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Research article

Addressing the recent transmission of H5N1 to new animal species and humans, warning of the risks and its relevance in One-Health

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Abstract

The primary natural reservoir of the H5N1 avian influenza virus is poultry and wild birds, particularly waterfowl. Nevertheless, the H5N1 virus subtype can potentially infect humans and other mammals, causing severe illness or even death. The virus is commonly transmitted through direct contact with infected birds or exposure to a contaminated environment. Sporadic cases of avian influenza subtype H5 have been reported in various new hosts, such as minks, otters, foxes, and sea lions, with the potential to cause human infection and trigger a global pandemic. Moreover, various hosts have the potential to act as mixing vessels for influenza viruses because they carry both avian and human-type receptors, not just pigs. Recently, highly pathogenic avian influenza H5 was reported in farmed animals such as dairy cattle, suggesting a new potential mixing vessel for influenza; thus, cattle are also widely infected with the genus influenza type D viruses. This underscores the imperative to adopt the One Health approach, fostering collaboration and integration across animal, planetary, and human health disciplines. Indeed, integrating biosecurity and One Health principles impacts the productivity of commercial poultry operations positively and could help mitigate avian influenza outbreaks. This review also provides an in-depth analysis of the contemporary and molecular epidemiology of H5N1 influenza viruses. Moreover, our examination delves into the potential risk of broadening H5N1 host tropism and its significant relevance within the One Health framework.

Keywords: Influenza, Virus evolution, Interspecies transmission, Mammals, Cattle, One Health

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Introduction

H5N1 is a subtype of the influenza A viruses. H5N1 belongs to RNA viruses, family *Orthomyxoviridae*, and can cause illness in birds, humans, and other mammals (Thomas and Noppenberger, 2007; Hutchinson, 2018; Van

Reeth and Vincent, 2019). Influenza A viruses are categorized into subtypes based on a combination of two groups of proteins on the surface of the viral envelope: hemagglutinin (HA) proteins, of which there are 18 types (H1-H18), and neuraminidase (NA) proteins, of which there are 11 types (N1-N11) (Dey et al., 2023). Each combination is considered a different subtype, and related viruses within a subtype may be referred to as a lineage (Hutchinson, 2018). According to the pathogenicity, avian influenza viruses are classified as low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) based on their genetic features and the severity of the disease they cause in poultry (CDC, 2023a). H5 and H7, with some exceptions, are classified as HPAI viruses that can cause high mortality rates in infected chickens, up to 90-100% (Alexander, 2000). The global recurrence and spread of the HPAI viruses, including subtypes H5N1, are a worldwide threat to the poultry sector, impacting the livelihoods of farmers and stakeholders and having severe consequences in food security and public health pandemic potential (WHO, 2023a). They also significantly affect the wild bird populations and threaten wildlife and biodiversity.

The replication of influenza viruses within the intestinal tract of wild birds establishes them as the primary reservoir for subsequent transmission to both domestic birds and various mammalian species on a global scale. In 2021, outbreaks of avian influenza subtype H5N1 caused 53 million bird fatalities. Despite its low transmissibility among individuals, the heightened concern arises from the observation that, within the past 26 years, 457 cases out of a total of 868 (53%) have culminated in fatality (CDC, 2023b). This review sheds light on the evolution of avian influenza viruses. Additionally, the potential risk of expanding H5N1 host tropism within the One Health framework as well as general recommendations, have been discussed.

Avian influenza in animals and mammals

Avian influenza in domestic birds

Avian influenza viruses have been found in various species of domestic and caged birds, such as chickens, turkeys, waterfowls, ostriches, pigeons, quails, pet birds, gamebirds, and zoo birds (Alexander, 2000). It is noteworthy that wild birds serve as the principal carriers of all avian influenza virus subtypes (H1-H16 and N1-N9), with a few exceptions. Various subtypes of avian influenza, particularly H1 to H11 and H13, have been isolated in domestic birds. The most frequently isolated subtypes in domestic birds are H5Nx, H6N2, H7N3, H7N9, and H9N2. However, subtypes H12 and H14-H16 have yet to be detected in domesticated birds. H17N10 and H18N11 subtypes have been identified only in bats (Gamblin and Skehel, 2010; Suarez, 2016; Kuhn et al., 2020). HPAI H5 and H7 can lead to 100% morbidity and mortality in poultry, mainly chickens and turkeys, within a few days.

Between 1997 and 2015, six outbreaks of H5N1 infection were documented, spanning multiple countries such as China, Indonesia, Egypt, Turkey, India, Bangladesh, and Nigeria, among others. The symptoms ranged from flu-like symptoms and pneumonia to death (Dey et al., 2023). H5N1, characterized as the most pathogenic strain of avian flu, has been associated with numerous outbreaks. The initial instances of human casualties were reported in Hong Kong in 1997, specifically in Guangdong, a southern province of China, where six cases resulted in mortality (Poovorawan et al., 2013; Dey et al., 2023). Furthermore, the 2003 outbreak resulted in significant losses in the poultry industry worldwide; the burden was particularly pronounced in developing countries (Kim, 2018; Rehman et al., 2022).

Notably, avian influenza viruses that are highly pathogenic in ducks are also highly pathogenic in chickens, but the reverse is not valid. Most HPAIV H5/H7 strains are non-virulent in ducks, unlike chickens and turkeys (Song et al., 2011; Grund et al., 2018; Scheibner et al., 2019). Mallard ducks are known to be the primary carriers of avian influenza viruses. Typically, they can withstand the sickness and death caused by AIV. Nonetheless, certain strains of the H5N1 and H5N8 viruses have proven extremely virulent, even to mallards (Tang et al., 2009). It has been observed that young ducklings are more susceptible to high morbidity and mortality after being infected with some HPAIV than older ducks (Pantin-Jackwood et al., 2012).

Mass culling of poultry incurs vast costs for government and industry, heavy economic losses for farmers, generating a long-lasting impact on their livelihoods and raising societal and environmental concerns. The World Organisation

for Animal Health (OIE) permits vaccination exclusively in countries where there is a potential risk that stamping out could lead to eliminating a significant food source. The decision to implement vaccination against avian influenza presents a complex dilemma, requiring careful consideration of various factors. It is important to note that only HPAI subtypes H5 and H7 are notifiable to the World Organization for Animal Health, and affected flocks must be culled (Swayne, 2013).

Intense debates have arisen regarding the permissibility of vaccination concurrent with eradicating infected flocks due to the substantial outbreaks and economic repercussions. Poultry farmers advocate for vaccination for various reasons: firstly, the stamping out approach alone falls short of containing the lethal AI virus. Secondly, the affected regions are too vast to prevent further virus transmission, and lastly, vaccination is essential in safeguarding valuable flocks, especially breeding stocks (Swayne, 2012; Ali et al., 2019; Sultan et al., 2019). Generally, it is recommended to vaccinate against influenza with vaccines that provide the differentiation between infected from vaccinated Animals (DIVA) principle to detect active infection in vaccinated animals. Of note, vaccinated birds can still become infected with field strains of the virus and serve as a source of virus to spread to other flocks. However, vaccinated birds are likely to have increased resistance to infection and reduced shedding of the virus (Capua et al., 2003).

Avian influenza in mammals

Reports of H5N1 infection in mammals and humans have surfaced globally. H5N1 reemerged as a human outbreak in 2003, originating in China and Asian countries before subsequently spreading globally (Cattoli et al., 2011). The recurrence of numerous small-scale H5N1 outbreaks consistently instills an ongoing concern for a potential "looming pandemic" (Wang et al., 2022). The outbreaks typically initiate in birds or wild birds and extend to other species through human contact and genetic mutations. Organizations such as the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) ensure the confirmation of human-to-human transmission before declaring a pandemic.

Notably, in the recent case involving the new clade 2.3.2.1c infecting Cambodian poultry, the WHO dismissed human-to-human transmission as the cause for infection in the two cases involving an 11-year-old girl and her father (Yang et al., 2007; Mahase, 2023). A WHO report in February 2023 identified Chile's new viral clade 2.3.4.4b with fear of human-to-human transmission (WHO, 2023b). There have been reports of H5N1 infection in 200 human cases in Indonesia since the 2003 outbreak, with morality reaching 84% (Pawestri et al., 2020). Since 2006, H5N1 has been endemic in Egyptian poultry, and the country reported the highest number of human cases globally (Elsobky et al., 2021). The actual burden of H5N1 in Egypt is believed to be underestimated. The initial human outbreak in the country occurred in 2009, with reports indicating a few human infections as early as 2006. Subsequently, another outbreak unfolded in 2014-2015, resulting in 31 and 88 confirmed cases, respectively, during those two years. Indeed, a significant proportion of the global cases confirmed in 2015, amounting to 292 out of 784 (37%), were reported in Egypt. The persistence of endemic infection among poultry contributes to continuous genetic variation. Unfortunately, efforts to contain the issue faced challenges, as authorities primarily relied on vaccination programs, neglecting other crucial measures such as zoning, movement restrictions, culling, and enhancing farmers' awareness of the disease (Kayali et al., 2016).

The replication of influenza viruses within the intestinal tract of wild birds establishes them as the primary reservoir for subsequent transmission to both domestic birds and various mammalian species on a global scale (CDC, 2023b). As the HPAI H5N1 viruses continue to evolve, they could infect other mammals (pigs, cats, horses, dogs, and ferrets) and humans, and the symptoms ranged from asymptomatic/mild illness to severe disease resulting in death. Recently, various mammals, including minks, otters, foxes, and sea lions, have sporadically contacted avian influenza subtype H5 (Agüero et al., 2023; Kupferschmidt, 2023; Sidik, 2023).

Recently, it has been shown that cattle might play a role in the epidemiology of influenza viruses. HPAIV. In 2024, confirmed cases with H5N1 clade 2.3.4.4b genotype B3.13 in dairy cattle were reported. Cattle exhibited a subclinical or clinical disease, including reduced

milk production, fever, dehydration, and reduced feed intake. Indeed, cats developed a fatal disease after drinking raw milk from infected cows. Additionally, workers in the dairy farm in Texas developed conjunctivitis. A recent study reported on the transfer of H5N1 from dairy cows to raccoons, as well as to wild and domestic birds (Nguyen et al. 2024). These findings have led to considerations about the role of cows in the evolution of influenza viruses

Consequently, the elevated pandemic risk linked to HPAI is substantial, and the heightened probability of HPAI H5N1 spillover from poultry to humans is a source of considerable apprehension. Nearly all instances of human infections involving influenza A subtype H5N1 have been linked to proximity with infected live or deceased birds or environments contaminated with the virus (WHO, 2023b). Consequently, the atypical nature of the virus's person-to-person transmission raises questions. As a result, rigorous avian influenza surveillance in both poultry and wild birds is imperative. Moreover, there is a pressing need to address strategic challenges hindering global progress toward disease control, consider alternative control measures, and foster consensus on science-based preventive and control strategies.

Evolution of H5N1

Genetic variations

Antigenic drift

According to the antigenic classification of influenza A virus, there are 18 HA and 11 NA subtypes (Dey et al., 2023). Both H5N1 and H7N9 cause severe morbidity and mortality in humans (Koutsakos et al., 2019). The minor variations over time in the antigenic components of NA or HA parts of the virus lead to shifting the antigenic sequence to that similar to humans across time through minor mutational changes to a few amino acids (Cattoli et al., 2011). This occurs due to the RNA viruses' lack of "proofreading" (Kim, 2018). The diagnostic antibodies evaded the viral detection when an antigenic new clade (2.1) occurred in H5N1 in poultry through "antigenic drift," producing new viral variants. This evasion from antibody detection occurred due to the mass vaccination of poultry. The molecular similarity of the virus to the seasonal human influenza virus increased (Koel et al., 2014). This antigenic drifting resulted in the phylogenetic diversity of 9 virus clades with different susceptibly and infective properties (Cattoli et al., 2011). Additionally, these antigenic variations can make the virus escape the humeral antibody immunity against influenza viruses in humans and cause outbreaks of infection with variable severity according to the viral parts mutated (Koel et al., 2014).

The most virulent mutations occur in the HA portion of the viral particle H5, either through substituting non-alkaline with alkaline amino acids or inserting an alkaline amino acid at the cleavage site during the replication of the viral RNA (Nancy et al., 2020). Phenotypic effects of the NA mutations (L204M substitution) of H5N1 include enhanced receptor binding, higher viral replication, and modulated viral virulence in mammals. This substitution resulted in lower NA viral particles and decreased NA activity. This could be a crucial site for molecular docking to study new human antivirals (Scheibner et al., 2023). After connecting the high fatality of the infected patients to the high viral load, viral genome sequencing of 35 human cases was performed to assess further the cause of this high fatality. Genetic analysis showed that these patients had high prevalent amantadine resistance with M2 mutations. Otherwise, they found low genetic diversity and genetic substitutions associated with viral adaptation to infect humans as polymerase basic two protein (PB2)-E627K (Pawestri et al., 2020). The PB2-E627K mutation has been present since the Spanish flu (H1N1) caused the 1918 pandemic (Scheibner et al., 2023).

For a pandemic to arise in humans, the virus must first bypass the animal-human barrier, and then human-to-human transmission ensues (Dey et al., 2023). PB2-E627K mutation allowed the avian flu to cross the "species barrier" through genetic adaptation; surprisingly, it is not present in the infected birds, only causing infection in the human species. It is the key mutation in all the human pathogenic avian flu variants, including H5N1, H7N7, and H7N9 (Jonges et al., 2014). PB2-E627K mutation causes increased infectivity through the airborne route and increased viral replication in mammals (Koutsakos et al., 2019; Suttie et al., 2019).

Antigenic shift

Another mutational change is the "*antigenic*

shift," an abrupt major change of the virus to another viral species, mainly through the whole genome of the HA and/or NA mutations, resulting from a completely different virus subtype. It occurs only in influenza A viruses due to a considerable animal reservoir (Kim et al., 2018). In the co-infection of a host cell by multiple viruses, genome segments may be rearranged, resulting in the emergence of new influenza viruses with unique genome combinations (Marshall et al., 2013).

Recently, researchers have identified potential hosts that could act as "mixing vessels" for spreading influenza viruses based on the distribution of avian and human sialic acid receptors. These hosts have been categorized into three groups: firstly, high probable mixing vessel hosts, including humans, pigs, minks, ferrets, seals, dogs, cats, and various birds such as turkeys, chickens, quails, and ducks; secondly, medium probable mixing vessel hosts, such as non-human primates, raccoons, camels, pikas, horses, and zoo animals like tigers and lions; and lastly, low probable mixing vessel hosts, including foxes, bats, and whales (Abdelwhab and Mettenleiter, 2023).

Turkeys are significant in the evolution of avian influenza viruses for several reasons. Firstly, they have avian and human-type receptors, making them highly probable mixing vessels for avian influenza viruses. These receptors are present in the nasal cavity, lung, kidney, esophagus, and intestine. Secondly, turkeys, especially turkey breeders, can contact swine influenza viruses like H1N1, H1N2, and H3N2, significantly reducing egg production and economic losses. This also raises the likelihood of reassortments and virus evolution in turkeys. Lastly, swine influenza viruses can be transmitted to turkeys, and the same can happen between ducks and turkeys, although less frequently. It is essential to note that mixing poultry and outdoor rearing could increase the chances of LPAIVs adapting and becoming a severe health risk (Abdelwhab and Mettenleiter, 2023). To summarize this part, different hosts have the potential to act as mixing vessels for influenza viruses because they carry both avian and human-type receptors, not just pigs. Detection of influenza viruses in new mammalian hosts highlights the crucial of high vigilance to prevent the potential future pandemic.

Molecular evolution of recent H5N1

To provide a comprehensive insight into the molecular evolution of H5N1, phylogenetic trees have been constructed, and the deduced amino acids have been summarized. Briefly, the sequence for the H5N1 HA gene of the original strain (1996 Gandong) was retrieved from the GenBank. Then nBlast was run for similarity sequences HOMOLOGS (the most similar 100 sequences were retrieved). Duplicates and incomplete sequences were removed. The most recent sequences were obtained by a separate search on the National Library of Medicine Databases with the filter “nucleotides” by adding the words “2022,” “2023," or "Chile" to retrieve some of the recent outbreak sequences. A second search was conducted using the terms “Human” and “Homo sapiens” to retrieve some human sequences. The GenBack similarity nBLAST, human sequences, and recent outbreaks were collected to build a single text FASTA file. The text was edited to carry the GenBank accession number, the organism, the country, and the year for each variant of the H5N1. The FASTA file was uploaded to the MEGAX program on Windows 10. We run multiple sequence alignment (MSA) in the program using the MUSCLE method. The aligned file was checked for any outliers. Using the Maximum Likelihood method, the aligned file was then used to build a phylogenic tree, with bootstrap 1000 and pairwise corrected. The tree was exported as a Text file, Tiff, and PNG pictures. The text file was uploaded to the iTOL online program to build a colored tree. The files are exported from the iTOL as Tiff, PNG, and SVG files. In the phylogenetic trees, the human strains are colored green, and the recent strains are colored red. We used the recent 2022- 2023 sequences (ONLY) to assess the mutations presented in those sequences using the GSAID site program Flusurver and exported them in the figure below. The program assesses the mutations based on the vaccines of the organism present in the system. The same was done separately for the HA protein (using the GenBank 1996 Gandong starting as the starting point) using the protein BLAST for similarity homolog.

Based on HA proteins, the most tested human strains (green) are clustered together, while the most recent strains are colored red, highlighting the continuous evolution of influenza viruses. Figures 1 and 2 summarize the most putative amino acid substitutions.

The HA protein portion of the virus is responsible for viral attachment to the host cell, while the NA protein portion is responsible for releasing virion particles from the host cells and preventing their intracellular aggregation. The human strains cause seasonal influenza symptoms or may progress to a viral pandemic according to the virulence of the virus in crossing the host-species barrier (Dai et al., 2016). Functional viral protein motifs are short segments of amino acids that correspond to certain important protein areas that induce various functions such as viral attachment and entry, immune response, viral virulence, viral structure, etc. (Sobhy, 2016). The HA protein binds to the cell surface's sialic acid (SA) glycan portion. The human strains bind to the 2,6 alpha SA (which requires fibronectin to initiate infection), while avian HA binds to 2,3 alpha SA (Kosik and Yewdell, 2019).

In our study, H5N1 human mutations were explored using the Flusurver online program on the GSAID site. There was no significant difference between the protein and the genetic presentation of the phylogenic analysis on the similarity index or the mutations. We found that the NA portion of the new viral strains in 2022-2023 (either protein or gene structure) is highly similar to the original strain of Guangdong 1996 H5N1, with 94% similarity. However, the HA portion of the new viral strains in 2022-2023 (both protein and gene structure) are of the new clade 2.3.4.4b in the H5N6 strain, which first appeared in the 2014 human strains outbreak, with 96% similarity. This agrees with a previous report (Bruno et al., 2023). Also, this indicates the risk of a human epidemic in the near future from the new strains. Clade 2.3.4.4 was first discovered in Eastern China in 2013-2014. H5N8, representing clade 2.3.4.4b, started to appear in mid-2016. By 2020, multiple variants were already causing European animal outbreaks (Lee et al., 2023).

In HA protein, the most presented mutations in our search led to antibody escape mutant and antigenic drift (T156A); this mutation was presented in human strains. There was also a host-specific shift caused by mutation (N110S) and mild drug resistance with antigenic drift in (L131Q) (Table 1).

Table 1: Showing some mutations in Hemagglutinin and Neuraminidase genes and their affected functions.

Function affected	Mutations HA gene	Mutations NA gene
Viral oligomerization interference	K3N, G16S, E284G, M285V	T76A, X78Q, V99I, H100Y, D460G, S434N
Antibody recognition site	R341K, K506Q, L120M	S339P, P340S
Antigenic drift and escape mutants and mild drug-resistant	L131Q, T156A	N366S, A395E, N366S, A395E
Host specificity shift	N110S, V226A	-
Binding small ligands	I298V, K492E, N252D	G336S, L269M, V338M, E287D, T289M
Strong drug resistance	-	H155Y

Most of the mutations linked to oligomerization were presented in the NA protein portion, including G336S, N366S, A395E, S339P, P340S, S434N, D460G, etc. The oligomerization is a prerequisite to enzymatic activity in the NA portion, and any change in this process will affect the viral enzymatic activity. Also, NA protein portion mutation can cause a decrease in viral particles, leading to decreased available particles for segmental incorporation and assembly (Kosik and Yewdell, 2019). However, all the new strains are similar to the original NA protein strain, i.e., The Guangdong 1996 strain (Table 2).

In NA protein, the most presented mutations in our search were mutations leading to antibody escape mutant and antigenic drift by (N366S, A395E), and strong drug resistance on the antibody recognition sites in (H155Y). This could be explained by the length of the stalk of the receptor binding site, which is coded on the NA portion; when deletion occurs, shortening leads to increased virulence and lethality of the virus (Kosik and Yewdell, 2019). After the Guangdong strain, the strains had a 20 amino acid deletion in their stalk motif protein from the 49th-68th stalk region (Zhou et al., 2009).

Table 2: Neuraminidase gene (NA gene) mutations in the new strains (Flusurver output).

Query	Best reference hit	%AA identity	% Length coverage	Number of mutations	List of mutations
OQ352542.1 (Peruviam-pelican/Chile/C61740/2022)	Goose/Guangdong/1/1996 (H5N1)	94.670	100	25	I8T, V17I, 120V, A46P, T76A, K78Q, A81T, V99I, H100Y, H155Y, T188I, M258I, L269M, E287D, T289M, G336S, V338M, S339P, P340S, N366S, G382E, A395E, I418M, S434N, D460G
OQ547433.1 (Panthera-leo/Peru/AIS0554/2023)	Goose/Guangdong/1/1996 (H5N1)	94.243	100	27	I8T, V17I, 120V, H44Y, A46P, T76A, K78Q, A81T, V99I, H100Y, H155Y, T188I, M258I, L269M, E287D, T289M, T332P, G336S, V338M, S339P, P340S, N366S, G382E, A395E, I418M, S434N, D460G
OQ683492.1 (Chicken/Colombia/Magdalena/3503/2022)	Goose/Guangdong/1/1996 (H5N1)	94.243	100	27	I8T, V17I, 120V, H44Y, A46P, T76A, K78Q, A81T, V99I, H100Y, H155Y, T188I, M258I, L269M, E287D, T289M, G336S, V338M, P340S, N366S, G382E, A395E, I418M, S434N, F445C, G447C, D460G
OR265944.1 (Domestic-duck/Araucania/240481-2/2023)	Goose/Guangdong/1/1996 (H5N1)	94.456	100	26	I8T, V17I, 120V, H44Y, A46P, T76A, K78Q, A81T, V99I, H100Y, H155Y, T188I, M258I, L269M, E287D, T289M, G336S, V338M, S339P, P340S, N366S, G382E, A395E, I418M, S434N, D460G
OR268568.1 (Pelican/Coquimbo/231946-1/2023)	Goose/Guangdong/1/1996 (H5N1)	94.456	100	26	I8T, V17I, 120V, H44Y, A46P, T76A, K78Q, A81T, V99I, H100Y, H155Y, T188I, M258I, L269M, E287D, T289M, G336S, V338M, S339P, P340S, N366S, G382E, A395E, I418M, S434N, D460G
OR269261.1 (Pelcian/Coquimbo/231946-1/2023)	Goose/Guangdong/1/1996 (H5N1)	94.456	100	26	I8T, V17I, 120V, H44Y, A46P, T76A, K78Q, A81T, V99I, H100Y, H155Y, T188I, M258I, L269M, E287D, T289M, G336S, V338M, S339P, P340S, N366S, G382E, A395E, I418M, S434N, D460G
OR269269.1 (Blackish-oysterchatcher/OHiggins/240628/2023)	Goose/Guangdong/1/1996 (H5N1)	94.456	100	26	I8T, V17I, 120V, H44Y, A46P, T76A, K78Q, A81T, V99I, H100Y, H155Y, T188I, M258I, L269M, E287D, T289M, G336S, V338M, S339P, P340S, N366S, G382E, A395E, I418M, S434N, D460G

In addition, the human strains in our study showed many mutations affecting viral oligomerization interfaces at the antibody binding sites (Tables 1 and 3), including (M285V, E284G, A201X, T156A, etc.).

Table 3: Hemagglutinin gene (HA gene) mutations in the new strains (Flusurver output).

Query	Clade	Best reference hit	%AA identity	% Length coverage	Number of mutations	List of mutations
OQ352548 (Blackskimmer/Chile/C61962/2022)	2.3.4.4b	Sichuan/26221/2014 (H5N6)	96.473	100	20	K3N, G16G, N110S, L120M, L131Q, T139P, T156A, Q185R, V194I, A201E, V226A, N252D, E84G, M285V, I298V, K492E, I526V, V538A, I547M, V548M
OQ352556.1 (Graygull/Chile/C61947/2022)	2.3.4.4b	Sichuan/26221/2014 (H5N6)	96.473	100	20	K3N, G16G, N110S, L120M, L131Q, T139P, T156A, Q185R, V194I, A201E, V226A, N252D, E84G, M285V, I298V, K492E, I526V, V538A, I547M, V548M
OR269134.1 (Chicken/OHiggins/24252-1/2023)	2.3.4.4b	Baikalteal/KoreaDonglim/3/2014(H5N8)	96.296	100	20	K3N, V10I, K30E, T110S, L120M, T130I, L131Q, S157A, A201X, D211T, V214I, V226A, N252D, E284G, I298V, R341K, K506Q, I526V, V538A, I547M, V548M
OR269259.1 (Whimbrel/Valparaiso/239946/2023)	2.3.4.4b	Baikalteal/KoreaDonglim/3/2014(H5N8)	96.296	100	21	K3N, V10I, K30E, T110S, L120M, T130I, L131Q, S157A, A201X, D211T, V214I, V226A, N252D, E284G, I298V, R341K, K506Q, I526V, V538A, I547M, V548M
OR269264.1 (Humboldtpenguin/Tarapaca/238744-2/2023)	2.3.4.4b	Sichuan/26221/2014 (H5N6)	96.296	100	21	K3N, G16S, N110S, L120M, L13Q, T139P, T156A, Q185R, V194I, A200X, A201X, V226A, A230T, E284G, M285V, I298V, K492E, I526V, V538A, I547M, V548M, I547M, V548M
Or269268.1 (Blackishoystercatcher/OHiggins/240628/2023)	2.3.4.4b	Sichuan/26221/2014 (H5N6)	96.473	100	20	K3N, G16S, N110S, L120M, L13Q, T139P, T156A, Q185R, V194I, A201X, V226A, N252D, E284G, M285V, I298V, K492E, I526V, V538A, I547M, V548M

Highly pathogenic human infecting strains show that H3N2 has the ability to spread in the cell culture of immunogenic cells (Westenius et al., 2018). This is confirmed by H3N2 high replication in human epithelial bronchial cells in another study. In addition, H3N2 induces a stronger type I interferon response in mammals with high cytokine and inflammatory responses (Hui et al., 2007).

In a previous study, some amino acid substitutions in H5N1 reduced the acid stability of the virus in chickens, increasing its virulence. In addition, some viral protein motifs (such as N-glycosylation motifs) were associated with increased viral virulence, and hence, their absence or absence of one of its amino acids causes a decrease in viral virulence (Wessels et al., 2018).

The HA cleavage site showed the protein motif -RRKR/G- in all the new strains in a previous study. This motif was associated with increased pathogenicity of the virus (Cui et al., 2022). Only one Egyptian isolated chicken strain from 2017 with clade 2.3.4.4b had the same protein motif and was accompanied by stalk deletion from the NA portion. Most strains didn't have the combined NA stalk deletion (Mosaad et al., 2023).

Effect of the mutations on the treatment outcome in humans

Oseltamivir (Tamiflu) is a neuraminidase enzyme inhibitor; it was the first available antiviral option when human cases broke out in 2005 of H5N1 infection. It blocks the NA responsible for cleaving new viral particles from the cells, thus stopping cellular spreading (Hariyono et al., 2021). In a study on a cohort of 215 cases from ten countries, the determinants of increased Oseltamivir effectiveness included early medication start before respiratory failure, young age <5 years old, and viral clade 2.2. No adjuvant therapy is known to be effective in improving mortality (Chan et al., 2012).

The escalating challenge of resistance to antiviral therapy directed against the viral

particles of H5N1 is a growing concern. An alternative solution is to target the host proteins. A study on one of the host proteins responsible for viral infection, cytochrome c oxidase subunit 4 isoform 1 (COX41), showed that knocking out or silencing COX41 by lycorine inhibited the replication of the viral particles and the absence of the pathological manifestations in the treated mice (Li et al., 2022).

A chimeric monoclonal antibody (C12H5), a cross-neutralizing antibody targeting the HA portion of H5N1 and H1N1, was discovered to regress viral entry and exit into the cell in vivo. This could influence the development of antivirals and vaccines against the H5N1 infection (He et al., 2022).

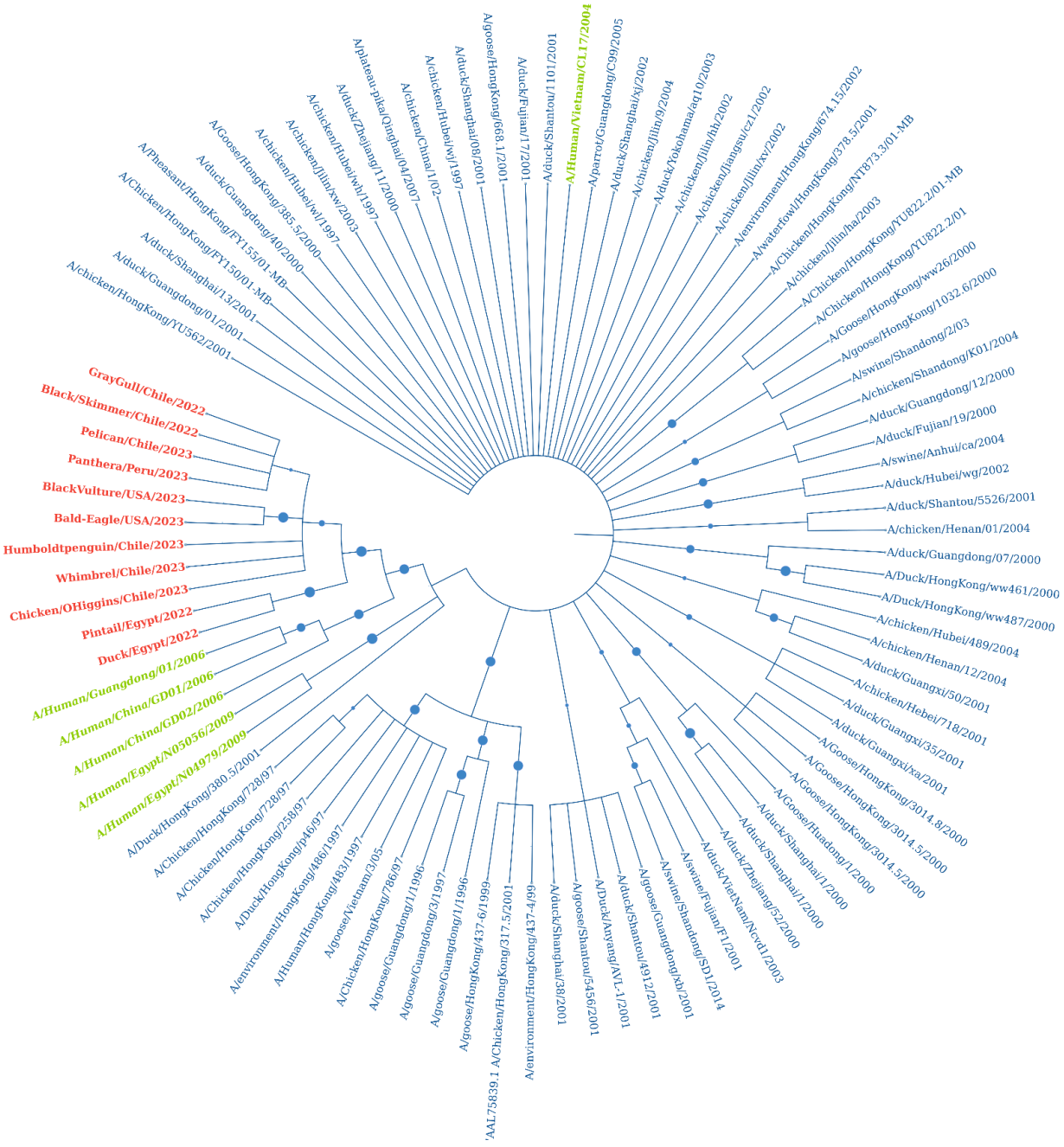

Figure 1: Hemagglutinin protein phylogenetic tree: it was generated using the maximum likelihood method, with bootstrap 1000 and pairwise corrected. Most tested human strains (green) are clustered together, while the tested recent strains are colored red.

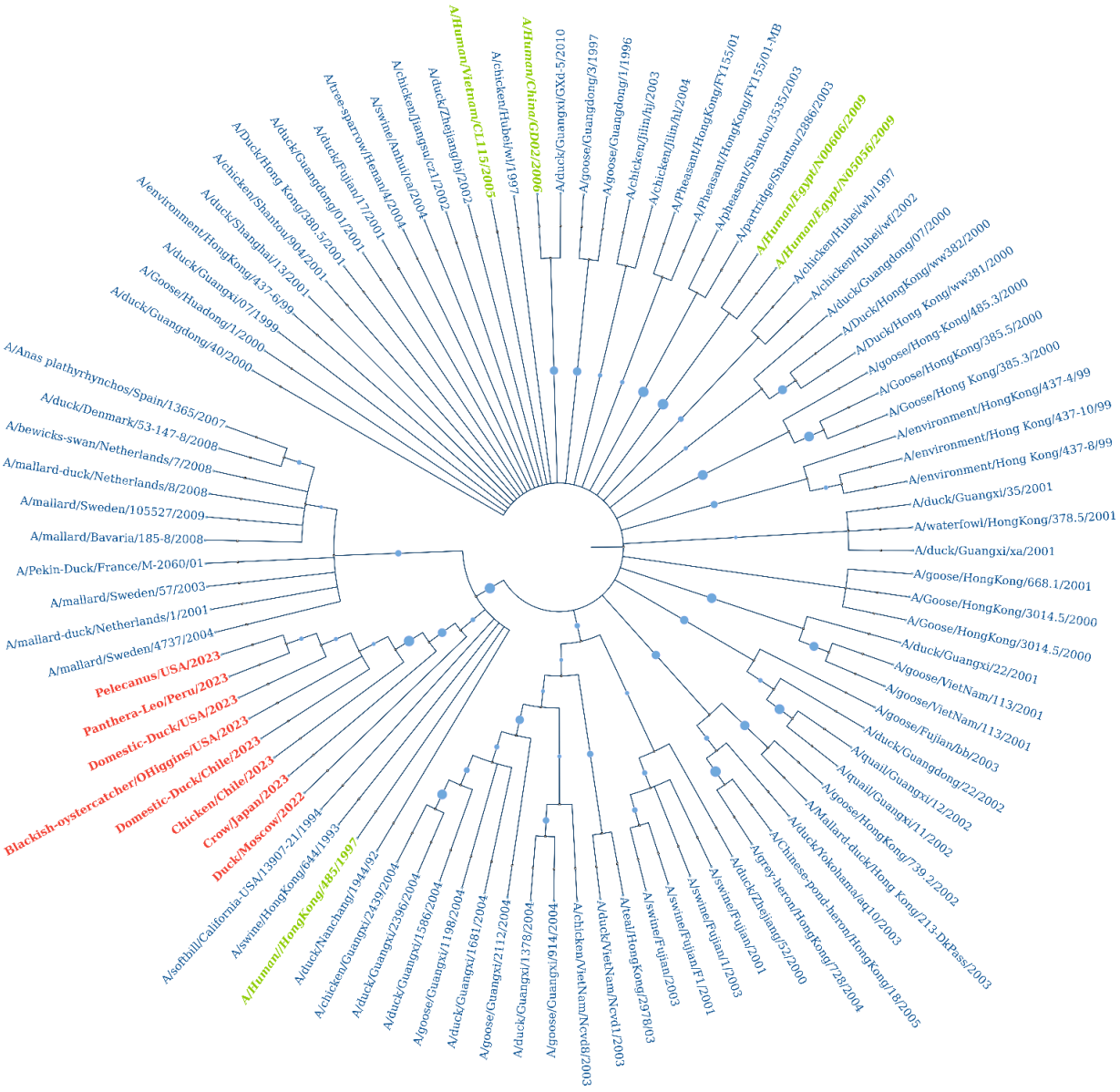

Figure 2: Neuraminidase protein phylogenetic tree: it was generated using the Maximum Likelihood method, with bootstrap 1000 and Pairwise corrected. Most tested human strains (green) are clustered together, while the tested recent strains are colored red.

Risks and surveillance

Surveillance

Molecular surveillance plays a crucial role in identifying the emergence of new viral clades. However, the presence of wild birds may contribute to a knowledge gap in understanding the mutational progression, potentially leading to the emergence of highly infectious viral subtypes in humans (Karo-karo et al., 2022). Surveillance for H5N1 is conducted using ELISA to identify viral-specific neutralizing antibodies. However, the monitoring of human H5N1 cases in Egypt reveals a discrepancy when compared to disease burden reports. Despite an 11% prevalence of neutralizing influenza antibodies in the growing poultry population (based on a 2015-2019 survey), most lack the specific H5N1 antibody. Notably, the reported cases predominantly focus on severe hospitalized instances, overlooking mild to moderate influenza cases. This observation underscores potential limitations in capturing the full spectrum of H5N1 infections in human populations (Badra et al., 2023).

The gold standard for viral detection is reverse transcriptase PCR (RT-PCR) and viral culture. However, these methods need complex techniques and a long time for preparation. A novel detection method has been introduced for distinguishing between H5N1 and H1N1 subtypes, even in low viremic states. This method

employs a fluorescence resonance energy transfer (FRET) assay combined with DNase I-assisted cyclic enzymatic signal amplification on the HA particles of the viruses (Wang et al., 2022). Additionally, a recently introduced method for rapidly detecting H5N1 and showing high specificity (approaching 100%) is surface-enhanced Raman scattering (SERS) using fingerprint sandwich immunomagnetic bead. This assay has a very low detection limit, reaching 5×10^{-6} $TCID_{50}$/ml (Wang et al., 2023).

Risks and clinical features

Clinical features in mammalian species range from asymptomatic infection to severe respiratory distress syndrome and death (Suttie et al., 2019). In a Vietnamese cohort comprising 93 patients, the primary symptomatic presentation was respiratory, with cough reported in 89%, dyspnea in 81%, and chest X-rays revealing bilateral pulmonary infiltration in 72% of cases. Additional manifestations, such as bleeding and hepatological symptoms, were associated with a heightened risk of mortality, reaching a 39% case fatality rate in this cohort (Liem et al., 2009). Fever >38 °C is a common presentation; early gastrointestinal symptoms include diarrhea, vomiting, and abdominal pain. Respiratory symptoms occurrence is comparatively lower than with seasonal influenza infection (Hui, 2008).

In addition to constitutional symptoms, H5N1 has the potential to induce complications such as encephalitis, conjunctivitis, pneumonia, acute respiratory distress syndrome (ARDS), or end-organ failure. These manifestations are mainly attributed to the induction of a cytokine response associated with the infection (Koutsakos et al., 2019; Dey et al., 2023).

According to the region, mortality reports from H5N1 infections are high, ranging from 50-85%. As of October 2020, four hundred fifty-five patients were deceased from the 861 infected patients, according to WHO reports (Pawestri et al., 2020; Hariyono et al., 2021). The cause of the high morbidity and mortality in H5N1 is the lack of immunity from previous exposures, as in other influenza viruses infecting only humans (Frey et al., 2023). Laboratory investigations usually show leucopenia lymphopenia; however, neutropenia is the most associated with a high risk of mortality (Hui, 2008; Liem et al., 2009).

The susceptibility of individuals aged between 10 and 40 years to severe morbidity and mortality resulting from H5N1 infection is a significant concern, given their heightened exposure to the virus through work in farming or the healthcare system. Conversely, pediatric and elderly populations are vulnerable due to their relatively lower immune response to viral infections. Therefore, the development of a vaccine that provides coverage across all age groups is imperative for preventing future pandemics. A recently developed vaccine, Audenz®, has successfully passed phase 2 single-blinded clinical trials for adults, the elderly (Frey et al., 2023) (and pediatric populations (6 months to 17 years old) (Chanthavanich et al., 2021). This inactive vaccine, derived from cell culture, demonstrates promising results, exhibiting an increased immunogenic response and cross-protection against various strains of H5N1 in both trials.

Laboratory investigations denoted the presence of a cytokine response/storm, including increased levels of interferon-gamma inducible protein-10 (IP-10) and high levels of inflammatory chemokines and cytokines, with occasional hemophagocytosis (Koutsakos et al., 2019). The cytokine storm that occurs with severe infections of influenza viruses, including H5N1, will require immunomodulatory therapy as corticosteroids, cyclooxygenase-2 inhibitors, anti-tumor necrosis factor alpha, and other potential therapeutic modalities (Liu et al., 2016).

Risk for H5N1 host tropism broadening and its relevance in the One-Health

A primary concern centers around the virus's capacity for genetic changes or reassortment events. Such alterations are likely linked to an elevated efficiency of infection and transmission within and between virus-host species (Sonnberg et al., 2013). Transmission typically occurs through close contact with infected birds or environments contaminated by their body fluids (Kaplan and Webby, 2013).

Previous studies focusing on live bird markets have also shown that different subtypes of AIV could be isolated from environmental swabs collected within such markets (Horm et al., 2012; Martin et al., 2018). Avian influenza viruses can survive outside the host for several days to several months, depending on environmental conditions and viral concentrations. However, in

tropical countries, virus inactivation can occur rapidly due to several factors, such as heat, salinity, dryness, ultraviolet radiation, and pH (Martin et al., 2018). Although viral particles may no longer be infectious, their RNA is still protected from matrix and core protein degradation and could consequently be detected. The detection of AIV RNA in sludge, aquatic plants, and aquatic animals suggests that these sites should be considered as a potential source of human and/or animal infection (Stallknecht and Brown, 2009; Kurmi et al., 2013).

Recognizing the shedding and prolonged survival of H5N1 in the environment, various transmission routes, both among animals and humans, are theoretically plausible (Li et al., 2014). Potential transmission modes include oral ingestion of contaminated water during swimming, direct intranasal or conjunctival inoculation during water exposure, and hand contamination by infected fomites, leading to subsequent self-inoculation (Abu Bakar et al., 2023). Another noteworthy risk factor is the widespread use of untreated poultry feces as fertilizer (Beigel et al., 2005).

The extension of H5N1 tropism to other animals could have several implications. First, it could complicate disease control efforts as it would involve multiple species and potentially increase the reservoirs for the virus. Second, it could enhance the chances of spillover events, where the virus could jump from animals to humans more frequently, increasing the risk of a pandemic. Third, it could impact ecosystem dynamics by affecting various animal populations.

Acknowledging the variety of transmission routes, the viral capabilities of expanding the host spectra, and the environmental implications, avian flu is a zoonotic infection of high relevance in the One Health context (Sims and Peiris, 2012). Indeed, this interdisciplinary approach accounts for the interconnectedness between human health, animal health, and the environment. As such, its actions involve the complimentary collaboration of a plurality of professionals ranging from physicians, veterinarians, public health officials, epidemiologists, and environmental scientists to achieve all-around solutions to complex and multifaceted issues, such as the avian flu. To mitigate the risk of tropism extension, surveillance systems are in place to monitor the presence of H5N1 in animal populations, not only in birds but also in mammals that may come into contact with infected birds (Vandalen et al., 2009; Hoye et al., 2010). Additional preventive measures for avian influenza can be summarized as follows:

i) Promoting biosecurity measures in agricultural settings, including proper hygiene practices, controlled access to farms, and separation of different animal species, can help reduce the risk of transmission and prevent the expansion of H5N1 tropism. Maintaining an efficient biosecurity program is essential. It is important to note that biosecurity is not limited to the farm gate but extends beyond it. This requires close collaboration with veterinarians, government agencies, and industry stakeholders to establish and maintain best practices and response plans.

ii) The One Health approach emphasizes the importance of vaccination measures in preventing avian flu outbreaks in poultry. Vaccinating poultry populations can help reduce the prevalence of the virus in birds, thereby decreasing the risk of environmental shedding and transmission to animals, including humans.

iii) Enhancing the welfare of commercial poultry is a crucial part of a comprehensive approach towards poultry production. This involves providing the right living conditions, ensuring access to clean water, balanced nutrition, and appropriate space.

iv) Enrichment strategies, such as environmental enhancements and behavioral opportunities, contribute to the overall well-being of the birds, reducing stress and enhancing their natural behaviors. Lower disease incidence leads to lower mortality rates and better flock performance, which translates to higher production efficiency.

v) It is urgently necessary to implement strict measures to prevent new waves of infection, especially in mammals, in order to prevent human infection ultimately. The further adaptation of HPAI H5 in cattle could completely alter the evolution and pathogenesis of the virus.

vi) It is crucial to update the epidemiology of avian influenza in mammals and wild birds to cope with the virus's evolution and take the necessary measures on time.

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