exigent. Other important mutations such as PB2 E627K and D701N which confer better ability to the virus to replicate in mammalian host, and a change in hemagglutinin receptor binding residues such as Q226L and G228S indicating an alarming possible adaptation to the human is also observed. Academic curiosities? These are genetic red flags. One thing SARS-CoV-2 should have taught us is that even small mutations, given the proper eco-environment, can become the tipping point of global turmoil.

Diagnostics continue to be unevenly distributed even though they are advancing at a rapid pace. Reverse transcription PCR and quantitative PCR are guideline but their use in deprived areas or on atypical hosts is erratic. New technologies such as CRISPR-based diagnostics (i.e., Sherlock and Detectr) and mobile [portable] sequencing devices promise, but future success will not be realized unless ambitious investment in strategies and concerted, distributed implementation. The only thing, molecular tools are never more than the measures of their availability and that is where the public health infrastructure frequently falls behind the viral ability. The importance of data and, what is more, the importance of data quality and context rather than the amount is also highlighted in this review. Patterns that cannot be observed by a naked eye could be exposed through the usage of visualization tools, AI-aided analytics, and geospatial mapping. They are temporal surges, transmission bottlenecks and emerging reservoirs. However, unless we have real-time, transparent and very strong data-streaming processes, particularly those that incorporate accumulation of data about wildlife and marine aggregations, we can still only read half the pages in the viral playbook. The purpose of the data must not only to enlighten us but also to engage us. The current spillovers that have affected mammals are more than that of statistics.

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Novelty Statement

This review provides a comprehensive and updated synthesis of the evolving molecular epidemiology of HPAI H5N1, with particular emphasis on the globally emerging clade 2.3.4.4b and its unprecedented cross-species transmission into mammals, including cattle and marine mammals. Unlike previous reviews, it integrates molecular mutation analysis, advanced diagnostic technologies, and global outbreak trend visualization (2004–2025) using temporal and spatial mapping approaches. Furthermore, the review highlights the emerging role of AI/ML-based spillover prediction, genomic surveillance, and next-generation vaccine strategies within a This review provides a comprehensive and updated synthesis of the evolv-

ing molecular epidemiology of HPAI H5N1, with particular emphasis on the globally emerging clade 2.3.4.4b and its unprecedented cross-species transmission into mammals, including cattle and marine mammals. Unlike previous reviews, it integrates molecular mutation analysis, advanced diagnostic technologies, and global outbreak trend visualization (2004–2025) using temporal and spatial mapping approaches. Furthermore, the review highlights the emerging role of AI/ML-based spillover prediction, genomic surveillance, and next-generation vaccine strategies within a

Author's Contribution

This review paper was conceptualized, designed, written, and revised with equal contributions from each author. The final manuscript has been read and approved by all authors.

Muhammad Wasif Gulzar: Conceptualization, methodology, formal analysis (epidemiological trend analysis using R, 2004–2025), visualization, writing original draft.

Sidra Nasir: Data curation, investigation, writing original draft.

Muhammad Nouman Khalid: Validation, supervision, writing review and editing.

Riffat Maqsood: Data acquisition, resources, reference management.

Sana Raiz, Sidra Zulfiqar: Writing original draft, interpretation of molecular determinants, editing.

All authors have read and approved the final version of the manuscript.

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Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors state that none of the work described in this study could have been influenced by any known competitive monetary objectives or personal relationships.

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