

Expanding Host Range of Avian Influenza Virus: H5N1 Subtype

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Table of Contents

Acknowledgement.....	2
List of Figures.....	5
List of Tables.....	6
List of abbreviations	6
Abstract.....	8
Introduction	9
Virology of avian influenza virus.....	12
Hemagglutinin	13
Neuraminidase.....	15
Matrix protein 1	16
Matrix protein 2	17
Polymerase complex.....	18
Nucleoprotein.....	22
Nonstructural protein 1.....	23
Nuclear export protein.....	24
Epidemiology and Evolution of AIV	25
Evolutionary perspective on AIV	25
Epidemiology of AIV and IAV	28
H7 subtypes (H7N9)	33

H5 subtypes (H5N1)	38
Signs of H5N1 pandemic risks	46
Conclusion	52
Future works	54
References:	55

List of Figures

Figure 1. AIV gene organization	10
Figure 2. AIV gene segments	10
Figure 3. AIV structure.....	11
Figure 4. HA homotrimer structure	14
Figure 5. AIV polymerase complex.....	22
Figure 6. Timeline of the emergence of swine influenza virus lineages and human infections in Europe and North America	29
Figure 7. The transmission map of influenza A virus between avian and mammalian reservoirs	30
Figure 8. Host range of IAVs	43
Figure 9. Evolution of H5N1	44
Figure 10. H5N1 global distribution	52

List of Tables

Table 1. Common AIV subtypes that infect humans	12
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List of abbreviations

AIV: Avian influenza virus

IAV: Influenza virus A

LPAI: Low pathogenicity avian influenza

HPAI: Highly pathogenicity avian influenza

HA: Hemagglutinin

NA: Neuraminidase

PB1: Polymerase basic 1

PB2: Polymerase basic 2

PA: Polymerase acidic

M1: Matrix protein 1

M2: Matrix protein 2

NP: Nucleoprotein

NS1: Nonstructural protein 1

NS2: Nonstructural protein 2 or NEP: nuclear export protein

RNP: Ribonucleoproteins

vRNP: Viral ribonucleoprotein

UTR: Untranslated regions

Abstract

The avian influenza viruses (AIV) belong to Orthomyxoviridae family and are classified as influenza A viruses. AIV has eight segments of single-stranded RNAs with a negative polarity that encode 11 proteins. Two groups of the most important subtypes of AIV are H5 and H7, which infect humans, and some of them are highly pathogenic. H5N1 and H7N9 were reported for the first time in Hong Kong in 1997 and China in 2013, and so far, have demonstrated 52% (H5N1) and 40% (H7N9) case-fatality in humans. Unlike H7N9, which has a narrow host range, including birds and humans, H5N1 has a broad host range, including birds, various mammals, and humans. This broad host range, along with mammalian adapting mutations (especially PB2-E627K), raises concerns about its potential to adapt to humans and cause pandemics. Evidence of mammal-to-mammal transmission, foodborne transmission, and the systemic infection of H5N1 in mammals has been observed in European and Canadian foxes, as well as marine mammals in South American countries. The main concern about AIVs in 2024 is the infection of dairy cattle in the US by H5N1. This outbreak occurred in cows, which are considered resistant to influenza A viruses. Some significant observations in this outbreak are transmission of H5N1 between cows, release of H5N1 into cows' milk and as a result milk contamination with H5N1 and transmission to humans. In conclusion, based on the patterns of mammalian infection in the past decade, particularly in the last four years in the Americas, we can anticipate further adaptations of H5N1 in different mammalian species and ultimately human hosts, leading to systemic infections and new pathways for transmission.

Key words: Avian influenza virus, H5N1, H7N9, AIV transmission, AIV epidemiology, AIV evolution, H5N1 mammalian infection, PB2-E627K, H5N1 cow infection, mammal-to-human transmission, mammal-to-mammal transmission, milk H5N1 contamination

Introduction

Avian influenza virus (AIV) belongs to the *Orthomyxoviridae* virus family. It is derived from influenza A and replicates in the nucleus. The virus is enveloped, pleomorphic, and has a spherical shape with spikes. Its size ranges from 80 to 120 nm. AIV has eight single-stranded RNAs with negative polarity, and it encodes 11 necessary proteins (Figure 1) (Spackman, 2020; AbuBakar et al., 2023). The eight genome segments of AIV are numbered from 1 to 8, based on their length, with 1 being the longest and 8 being the shortest (Figure 2) (El Zowalaty et al., 2013; AbuBakar et al., 2023). Generally, the proteins that the AIV genome codes are polymerase basic 2, polymerase basic 1, hemagglutinin [HA], neuraminidase [NA], nucleoprotein, polymerase acidic, matrix, and nonstructural proteins which can be seen in Figure 3 (AbuBakar et al., 2023). AIV comprises three main subviral components: an envelope, a layer of matrix 1 proteins (M1 protein), and a viral ribonucleoprotein (vRNP) core. The viral envelope, made of a lipid bilayer membrane, is derived from the host cell membrane. Spikes derive from 2 integral transmembrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and a transmembrane ion channel matrix 2 (M2) (AbuBakar et al., 2023; El Zowalaty et al., 2013).

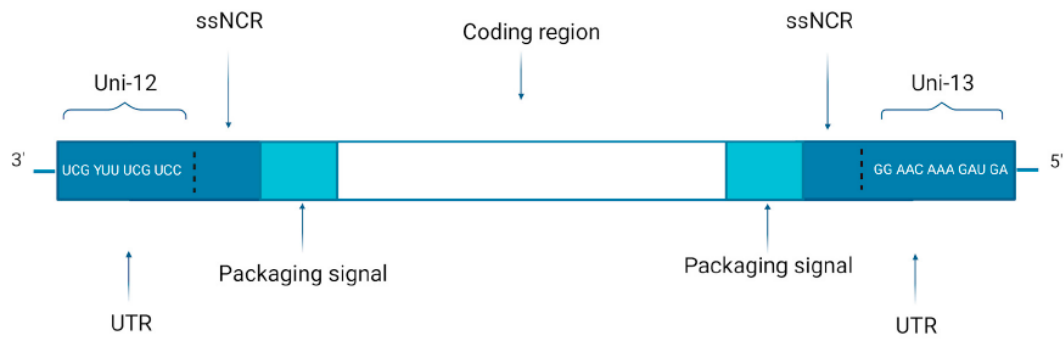


Figure 1. AIV gene organization: AIV genes consist of one UTR (Untranslated regions) at each end of the gene (universal primers Uni-12 at 3' end and Uni-13 at 5' end), ssNCRs (the segment-specific non-coding regions) next to the UTRs, a coding region in the middle, and two packaging signals at two ends of the coding region (AbuBakar et al., 2023).

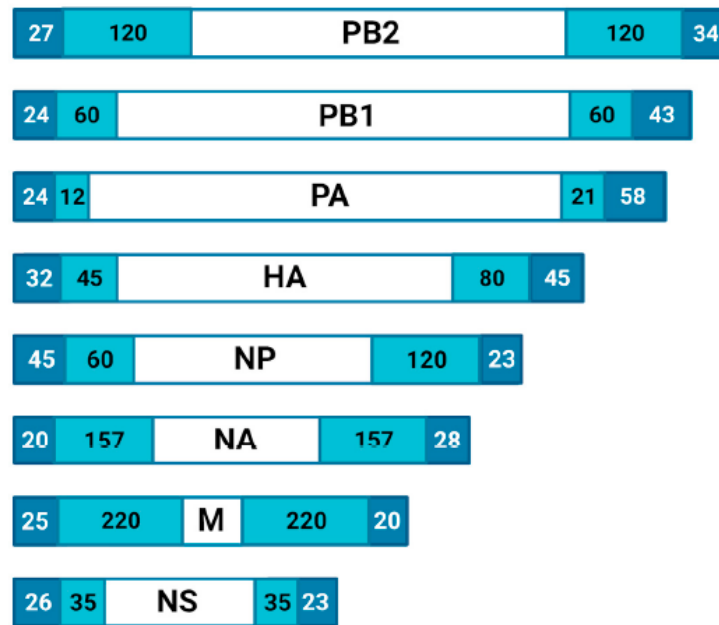


Figure 2. AIV gene segments: AIV has eight negative-sense, single-stranded RNA gene segments that can be translated to PB2, PB1, PA, HA, NP, NA, M, and NS (white boxes). The UTRs of each gene segment and their nucleotide number are shown in dark blue boxes, and their packing signals with their nucleotide number are indicated in light blue boxes (AbuBakar et al., 2023).

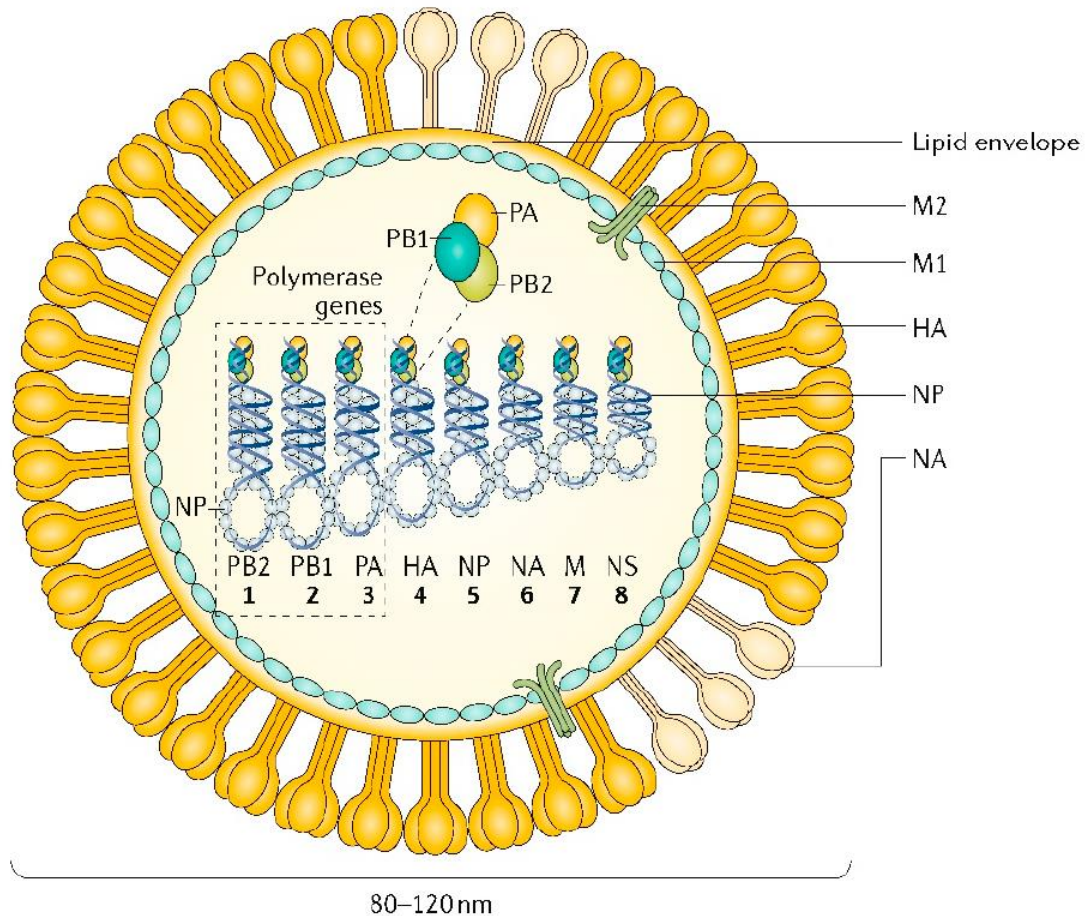


Figure 3. AIV structure. AIV has three main components: 1. Its envelope consists of a lipid bilayer and HA, NA, and M2 ion channel proteins; 2. An inner surface envelope layer made of M1 proteins; 3. Eight vRNA segments. In this figure, RNA polymerase is also shown, which its three proteins produce and assemble in infected cells (Bisset & Hoyne, 2020).

Hemagglutinin (HA) and neuraminidase (NA) are two critical proteins in the pathogenicity and tropism of the virus based on their attachment and detachment of the virion to and from host cells. Among these two proteins, HA plays a vital role in the pathogenicity of the virus, as the proteolytic cleavage site (PCS) sequence of HA protein can determine whether the virus is highly pathogenic or low pathogenic by processing the HA and releasing fusion requirements (Verhagen et al., 2021; Perdue & Suarez, 2000). There are 18 subtypes of HA and 11 subtypes of NA, and their natural combinations are more than 130. It is anticipated that future mutations can produce additional types that

can be indefinite (AbuBakar et al., 2023). Moreover, the importance of HA and NA influenced the nomenclature of all the different types of influenzas, and nowadays, the types of AIV, same as others, are called by the H (type number) and N (type number); for instance, the H1N1 virus, also known as the Spanish flu, caused a pandemic during 1918 and 1919 (El Zowalaty et al., 2013). Some of the common subtype cases that infected humans are shown in table 1.

Table 1. Common AIV subtypes that infect humans (Sha et al., 2016; Van Kerkhove, 2013; Su et al., 2015; Kutter et al., 2023; Friedman et al., 2017; Husain, 2014; Malik Peiris, 2009; Zhu et al., 2022; Noisumdaeng et al., 2022; WHO-H5N8, 2021; Jonges et al., 2014; Tan et al., 2023).

Subtype	Year (first observation (f)/pandemic (p))	Place	Known as
H5N1	f. 1997	Hong Kong	Avian flu
H7N9	f. 2013	China	Avian flu
H2N2	p. 1957	China	Asian influenza
H3N2	P. 1968	Hong Kong	Hong Kong influenza
H1N1	p. 1918 and 2009	North America	Swine flu
H5N6	f. 2014	China	Bird flu
H7N7	p. 2003	Netherlands	Avian flu
H5N8	f. 2021	Russia	Bird flu
H9N2	f. 1998	Hong Kong	Avian flu

Virology of avian influenza virus

Avian influenza virus (AIV) is classified into two groups based on pathogenicity: low pathogenicity avian influenza (LPAI) viruses and highly pathogenic avian influenza (HPAI) viruses. HPAI viruses can cause high mortality rates in birds, become systemic, attack their nervous systems, and can even cross species barriers. On the other hand, LPAI viruses usually cause local infections in the respiratory tract, remain within their species and do not cause pandemics (AbuBakar et al., 2023; Spackman, 2020; Chaves et al., 2011).

Hemagglutinin

RNA number 4 of AIVs' genome corresponds to Hemagglutinin (HA) protein, an essential homotrimer integral membrane glycoprotein located in the virus envelope that plays a significant role in AIV pathogenicity by recognizing the receptor (El Zowalaty et al., 2013; Isin et al., 2002). The glycoprotein part of HA attaches to sialic acid-containing moieties on the host cell, causing virus and host cell membrane fusion (endocytosis) and releasing the virus genome into the host cell cytoplasm (Isin et al., 2002). In addition, HA is a major antigenic determinant. Its genome, mRNA, and protein consist of 1778 and 1757 nucleotides and 550 amino acids, respectively. Each virion contains 500 HA on its surface, with each protein weighing 220 kDa (El Zowalaty et al., 2013). HA is comprised of three identical monomers that attach to a central α -helical coiled-coil, forming a stem-like domain with three globular heads (El Zowalaty et al., 2013; Isin et al., 2002; Gamblin et al., 2021). The sialic acid-binding sites are located within HA's globular heads. HA has a cylindrical trimer shape with an approximate length of 135 Å and a width that ranging from 30 to 50 Å in diameter (Gamblin et al., 2021; El Zowalaty et al., 2013). There are 18 types of HAs, which are divided into two groups. The first group consists of three clades while the second group consists of two clades (Gamblin et al., 2021; AbuBakar et al., 2023).

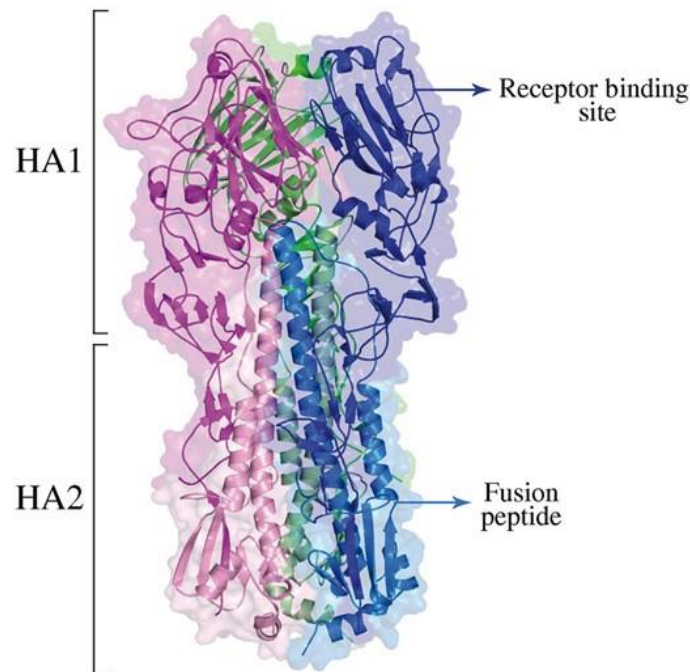


Figure 4. HA homotrimer structure. The HA protein of IAVs comprises three monomers, each of which is made of the globular HA1 domain and the stalk-like HA2 domain, and they represented by the colors purple, green, and blue. The receptor binding site and fusion peptide are also indicated (Lazniewski et al., 2018).

In producing HA monomers, a fusion-inactive chain called HA0 is cleaved into two polypeptides - HA1 and HA2. During this process, two disulfide bonds are formed between them, and the resulting HA0 folds in the endoplasmic reticulum (Isin et al., 2002). Then, each C-terminus of HA1 and the N-terminus of HA2 split from each other, and helical transmembrane peptides of each HA2 chain are anchored into the viral membrane for monomer placement. HA1 residues fold to form globular heads, whose distal tips are the receptor binding sites (Isin et al., 2002). The mentioned parts form four subdomains generally: the membrane-distal receptor binding subdomain (subdomain R), the vestigial esterase subdomain of HA1 (subdomain E), the membrane-proximal fusion subdomain (subdomain F) formed by parts of HA1 and HA2 and the membrane anchor subdomain, which is a part of membrane-associated residues of HA2 (Gamblin et al., 2021).

The glycoprotein component of HA is crucial for HA dual functionality, which necessitates a significant degree of conformational flexibility for both of HA's responsibilities. The first 20 amino acids of HA2 constitute the fusion peptide responsible for the endocytosis process. This nonpolar peptide is rich in glycine amino acids, imparting high conformational flexibility (Isin et al., 2002). Typically, the fusion peptide is located between the subunits of the HA trimer at a normal pH. However, when the pH decreases to 5 or 6.5, the fusion peptides undergo a conformational change and become exposed (Isin et al., 2002; Gamblin et al., 2021). This exposure of the fusion peptides enables the hydrophobic association between the HA molecule and the target membrane (Isin et al., 2002).

Hemagglutinins (HAs) possess a restricted binding affinity towards the receptors present on host cells. Thus, during the fusion process, the fusion site's formation requires eight HA molecules, out of which only two or three molecules are required to undergo the crucial conformational changes (Isin et al., 2002; Gamblin et al., 2021).

Neuraminidase

Neuraminidase (NA) is a glycoprotein that is mushroom-shaped and homotetrameric in structure. It is a surface protein that plays a crucial role in the pathogenicity of AIV. NA is also an antigenic determinant and a sialidase (exosialidase) that cleaves the α -ketosidic linkage between the terminal sialic (N-acetylneuraminic) acid residues from glycoconjugates (an adjacent sugar residue) (Shtyrya et al., 2009; El Zowalaty et al., 2013). NA executes three functions in different stages of infection. Initially, due to its sialidase activity, NA aids in the digestion of mucin by cleaving sialic acids from it, allowing the virus to reach its target epithelium (Shtyrya et al., 2009; El Zowalaty et al.,

2013; Liu et al., 2023). Secondly, it contributes to viral and cell membrane fusion by cleaving the terminal SAs from virus receptors. Lastly, NA prevents the accumulation of new virions by attaching the HA of the first virion and the sialylated glycans of the second virion and removes the cell surface's SA receptors to facilitate the budding process of newly formed virions (Shtyrya et al., 2009; El Zowalaty et al., 2013; Liu et al., 2023).

NA is derived from a 1413 nucleotide genome, 1392 nucleotide mRNA, and 470 amino acid residues. Each NA monomer weighs 60 KDa, and the tetramer molecular mass is 240 KDa (Shtyrya et al., 2009; El Zowalaty et al., 2013). Each virion is comprised of 100 neuraminidase molecules, which make up approximately 10–20% of the total virion surface glycoprotein (El Zowalaty et al., 2013; Liu et al., 2023). Neuraminidase is also localized in the virion envelope, infected cell surface, cytoplasmic tail, transmembrane span, extracellular stalk, the globular head bears antigenic sites and enzyme active site (El Zowalaty et al., 2013). NA consists of a cytoplasmic domain, a transmembrane domain, a head (with an enzyme active site and calcium binding domain), and a stem that connects the head and the transmembrane domain. The enzyme active site and calcium binding domain are responsible for the enzyme structure stability at low pH values (Shtyrya et al., 2009).

Matrix protein 1

Matrix protein 1 (M1) is the seventh RNA sequence consisting of 1027 nucleotides. Its primary function is replicating and assembling the virus, creating a new virion (El Zowalaty et al., 2013; Hilsch et al., 2014). M1 is found underneath the lipid bilayer of the virion envelope, which associates with vRNPs in mature virions to create the nucleocapsid. By recognizing the viral glycoproteins and forming a domain on the inner

surface of the plasma membrane, M1 establishes a binding site for vRNA (Hilsch et al., 2014). As a result, it modulates the nuclear transport of vRNP in early infection by binding to vRNP prior to RNP transport to the nucleus. In late infection, M1 proteins bind RNP and produce RNP transport signals from the nucleus to the cell surface (Hilsch et al., 2014). Furthermore, M1 acts as an endoskeleton, determining the 3-dimensional structure of the virion (Hilsch et al., 2014). M1, the most abundant virion protein, associates RNP with hemagglutinin and neuraminidase at the cell membrane and promotes virion formation and budding (El Zowalaty et al., 2013; Lamb & Choppin, 1983). M1 is also pH sensitive, and the structural change induced by pH may affect virion entry and disassembly (Peukes et al., 2020). The mRNA for M1 has 1005 nucleotides, which translates to 252 amino acids with a weight of 28 kDa, and each virion contains 3000 M1 proteins (El Zowalaty et al., 2013).

Matrix protein 2

Matrix protein 2 (M2) originated from the same RNA sequence as M1. M2 is a 15-kDa membrane cation channel homotetramer that assembles and disassembles the virus and recruits M1 to the plasma membrane, which does not have an apical transport signal (El Zowalaty et al., 2013; Petrich et al., 2021). The three primary roles of M2 are its ion channel activity, assisting in virus assembly and budding, as well as disrupting several host cell functions. Specifically, as an ion channel, M2 pumps protons into the virion interior when it enters an endosome at a pH of 6.5-6.0, disturbing M1 and vRNPs interactions (Saelens, 2019). As the pH decreases to 5.4-6.0 in the late endosome, M2 begins to flux potassium ions into the virion, resulting in the breakage of vRNP-vRNP interactions and releasing it into the cytoplasm to migrate to the nucleus for replication

and transcription (Saelens, 2019). Moreover, it increases the pH of the host cell's Golgi apparatus to protect from hemagglutinin, which is sensitive to pH. Generally, it acidifies the virus interior until it can pass through the endosomal pathway to dissociate vRNP from M1 (El Zowalaty et al., 2013). M2 recruits M1 to the plasma membrane and assembles virion with their interaction (The membrane distal end of the cytoplasmic part of M2 interacts with M1) (Saelens, 2019). In infected cells' plasma membrane, M2 accumulates at the periphery of HA and NA-containing lipid rafts, altering the membrane curvature and releasing newly assembled virions. An example of cell function disruption by M2 function is through the M2 ion channel, which can activate inflammasomes and interrupt autophagy in myeloid cells (Saelens, 2019). M2 is found in the virion envelope, infected cell surface abundantly, and extracellular regions such as transmembrane span, cytoplasmic tail, and tetramers form a cation-selective channel. It is a 97 amino acid long protein, and the approximate number per virion is not applicable (El Zowalaty et al., 2013).

Polymerase complex

The polymerase complex in influenza viruses is RNA-dependent RNA polymerase complex functions as both transcriptase and replicase. The polymerase complex and its units are among influenza viruses' most conserved structures and proteins (Takashita, 2021). The active polymerase complex consists of 3 polymerases and Nucleoproteins: Polymerase basic 1, Polymerase basic 2, and Polymerase acidic (Biswas & Nayak, 1996). This complex produces three types of viral RNAs in the infected cells: mRNAs, complementary RNAs (cRNAs) anti-genomes, and vRNAs genomes (products of replication) (Biswas et al., 1998).

Polymerase basic 1 (PB1) protein is the core subunit of the heterotrimeric polymerase complex. It associates with RNP (Ribonucleoproteins) and virion RNA and initiates transcription and RNA elongation. It also possesses endonuclease activity and participates in RNA transcription and replication processes (El Zowalaty et al., 2013). The genome of PB1 consists of 2341 nucleotides, and its mRNA also comprises of 2341 nucleotides. PB1 translates to 757 amino acids, with a total weight of 87kDa. PB1 is located in the virion interior and nuclei of the infected cells, and the count of PB1 per virion ranges from 30 to 60 (El Zowalaty et al., 2013). In addition, PB1-F2 encodes by alternative ORF (open reading frame) overlaps with PB1 genes ORF during an open reading frame by +1 frame shifting (Varga et al., 2012). It has various effects on host cells, such as pleiotropic immunopathological effects that are strain and host-specific (for example, by inducing the local production of IFN- γ in the respiratory tract of specific hosts such as pigs), proapoptotic and apoptotic activity, inflammation, secondary bacterial infection (severe bacterial pneumonia in mice), and in some cases, cellular localization (such as H5N1 type of AIV) (El Zowalaty et al., 2013; Pena et al., 2012). PB1-F2 is approximately 11kDa in weight and is formed from 57 to 101 amino acids. It localizes to both sides of mitochondrial membranes and co-localizes with PB1 in the nucleus of IAV-infected cells. The count of PB1-F2 per virion is not applicable (El Zowalaty et al., 2013; Pena et al., 2012).

The Polymerase basic 2 (PB2) protein is essential to AIV polymerase and RNA transcriptase. It is responsible for recognizing host cell mRNA caps and plays a crucial role in viral pathogenicity and virulence (El Zowalaty et al., 2013). The genome of the PB2 protein comprises 2341 nucleotides, while its mRNA has 2320 nucleotides. It consists of

759 amino acids and weighs 96 kDa. PB2 protein is found in the virion interior and nuclei of the infected cells, with 30 to 60 number of them per virion (El Zowalaty et al., 2013). The nuclear localization of PB2 protein is facilitated by two sections - aa 448 to 496 and aa 736 to 739, and deletion or substitution of each one disturbs nuclear localization and turns it into a cytoplasmic protein (Mukaigawa & Nayak, 1991). Mutations in PB2 protein can lead to high virulence and intra- and inter-host transmission (Yan & Wu, 2011). For example, in H5N1 viruses, mutations in amino acids at positions 627 and 701 of the PB2 are significant in replication efficiency. Amino acids at these positions in AIVs that infect birds are glutamic acid, while for viruses that infect humans, they are lysine (Le et al., 2009). PB2s with lysine grow better at low temperatures, but glutamic acid subtypes work better at higher temperatures. The human's upper respiratory tract has a lower temperature, around 33°C, compared to the lower respiratory tract, which its temperature is around 37°C (Le et al., 2009). At the same time, in avians, the virus grows in their intestine, which has a temperature of around 41°C. Therefore, mutations in these positions can lead to the virus's high virulence and intra-host transmission from the lower to the upper respiratory tract (Le et al., 2009).

Polymerase acidic (PA) is the third polymerase gene in AIV that comprises 2233 nucleotides that produce a 2211-nucleotide-long mRNA. This gene translates to a 716-amino-acid protein weighing 85.5 kDa. The PA protein localizes in the Virion interior and infected cell nuclei, occurring in 30 to 60 molecules per virion (El Zowalaty et al., 2013). PA is not only a polymerase subunit and RNA transcriptase but also a protease and endonuclease. Consequently, by its proteolytic degradation activity, PA reduces its steady-state level and coexpressed proteins' steady-state level (Wang et al., 2021;

Perales et al., 2000). The PA subunit interacts with PB1 to construct a polymerase complex through the NH2 terminus of PB1. Furthermore, PA can effectively break host species barriers and adapt to these new hosts. Additionally, PAs exhibit polymorphic characteristics that contribute to the pathogenicity and host adaptation of the influenza virus (El Zowalaty et al., 2013; Wang et al., 2021).

The 3P heterocomplex free from viral RNP can be formed even in the absence of other viral proteins by three types of polymerases. The active polymerase complex, known as the viral ribonucleoprotein polymerase complex, is responsible for several crucial functions, such as the translation and replication of viral genome, host selection, pathogenicity, and transmission. It is made up of nucleoproteins, three polymerases, and viral RNA, which can assemble in the presence of other viral proteins (Figure 5) (Biswas & Nayak, 1996; Wang et al., 2021). PB1 and PB2 can also form a stable complex without viral RNA, viral RNP, or other viral proteins. The dynamicity of the first complex during assembly allows for a range of interactions based on the viral genome's transcription and replication modes (Biswas & Nayak, 1996). Experiments have shown that the expression and presence of all three polymerase types are necessary until polymerase basic 2 recognizes and cleaves the cap of host mRNA, and allowing for cap-dependent transcription of viral mRNA. PB1 and PB2 also collaborate in cap-dependent mRNA transcription, with PB1 leading the RNA chain elongation through its polymerizing activity (Biswas & Nayak, 1996).

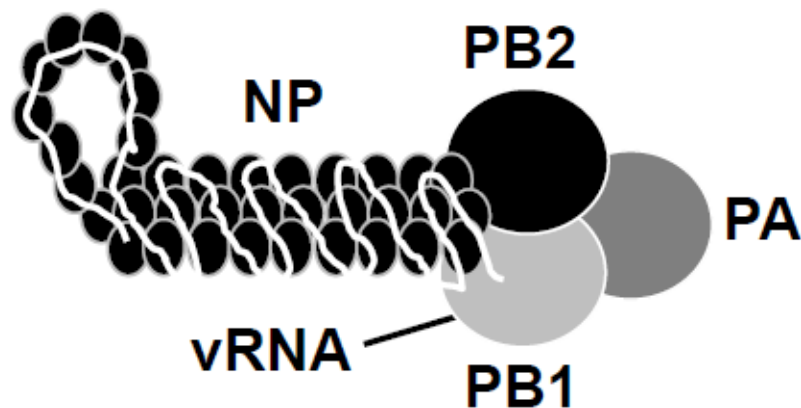


Figure 5. AIV polymerase complex. AIV polymerase complex composed of three components, PB2, PB1, and PA, is responsible for replicating the single-stranded negative-sense viral RNA coated with nucleoproteins (NP) (Martínez-Sobrido et al., 2018).

Nucleoprotein

Nucleoprotein (NP) is a major structural protein in the virus that coats the viral genomic RNA. It plays a role in RNA synthesis and regulates RNA nuclear import by assembling with polymerase complex and the budding process of virus particles by interaction with M1 (El Zowalaty et al., 2013; Biswas et al., 1998). It interacts with RNAs and polymerase proteins and is responsible for the maturation and packaging of AVIs. It is also responsible for determining the transcription or replication mode of the viral RNA (El Zowalaty et al., 2013; Biswas et al., 1998). NP is the fifth RNA sequence in length with 1565 nucleotides and translates to 1540 nucleotides mRNA. NP is a 55kDa protein comprising 498 amino acids that localize in the virion interior and associate with the P complex and viral RNA. Each AIV virion contains 1000 NP proteins (El Zowalaty et al., 2013).

Nonstructural protein 1

Nonstructural protein 1 (NS1) is a 25KDa multifunctional and viral interferon antagonist dimer protein that codes by 890 nucleotides. It is found only in the nuclei of infected cells as it is expressed in host cells but is not packaged into the virion. NS1 is responsible for viral fitness and the process of interspecies adaptation (El Zowalaty et al., 2013; Evseev & Magor, 2021). NS1 regulates host gene expression by binding to host RNA to inhibit its mRNA translation and suppress host pre-mRNA maturation by inhibiting cleavage and polyadenylation specificity factor 4 (CSPF4) and poly(A)-binding protein II (PABP2) (Evseev & Magor, 2021). It also regulates and promotes viral pre-mRNA splicing and translation and viral polymerase activity by recruiting the host eukaryotic initiation factor 4G1 (eIF4G1) and poly(A)-binding protein I (PABP1) to viral translation initiation complexes. It also downregulates dsRNA-induced antiviral responses by multiple protein-protein interactions and inhibiting type I interferon (IFN) signaling and its effector functions (Hao et al., 2020; El Zowalaty et al., 2013; Evseev & Magor, 2021). Notably, NS1 also activates the PI3K/Akt apoptosis pathway to delay virus-induced apoptosis in the host cells. This apoptosis inactivation provides the virus enough time to replicate sufficiently (Hao et al., 2020). NS1 also promotes cell survival by inhibiting p53-mediated transcriptional activity and apoptosis. On the other hand, NS1 interacts with heat shock protein 90 and alpha-tubulin to facilitate apoptosis (Hao et al., 2020). Its mRNA has 868 nucleotides and translates to 230 amino acids (El Zowalaty et al., 2013). Each NS1 protein has two domains and a flexible linker region. NA has three main parts or domains. The smaller domain, the RNA-binding domain (RBD), is an N-terminal domain with three

alpha-helices. The other domain is the effector domain (ED) in the middle, and the last part of NS1 is a C-terminal tail (Evseev & Magor, 2021).

Nuclear export protein

The nonstructural protein 2 (NS2), also known as nuclear export protein (NEP), is a 14 kDa protein composed of 121 amino acids encoded from the same sequence as NS1 and its location is associated with core components of the virion and cytoplasm of infected cells (El Zowalaty et al., 2013). The nuclear pore complex (NPC) controls the nucleocytoplasmic transport of macromolecules (Paterson & Fodor, 2012). NEP is an adaptor between vRNP complexes and the nuclear export machinery of the cell, causing the export of virion RNAs out of the nucleus and into the cytoplasm. NEP consists of two domains: an N-terminal domain and an amphipathic C-terminal domain of two-helices (Hao et al., 2020). The nuclear export signals (NESs) are related to the N-terminal domain. The N-terminal NES interacts with the chromosome region maintenance 1 (CRM1), and the C terminus binds the M1 protein to form the CRM1–NS2–M1–vRNP nuclear-export complex that can leave the nucleus (Hao et al., 2020). NEP also can positively or negatively affect the replication process, as high number of NS2 proteins can decrease viral RNA replication in some cases, while their presence is necessary for replication in mammalian cells infected with avian H5N1 influenza viruses. Therefore, NEP can be significant in host adaptation, and its expression determines levels of virus replication (Hao et al., 2020).

Epidemiology and Evolution of AIV

Evolutionary perspective on AIV

Over the past two decades, avian influenza viruses have demonstrated significant epidemiological and evolutionary success. Along with some less common evolving processes, two primary evolutionary processes, antigenic drift and antigenic shift, have been identified as the continuous and primary drivers of the evolutionary success of AIV (El Zowalaty et al., 2013; Husain, 2014). Antigenic drift is characterized by point mutations in the genome of the virus's surface glycoproteins, determining whether AIV is HPAI or LPAI. This process often results in the formation of new seasonal influenza virus strains and is highly likely due to AIV's highly error-prone replication process (Husain, 2014). On the other hand, an antigenic shift or reassortment occurs when two different genetic materials from two distinct subtypes are exchanged during co-infection, allowing the virus to adapt to different hosts and re-emerge each year (El Zowalaty et al., 2013; Husain, 2014). This process is responsible for creating pandemic strains and can convert low pathogenic AIVs to highly pathogenic ones, resulting in new substantial ecoepidemiology and zoonotic effects (El Zowalaty et al., 2013). For example, H9N2 and H5N1 subtypes have caused infections with low pathogenicity, though they have caused widespread infections in Hong Kong and Asia, respectively (El Zowalaty et al., 2013; Alexander, 2007; Husain, 2014).

In all influenza A viruses, including AIV, the haemagglutinin glycoprotein is initially produced as a precursor. This precursor needs to be cleaved by host enzymes while attached to host surface receptors (Gamblin et al., 2021; El Zowalaty et al., 2013). As mentioned before, this cleavage is crucial for the virus to enter the host cell and

determines its virulence. The main difference between LPAI and HPAI viruses is that the LPAI virus replicates locally, while the HPAI virus replicates systemically. This is one of the reasons why HPAs are more dangerous, as they can infect different organs and lead to mortality (El Zowalaty et al., 2013; Alexander, 2007). Observations of AIV infections in humans and birds have shown that cleavage efficiency depends on HA subtypes and proteases' stringent selectivity (El Zowalaty et al., 2013). The H5 and H7 subtypes are two types of glycoproteins that make up an extensive spectrum of HPAI, and some of their strains will be discussed later (El Zowalaty et al., 2013; Alexander, 2007).

The precursor molecule HA0 needs to be cleaved post-translationally and extracellularly by host proteases to make the virus infectious. The HA0 proteins of LPAI viruses have a single arginine residue at the cleavage site and another basic arginine or lysine residue at position 3 or 4 from this site (El Zowalaty et al., 2013; Alexander, 2007; Gamblin et al., 2021). This requires the presence of extracellular host proteases such as trypsin-like enzymes, and this brings some problems and restrictions for virus replication because these enzymes are found in a narrow range of organs. For instance, avian extracellular proteases are found only in their respiratory and intestinal tracts (Alexander, 2007). Similarly, the HA of mammalian viruses has a single arginine residue and, infrequently, a single lysine at the cleavage site. Therefore, the limitation mechanism of spreading the virus between mammalian organs are the same because the extracellular host proteases do not exist in various mammalian organs (El Zowalaty et al., 2013; Alexander, 2007).

Conversely, HPAI viruses have a distinctive feature in their hemagglutinin (HA) cleavage site. This feature includes the presence of basic arginine and lysine amino acids,

which are either inserted or substituted (El Zowalaty et al., 2013). This unique feature enables the cleavage site to be cleaved by intracellular ubiquitous proteases or proteases, which are believed to be proprotein-processing subtilisin-related endoproteases. The primary hypothesized protease is furin (El Zowalaty et al., 2013; Alexander, 2007). Accordingly, this enables the HA of HPAs to be cleaved by a wide range of host cells, allowing the virus to replicate in different vital organs of avians, causing significant damage and mortality (Alexander, 2007).

These HPAs have evolved from LPAs due to mutations. These mutations are postulated to occur from spontaneous duplications of purine triplets that lead to the insertion of basic amino acids by the polymerase complex during transcription (Alexander, 2007). Moreover, in some cases, nucleotides are not repeating, and it appears that nucleotide substitution can also occur to convert LPAs to HPAs. Furthermore, recombination has also been observed to change the cleavage site, as seen in the H7N3 HPA virus (Alexander, 2007). In 2002 and 2004, this virus was observed in Chile and Canada, respectively, and its cleavage site contained 11 amino acids from the nucleoprotein gene of the Chile virus and 7 amino acids from the matrix gene of the Canadian virus (Godoy et al., 2023; Alexander, 2007). Similarly, reassortments can also lead to the creation of a subtype that infects both humans and poultry, as observed in China, other parts of Asia, and Africa since 1996 in the process of H5N1 subtype creation. H5N1 has been created from Goose/Guangdong/1/96 virus (Gs/GD/1/96) and H9N2 or H6N1 viruses (Guan et al., 2009).

Epidemiology of AIV and IAV

During the previous century, the influenza A virus (IAV) subtypes have infected birds, humans, and other mammals and caused many pandemics. While the primary source of most IAV infections is avian, nowadays, some have evolved just to infect humans and a wide range of mammals (Bisset & Hoyne, 2020). For instance, H1N1 (Spanish flu), H3N2 (Hong Kong flu), and H1N2 (Asian flu) are three of the most common avian-originated subtypes that have emerged from swine and infected humans. The pandemic timeline of these subtypes in North America and Europe and their main host range is shown in Figure 6 (Freidl et al., 2014). Swine, a mixing vessel for influenza viruses, is pivotal in creating conditions for viral reassortment and cross-species transmission (Bisset & Hoyne, 2020). This is due to the distribution of viral receptors in their respiratory tracts, which feature both α -2,3 and α -2,6 sialic acids linkages, enabling bidirectional transmission at the pig–human interface (Sreenivasan et al., 2019).

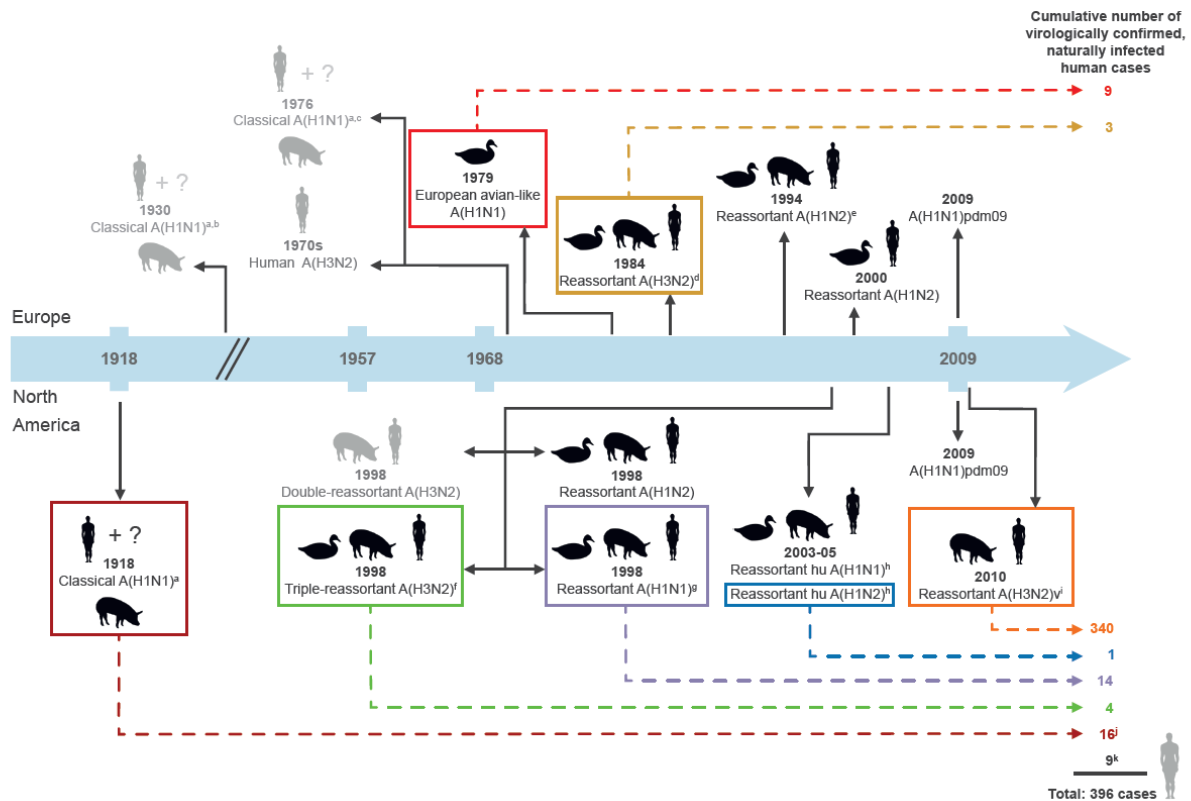


Figure 6. Timeline of the emergence of swine influenza virus lineages and human infections in Europe and North America. H1N1 pandemics occurred in 1918, 1979, 1998, 2003-2005, and 2009, with birds, humans, and swine as their main reservoirs. H1N2 pandemics occurred in 1994, 1998, 2000, and 2003-2005, with the same reservoirs as H1N1. H3N2 pandemics occurred in 1984, 1998, and 2010, with the same reservoirs as the others. Viruses in grey fonts did not spread efficiently in the swine population and did not lead to further infections (Freidl et al., 2014).

Furthermore, wild aquatic birds are the principal reservoirs for influenza A viruses. These birds can carry subtypes H1-H16 and spread them to other species, including domestic birds like chickens, turkeys, ducks, and geese, as well as other animals such as cats, horses, dogs, swine, humans, and marine mammals. The various host ranges and reservoirs, subtypes that infect them, and their cross-species transmission are demonstrated in Figure 7 (Bisset & Hoyne, 2020).

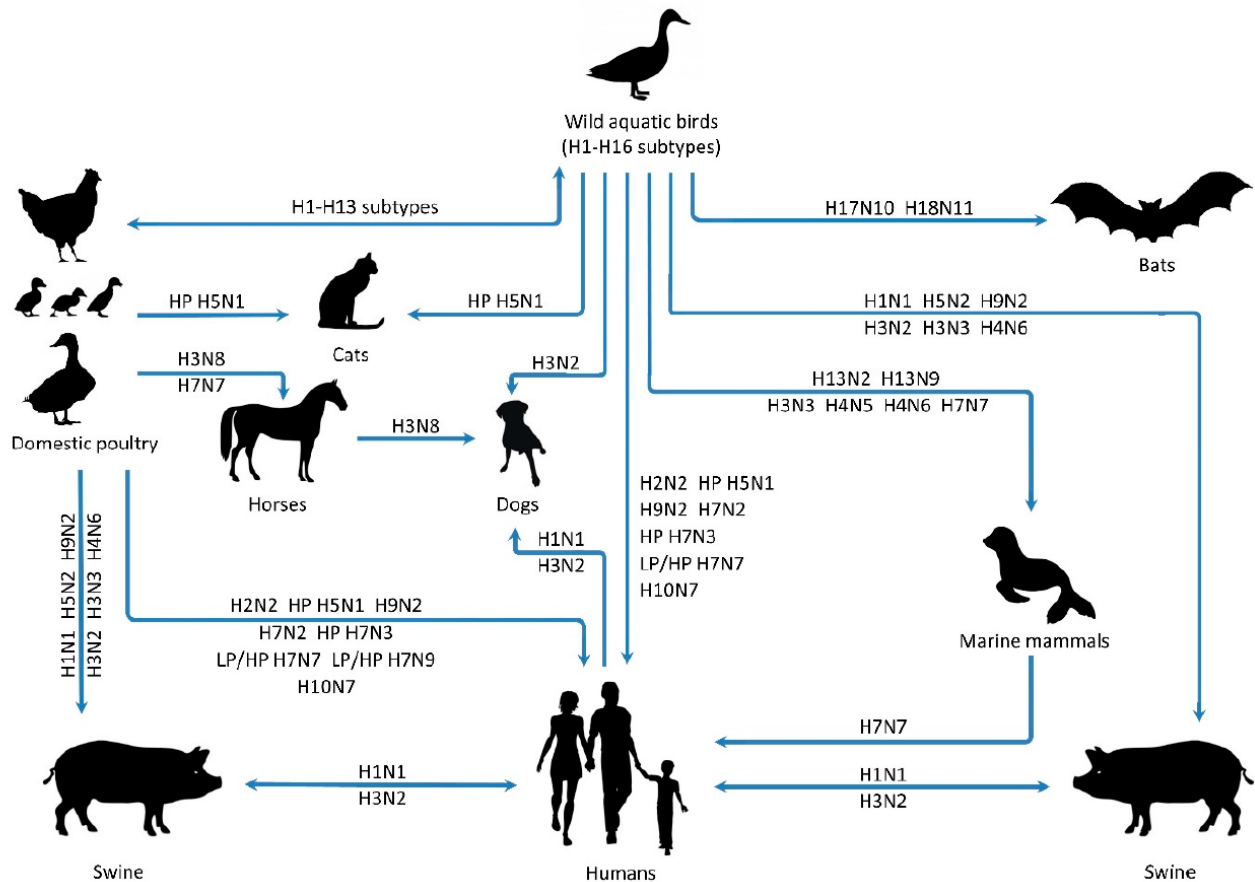


Figure 7. The transmission map of influenza A virus between avian and mammalian reservoirs. Domestic birds, cats, horses, dogs, swine, humans, and marine mammals can get infected by coming in contact with aquatic birds. Aquatic birds can be infected by having contact with domestic poultry. Domestic poultry can also transmit the influenza virus to most mammals. Humans can transmit two influenza subtypes to dogs, and dogs can get infected with domestic poultry subtypes by horses as intermediate hosts. The transmission pathways and the subtypes that transmit between these reservoirs are presented on the arrows. The H17N10 and H18N11 are just observed in the bats (Bisset & Hoyne, 2020).

As mentioned, the H5 and H7 subtypes are of great concern because of their potential to cause human pandemics and because they include some of the most famous HPAI subtypes. Less than ten of the subtypes of these two groups have infected humans (Bui et al., 2016). Two subtypes, H5N1 and H7N9, have caused the majority of AIVs infections in people and are the subtypes with 52% and 40% case-fatality rate in large numbers of humans (Chang et al., 2023; <https://www.who.int/emergencies/disease-outbreak-news/item/2024-DON512>; <https://www.cdc.gov/flu/avianflu/inhumans.htm>).

Consequently, we will focus mainly on these types of avian influenza viruses. The primary transmission mode of zoonotic AIV to humans is through domestic poultry (Bui et al., 2016). A bird's excretion, including saliva, nasal secretions, and feces, can potentially carry viruses and be a source of contamination (Skeik & Jabr, 2008). This transmission can occur through direct or indirect contact, such as direct contact with the feces of sick birds or just visiting a bird's market, coming to contact with infected carcasses. It has been observed that human infections with H7N9 are mainly caused by direct and indirect contact with poultry, whereas direct exposure to poultry is the primary transmission mode for H5N1 (Bui et al., 2016; Bethmont et al., 2016). Additionally, the rate of H7N9 infection in birds is lower than that of H5N1, while the rate of human infection with H7N9 is higher than that of H5N1 (Bui et al., 2016; Bui et al., 2017). This paradox has increased the importance of research on these subtypes. Furthermore, the incidence of H7N9 has largely been reported in China and other Asian countries, while this incidence of H5N1 has been reported in Asia, Europe, Africa, the Middle East, and Americas (Bui et al., 2016; Shi et al., 2023). This makes us pay more attention to the reservoirs of these viruses. The most significant factor in the prevalence rates of H7N9 and H5N1 is what reservoirs they habit. For instance, H7N9 mainly infects poultry, while H5N1 can infect both poultry and wild birds (Bui et al., 2016; Bui et al., 2015). Hence, the migratory patterns and migratory birds are significant factors in the incidence of the virus by infecting poultries and domestic birds. A clear illustration of that is the infection of farmed ducks by migratory ducks through the sharing of water sources or through contamination of feed (Bui et al., 2016; Bui et al., 2015).

Some H5 and H7 subtypes can transmit directly from the avian host to humans. Therefore, studying the migratory patterns of birds and how they become infected can help understand the transmission rate of AIVs and their potential to infect humans or other mammals (Blagodatski et al., 2021; Brown et al., 2007). Fecal–oral transmission is one of the ways that birds can be infected with AIVs, as AIVs can enter into the cells of birds' respiratory and gastrointestinal tract (Brown et al., 2007). As a result, aquatic environments may increase the infection rate in birds by water-borne transmission that can lead to a pandemic in poultries (ducks' example) and eventually a higher risk of human infections and a pandemic in humans. Experiments showed that H5 and H7 subtypes can persist in water for long periods, and their persistence depends on the water's temperature, pH, and salinity (Brown et al., 2007; Roche et al., 2009). For example, these viruses can persist in fecal materials in water for up to 30 days at 4 degrees Celsius but only 7 days at 20 degrees Celsius, and under neutral pH conditions, different subtypes were observed in the water from 2 to 7 weeks. In general, waters with neutral or basic pH levels showed the most persistence of AIVs (Blagodatski et al., 2021). However, the persistence of the viruses of the same subtypes in water is variable, and further studies are needed for a better understanding of the persistence of AIVs in water. The persistence of AIVs in water can cause birds to become infected or spread the virus during their migration worldwide (Brown et al., 2007).

Furthermore, it has been observed that in addition to direct and indirect contact with infected birds, there are other potential transmission pathways for H5N1 infection in humans. To date, there is no concrete evidence for sustained human-to-human transmission of H5N1 (Skeik & Jabr, 2008). However, Ungchusak et al. (2005) found 2

cases in Thailand that the most likely explanation for their infection was human-to-human transmission. An 11-year-old girl and her mother died from H5N1, with the mother having had close contact with her daughter while providing bedside care despite not having contact with birds, unlike her daughter. Also, her aunt, who lived with her, got infected with H5N1 and survived (Ungchusak et al., 2005). However, in this case the fomite-transmission cannot be ruled out. Additionally, the consumption of dishes containing raw contaminated poultry blood has been identified as another potential transmission pathway for H5N1 in humans ([https://www.who.int/news-room/fact-sheets/detail/influenza-\(avian-and-other-zoonotic\)](https://www.who.int/news-room/fact-sheets/detail/influenza-(avian-and-other-zoonotic))). Furthermore, raw poultry or eggs can transmit H5N1 to mammals that consume them (Skeik & Jabr, 2008).

H7 subtypes (H7N9)

As mentioned before, one of the subtypes of AIV that has caused several pandemics is the H7 subtype. The H7 subtype caused numerous outbreaks in poultry all around the globe, and three specific subtypes, H7N9, H7N7, and H7N3 subtypes, infected humans and caused outbreaks in human populations in 2013, 2003, and 1963. These outbreaks have mostly been seen in the Netherlands, Canada, Britain, China, and some other Asian Southeast countries (Husain, 2014; Kaye et al., 2023; Stegeman et al., 2004). This paper will focus on H7N9 as an exemplary case to provide a more detailed understanding of the H7 subtypes.

Three cases of human infections with the H7N9 subtype were reported for the first time in China (Shanghai and Anhui province) in 2013. These patients had respiratory symptoms such as breathing difficulties and severe respiratory tract infection, which led to severe pneumonia (Husain, 2014). Two of these patients died, and the other one was

in critical condition. Approximately one month later, four additional human cases were observed in eastern China with no epidemiological link with the three previous infections of the H7N9 virus subtype (Husain, 2014). In 2014, 436 cases in East China, 10 in Hong Kong, 4 in Taiwan, and 1 in Malaysia were laboratory-confirmed. In total, 451 were reported, of which 165 cases of them died, and the amounts of fatality rate were over 36%, while some patients were hospitalized in critical condition (Husain, 2014). The H7N9 virus demonstrates age-specific and sex-specific morbidity and mortality. While a few cases of children, teenagers, and young adults were listed, most of the patients were middle-aged people with a median of 58 years old. In addition, mostly middle-aged male patients indicated the most severe condition and most fatality rate in comparison with females (Husain, 2014; Lu et al., 2014). The incubation period of H7N9 infection was approximately 2 to 3 days, the duration from the onset of illness to death ranges from 12 to 21 days, while the time from hospitalization to discharge for surviving patients fluctuates between 21 to 41 days (Husain, 2014). Furthermore, H7N9 tests on people who had contact with patients were negative, and it showed H7N9 patients were epidemiologically unrelated. Consequently, the H7N9 virus could not infect humans by human-to-human transmission (Husain, 2014). Furthermore, infection with H7N9 continued and caused five waves of infections till 2017; the fifth one during 2016 and 2017 had the highest peak (Su et al., 2017). Like previous waves, most patients were men in the great fifth wave (in total, it is considered that 69% of individuals infected with H7N9 were male in all the waves of infections). However, instead of older men, middle-aged adults who lived in semi-urban and rural areas were mostly infected in the fifth wave (Su et al., 2017). In general, about 1560 people were infected with H7N9, and more than 600

of them died during these five waves of H7N9 (Li et al., 2021). During the first four waves, 85% of patients had close contact with poultry. In the fifth wave, 90% of them had close contact with poultry, and all people with the highly pathogenic version of the virus had close contact with poultry (Su et al., 2017). The most recent infection with H7N9 was in China in 2019 (<https://www.cdc.gov/flu/avianflu/reported-human-infections.htm>).

The groups of infected people with H7N9, as mentioned, are epidemiologically unrelated, and besides fever and cough, which are their most common symptoms, they also experienced other symptoms (Husain, 2014). The first three fatal cases showed severe pneumonia and acute respiratory distress syndrome (ARDS). In the first three cases and also in further waves' cases these pre-existing conditions were observed; chronic obstructive pulmonary disease (COPD), hepatitis B, hypertension, diabetes mellitus, chronic cardiovascular diseases, chronic liver disease, chronic respiratory diseases, and depression. Moreover, most of the cases involved severe lower respiratory tract illnesses. Also, shock, multi-organ failure, congestive heart failure, myelitis, acute kidney injury, diarrhea, vomiting, rhabdomyolysis, and liver, pancreas, and muscle injury were diagnosed in these H7N9 cases (Lu et al., 2014; Husain, 2014; Su et al., 2017). Therefore, these showed that underlying medical conditions were the significant risk factors for cases' symptoms, besides age and sex (Su et al., 2017; Husain, 2014). Some severe cases of patients from Shanghai in 2013 showed pneumonia, hemorrhagic bronchitis, multiple organ dysfunction, and central nervous system involvement (Lu et al., 2014).

This novel H7N9 virus was created through a reassortment process between H9N2, duck's H7N3 virus, and H2N9, H7N9, H10N9, or H11N9 viruses. All of the six

internal gene segments of H7N9 originated from H9N2, which M, NP, and NS segments believed to be circulating in chickens and polymerases' genes were from the brambling bird, while the HA gene came from the H7N3 virus and the NA gene could have come from one of these four subtypes H2N9, H7N9, H10N9, or H11N9 viruses from wild birds or chickens (Lee et al., 2013; Husain, 2014; Wang et al., 2014). At least two steps of sequential reassortment generated H7N9. It seems the first reassortment occurred in wild birds and the second in the Yangtze River Delta region domestic birds. Then, interspecies transmission happened, and H7N9 was transmitted to chicken population (Lee et al., 2013; Husain, 2014). Research showed that most of the patients had direct contact with chickens. Therefore, researchers concluded that introduction of these infected chickens into live-bird markets spread the virus (Li et al., 2021; Husain, 2014; Lu et al., 2014). Moreover, direct contact could explain why the virus was sex and age-specific, as mostly elderly and retired males visited or bought poultry from the live-bird markets (Husain, 2014).

Some main mutations in the H7N9 genome were illustrated at the protein level. The first change is at the HA cleavage site of the virus, which has just one basic amino acid, arginine. This arginine indicates low pathogenicity of the H7N9 virus in poultry (Li et al., 2021; Husain, 2014). A T160A substitution, which occurred during H7N9 HA evolution, also eliminated the N-glycosylation site. This elimination, which also have been seen in H5N1, led to the increase of H7N9 HA and H5N1 HA binding to the human-type receptor, $\alpha 2$, 6-linked sialic acid, which is expressed by upper respiratory tract epithelial cells, before (Husain, 2014; Broszeit et al., 2019). Three other mutations (G186V, Q226L, and A138S substitutions) were observed in the HA receptor-binding site that their observation

in previous new subtypes formations showed to be responsible for converting avian-type receptor, $\alpha 2$, 3-linked sialic acid to human-type receptor, $\alpha 2$, 6-linked sialic acid, which created high-affinity binding of the virus to human cells (Husain, 2014; Lu et al., 2014; Su et al., 2017).

Several genetic mutations have been identified in the H7N9 NA genome. One such mutation is the deletion of five amino acids (residue 69-73) in the stalk region of NA, which led to increased virulence (Lu et al., 2014; Husain, 2014). Another mutation, known as R292K substitution, has been indicated to cause resistance to NA inhibitors. Additionally, mutations in NA and M2 proteins have been found to cause antiviral drug resistance. Mutations in PB2, such as E627K, L89V, and D701N, have also been linked to the adaptation and virulence of avian influenza A virus in mammals (Li et al., 2021; Husain, 2014). Other mutations that have been identified in H7N9 are N30D and T215A in M1 and P42S in NS1, which have been shown to increase virus virulence in the H5N1 strain in mice (Husain, 2014; Su et al., 2017).

The host range of H7N9 does not include a wide variety of mammals, unlike H5N1's host range, which will be discussed (Yin et al., 2023). The H7N9 subtype primarily infects poultry such as chickens, ducks, geese, and pigeons, as well as humans (Uchida et al., 2017). However, most other mammalian infections were in laboratory animal models and not common in nature, such as camels, pigs, mice, and ferrets (Yin et al., 2023; Husain, 2014; Wu et al., 2020). The main mutations in H7N9 that enable infection in these animal models are PB2-E627K and HA-Q226L (Liu et al., 2014; Yin et al., 2023).

H5 subtypes (H5N1)

As H7 AIV subtypes, H5 subtypes are highly successful in infecting various species and causing pandemics. Some H5 subtypes, such as H5N2, H5N3, H5N6, and H5N8, have infected birds over the years (except H5N6, which infected three human cases in China in 2014-2015). However, H5N1 is one of the most significant subtypes in this group, as it has infected humans abundantly, causing several pandemics and outbreaks in both humans and birds over the years (Lai et al., 2016). Even this year, in 2024, we are witnessing humans and cows-infected cases with H5N1. Human case in the United States of America were exposed to infected cows and human case in Vietnam were exposed to infected bird. This virus infected 889 humans around the world in 23 countries from 2003 to April of 2024 and killed 463 of them. Therefore, the case fatality rate of H5N1 is 52% to this day (<https://www.who.int/emergencies/disease-outbreak-news/item/2024-DON512>).

In May 1997, H5N1 emerged in humans for the first time in Hong Kong (Bethmont et al., 2016; Lamb, 2008). A 3-year-old boy became infected with H5N1 (entirely of avian origin), which caused death. He was the first human case who died from H5N1 avian influenza. In the following month, 17 other cases of human infections with H5N1 were reported in Hong Kong, and 5 of them died from the H5N1 infection (Lamb, 2008; Uyeki, 2008; Yee et al., 2009). The 33% mortality rate of H5N1 made it an important subtype for further studies since 1997 (Lamb, 2008). Six years later, in July 2003, a new H5N1 outbreak occurred in the avian population in Thailand, Vietnam, and Indonesia, and it spread over Asia, southeast Europe, and Africa (Lamb, 2008; Uyeki, 2008; Yee et al., 2009). This outbreak brought fatal outcomes to over 100 million poultries (Lamb, 2008).

Moreover, during the wave of H5N1 between December 2004 and May 2006, Indonesia showed the highest human case-fatality rate that had been observed, with nearly 80% fatality (Yee et al., 2009). As of 2007, there had been 278 human cases of H5N1, resulting in 168 deaths (Lamb, 2008). By the end of 2007, that number had risen to 349 human cases, which mostly were younger than 40 years old, with a mortality rate of 62%. Until 2007, the most common first symptoms of H5N1 cases were high fever, cough, shortness of breath, dyspnoea, evidence of pneumonia on chest X-ray, and nearly one-third of cases had diarrhea (Uyeki, 2008). Other symptoms or diseases in cases when they were in hospital or experiencing their more advanced phase of disease were respiratory failures, multi-organ failure, sepsis, ARDS, shock, reactive haemophagocytosis, leucopenia, lymphopenia, and mild-to-moderately decreased platelet counts (Uyeki, 2008).

From 2010 to 2013, 181 individuals became infected with N5H1. Then, one of the largest H5N1 pandemics occurred from November 2014 to April 30, 2015, with 165 confirmed human infection cases, of which 51 of them died. By the end of 2015, the WHO had reported 213 human cases (Lai et al., 2016; Refaey et al., 2015). Egypt had the most cases of infection in two months, with 88 cases in January and February 2015 in this pandemic (Kayali et al., 2016). The H5N1 virus clade 2.2.1.2 spread in Egypt between poultries in the middle of 2014 and started to cause a pandemic in avians and afterward in humans. The male-to-female ratio of infected human cases was 1:1.2 in this pandemic, which is close to the percentage of female cases, 61% from 2006 to 2016 (Lai et al., 2016; Refaey et al., 2015). The median age of cases was 19, with recovered and fatal cases reporting different median ages globally (a general median reported number is 22 years old for fatal cases and 10 years old for recovered cases). Until 2015, most human cases

of H5N1 were infected by having contact with avians (Lai et al., 2016). The outbreak's geographical distribution of human H5N1 cases highlighted the role of bird migration in human AIV infections more than previous subtypes. Research on monthly and seasonal infection rates and bird migration patterns revealed that weather and migration play a vital role in virus prevalence (Lai et al., 2016; Tian et al., 2015). In the winter, the weather is dry and cold, and birds' immune systems are weaker, which leads to a higher incidence of poultry infection. In addition, birds' migration patterns coincide with this season and spring (the second season with the highest infection rate), leading to more human infection (Durand et al., 2015; Mathur et al., 2014). For instance, the onset of the H5N1 pandemic in Egypt from 2014 to 2015 coincided with the migration of birds to that region, based on the months with the highest peak of infection. In general, people from 17 countries were infected with H5N1 from 1997 to 2015, mainly from Asian and North African countries. The overall case fatality risk during this period was 53.3%, with 95.8% of cases having contact with poultries and 6.2% having contact with infected humans (Lai et al., 2016).

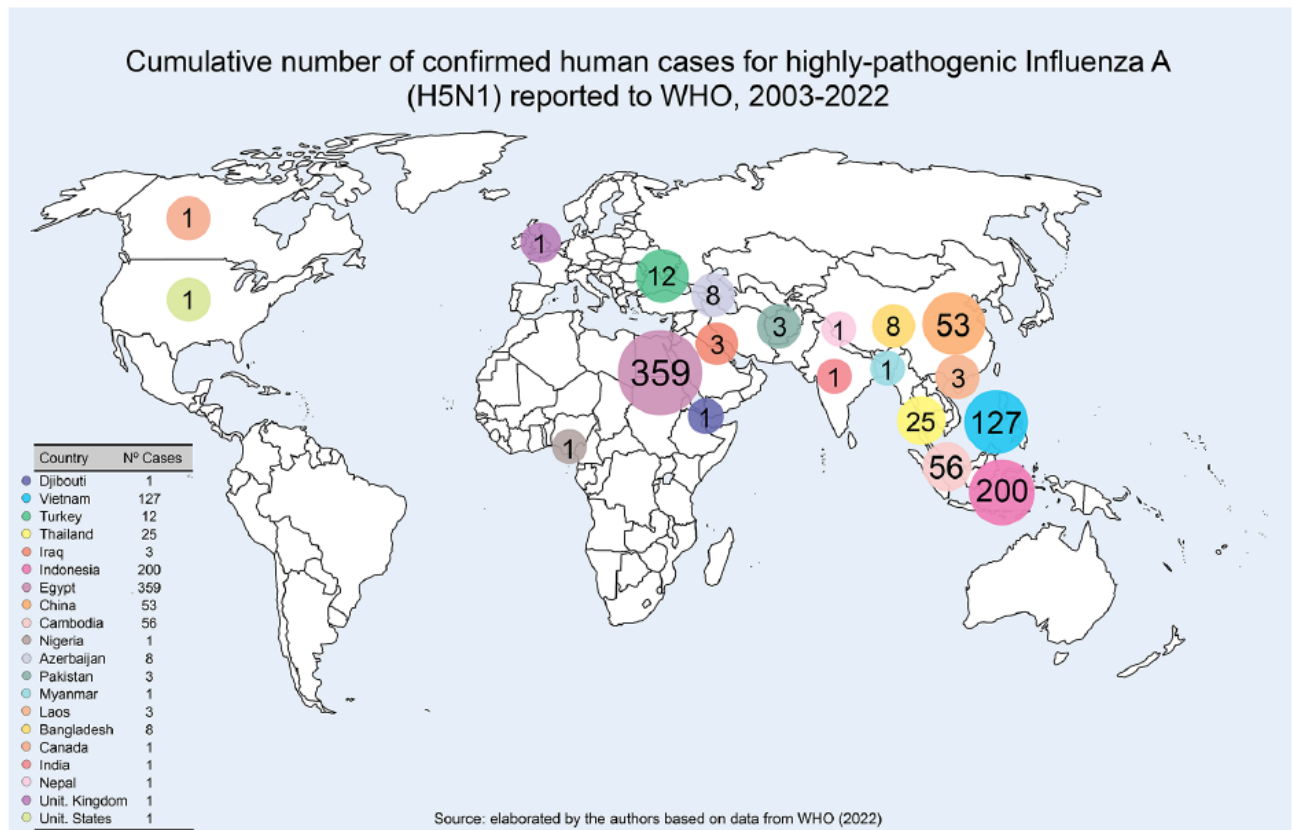


Figure 6. The distribution map of H5N1 AIV from 2003 to 2015. The figure highlights the variable distribution of human infections across different regions of the world during this period (Possas et al., 2023).

Since 1997, more than 890 cases of human infection with H5N1 have been reported in 23 countries around the world, with a mortality rate of over 50%. However, there has been a significant decrease in the number of human infections from 2016 to 2022, with only a few cases being reported during this time (<https://www.cdc.gov/flu/avianflu/reported-human-infections.htm>). From July 2022 to July 2023, WHO reported 14 human cases of H5N1, 2 of which were fatal (Charostad et al., 2023). In 2022, the first American human infection with H5N1 was reported in Colorado, in a patient who had contact with poultries. No other human infections were reported in the US for the next two years until March 27, 2024, when a farm worker in Texas tested positive for H5N1, and dairy cattle and wild birds in the area were also infected. Viral

genome sequencing of human and cattle specimen samples showed minor changes in the H5N1 genome, with no observed changes that would make the virus more efficient at adapting to mammals (<https://emergency.cdc.gov/han/2024/han00506.asp#:~:text=Since%201997%2C%20more%20than%20900,been%20reported%20worldwide%20since%202022>). In addition, the first case of human infection in South America was reported in Ecuador in 2023, with a 3-year-old girl being infected with the 2.3.4.4b clade, the same clade that caused another case in China in the same year. In 2023, two human cases were reported in Cambodia, and one case was reported in Chile (Castillo et al., 2023).

Since the first observation of avian influenza virus (AIV) H5N1, different mammals become infected with H5N1, which are noteworthy. In early 2004, tigers were infected for the first time in a zoo in Thailand (Amonsin et al., 2006). These tigers were infected with H5N1 by consuming infected birds or by tiger-to-tiger transmission. Therefore, these cases demonstrated the possibility of transmitting H5N1 to a mammal by consuming a sick bird or contacting other mammals for the first time (Yee et al., 2009; Amonsin et al., 2006). Some cat cases were also observed, which their tests revealed the virus in their gut, stool, and trachea. Hence, it can be concluded that cat infection by consuming infected birds is also possible (Yee et al., 2009). In 2023, over 280 outbreaks in mammals were observed in the continent of America, with skunks and red foxes being among the most infected mammals (<https://www.paho.org/en/documents/epidemiological-update-outbreaks-avian-influenza-caused-influenza-ah5n1-region-americas-1>). Besides these mammals, some other mammalian cases were observed in the nature commonly such as

bobcats, cats, pigs, dogs, lions, minks, seals, whales and bears (Figure 8) (Kandeil et al., 2023; Uyeki, 2008).

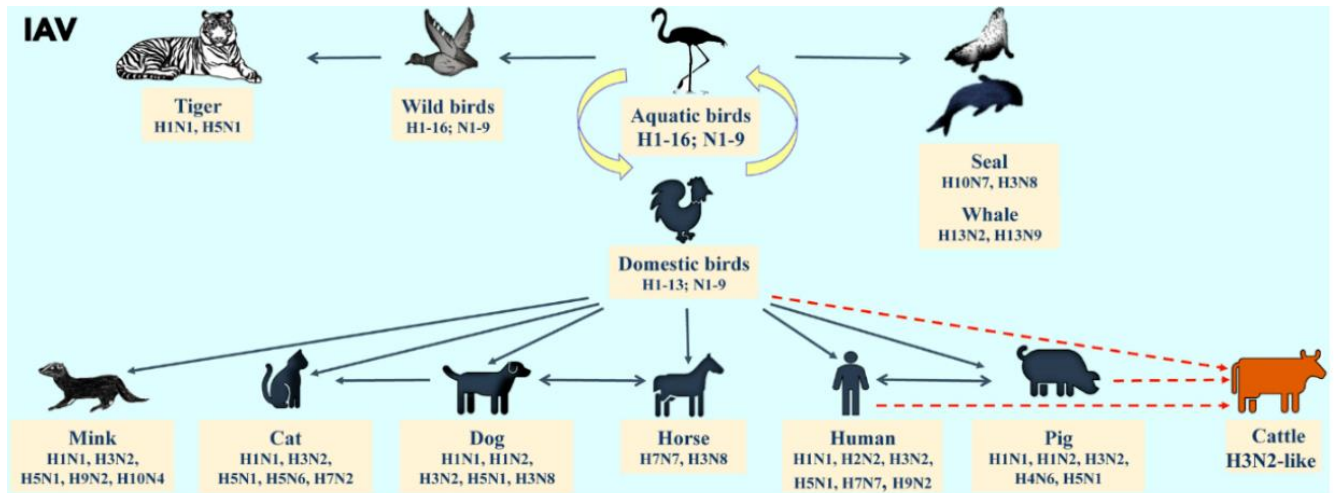


Figure 8. Host range of IAVs. Influenza viruses have a broad host range, and various subtypes can infect mammals and birds. The figure shows each host and the subtypes that infect it (Sreenivasan et al., 2019).

In summary, the host range of H5N1 virus are widely diverse and include wild birds, domestic birds, cats, dogs, foxes, seals, leopards, tigers, lions, bears, pikas, otters, polecats, porpoises, pigs, Virginia opossums, civets, badgers, dolphins, stone/beechn martens, coyotes, raccoons, raccoon dogs, Mustelidae (minks and otters), skunks and fish (Charostad et al., 2023).

The initial H5N1 subtype virus that caused human infection in Hong Kong in 1997 evolved from the reassortment of three subtypes of viruses. This particular virus's HA gene originated from a Gs/GD/1/96 (H5N1)- like virus, its NA gene from an A/teal/Hong Kong/W312/97(H6N1)- like virus, and its internal genes from either an A/quail/Hong Kong/G1/97(H9N2)- like virus or an A/teal/Hong Kong/W312/97 (H6N1)- like virus (Noisumdaeng et al., 2022).

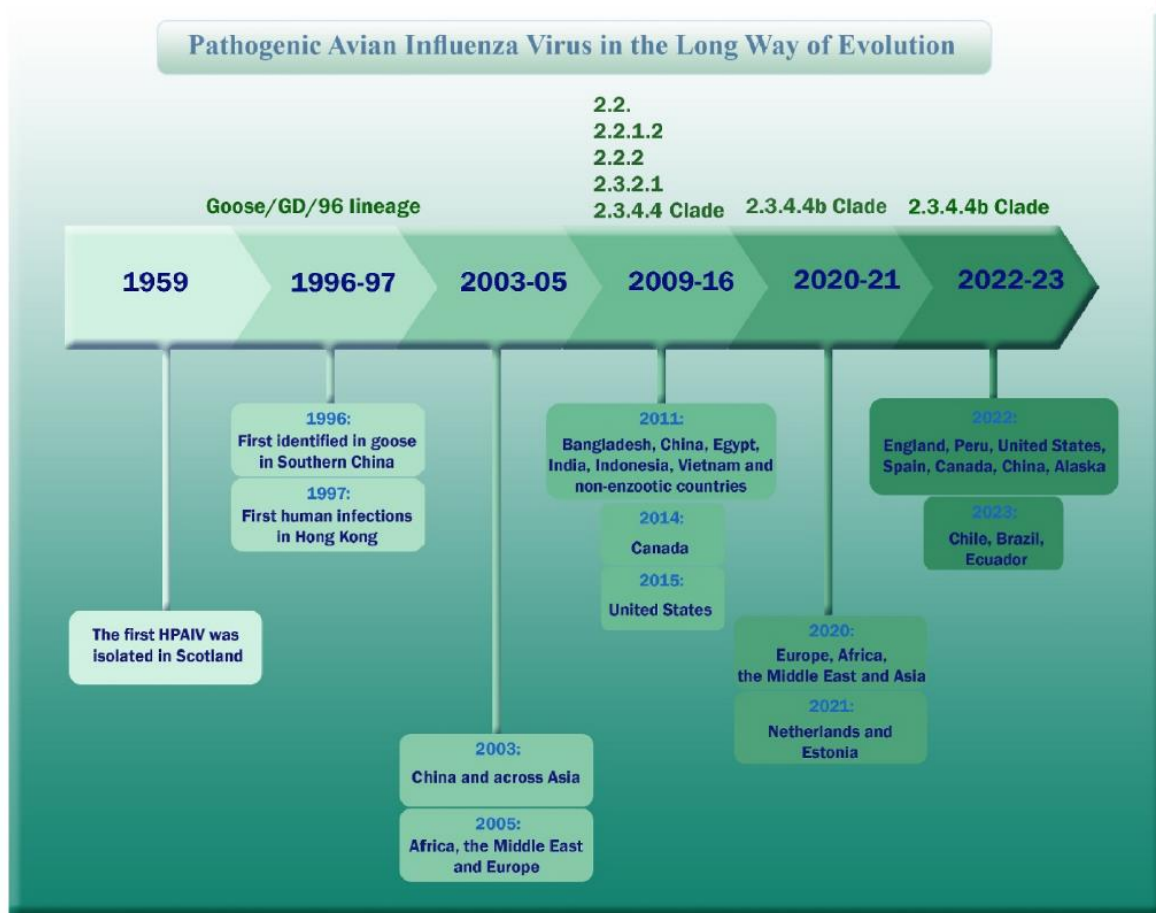


Figure 9. Evolution of H5N1. The H5N1 subtype has been classified into ten primary clades, labeled 0 to 9, each with multiple subclades. This figure illustrates the various clades of H5N1 that have infected humans and other mammals and are the most famous ones, providing details on their emergence year and location (Charostad et al., 2023).

To date, H5N1 has demonstrated the ability to infect various species, including mammals, due to different point mutations. One of the crucial substitutions in H5N1 genes, which facilitated its ability to infect humans, is the T160A substitution. This substitution is further elaborated in the context of H7N9 substitutions (Husain, 2014). Another substitution, S159N (H3 HA numbering), can also confer the ability for H5N1 to infect humans by decreasing its affinity to $\alpha 2-3$ linked receptors and increasing its clear binding to $\alpha 2-6$ linked sialosaccharide (Gambaryan et al., 2006). The S227N substitution is another mutation that enhances H5N1's affinity to $\alpha 2-6$ linked sialosaccharide

receptors. This substitution occurs between amino acids 226 and 228, which play a critical role in receptor specificity and host range restrictions not only in H5N1 but also in other influenza A viruses (Gambaryan et al., 2006). S133A, S154N, S107R, T108I, K218Q, S223R, Q222L, and G224S in the HA sequence have been shown to enhance virus binding to $\alpha 2-6$ receptors while also reducing $\alpha 2-6$ receptor binding or increasing virus binding to both $\alpha 2-3$ and $\alpha 2-6$ receptors or leading to increased virus transmission and virulence (Cruz et al., 2023; Charostad et al., 2023). Additionally, the S369I and I396M substitutions in the NA protein play a key role in the adaptation of the virus to mammals (Cruz et al., 2023). It is important to note that mutations in the HA and NA genes alone are not sufficient for efficient mammalian infection (Gambaryan et al., 2006).

The L89V, G309D, T339K, R477G, I495V, K627E, A676T, and E627K substitutions, as well as some rare mutations such as Q591K, D701N, and T271A are mutations in the PB2 protein. These mutations are associated with mammal adaptation by enhancing polymerase activity, improving replication efficiency, and increasing the virulence of H5N1 (Cruz et al., 2023; Charostad et al., 2023). The mutations H99Y, D3V, and D622G identified in PB1, N66S in PB1-F2, and N383D in PA similarly affect mammalian adaption. The N30D, I43M, and T215A mutations in M1 observed in mice are associated with increased virulence (Cruz et al., 2023; Cui et al., 2022). Furthermore, specific mutations in NS1, including P42S, L103F, and I106M, have been found to enhance virulence and reduce the antiviral response in mice. Moreover, mutations K55E, K66E, and C138F in NS1 have been linked to increased replication in mammalian cells and reduced interferon response (Cruz et al., 2023; Cui et al., 2022).

The mutation identified as E627K has been found in the PB2 gene and is associated with heightened pathogenicity of H5N1, H7N9, and H9N2 viruses. It also facilitates respiratory droplet transmission of H5N1, H7N9, H1N1, and H9N2 viruses. This mutation has been detected in viruses that have infected humans and foxes in the USA and certain European countries (Cui et al., 2022).

Kandeil et al. (2023) illustrated that the reassortment of Asian-originated H5N1 clade 2.3.4.4b in America has the potential to infect ferrets. However, even though their experiment showed that the neurological virulence of the reassortant clade has increased in mammalian models, they still retain avian-like receptor specificity and molecular markers of mammalian adaptation and ferret-to-ferret transmission were not observed (Kandeil et al., 2023). Another study conducted by Herfst et al. (2012) revealed that a mutant version of A/H5N1/Indonesia/5/2005 has the ability of ferret-to-ferret transmission and that 5 to 9 mutations can lead to mammal-to-mammal transmission. These substitution mutations were Q222L, G224S, E627K, H103Y, T156A, I368V, H99Y, R99K and S345N, which are in PB2, PB1, HA and NP proteins (Herfst et al., 2012).

Signs of H5N1 pandemic risks

One of the H5N1 hosts that recently had new mutations and neurotropism observed in them are foxes, one of the mammalian cases that highlight the risks of this virus for mammalian species (Bordes et al., 2023). Lagan et al. (2023) have reported a new mutation, PB2-M535I, as a significant adaptation for mammalian hosts, with documented cases in at least 5 European countries. PB2-T271A, PB2-E627K, and PB2-D701N are other mutations in PB2 that have been reported in foxes. PB2-M535I mutation,

which is present in the conserved European avian H5N1 clade 2.3.3.4b strains, was also observed in H7N9 human cases in 2013 along with PB2-Q591K and PB2-D701N, leading to restoring viral polymerase activity without the existing PB2-E627K mutation (Lagan et al., 2023). PB2-E627K mutation is one of the most essential mutations observed in AIVs infected mammals because it increases virus replication in the lower temperatures of the mammalian upper respiratory system. Bordes et al. (2023) conducted research on three fox cases in the Netherlands, which became infected by the consumption of infected birds, not only exhibiting a combination of these mutations but also illustrating neurotropism. H5N1 RNAs were extracted from the brains of all three foxes, and one of them showed neurological symptoms such as blindness, opisthotonos, head shaking, and falling over (Bordes et al., 2023). Moreover, a study in Canada showed similar mutations as the mentioned experiments and neurotropism in 40 cases of H5N1-infected free-living mesocarnivores, including red foxes, striped skunks, and minks. H5N1 viral antigens were found in all brain structures of 40 cases; in the neurons and neuropil staining, viral antigen was predominant, and in the cerebral cortex of red foxes, including multifocal necrotic neurons and glial cells, neutrophilic infiltrates into the cortex and perivascular cuffing reassortant H5N1 virus were found. Also, gliosis, hemorrhage, neuronophagia and meningitis, neuropil rarefaction, and vasculitis were observed in their brain sections (Alkie et al., 2023).

The other H5N1 hosts where H5N1 demonstrated neurotropism and mammalian adaptations are seals and sea lions (carnivorous marine mammals). Mainly, H5N1 transmits to seals through consumption of infected birds, environmental exposure like ingesting infected birds' feces or feathers and drinking fecal-contaminated water (Lair et

al., 2024; Mirolo et al., 2023). In a study conducted in 2022 in Quebec, 209 seals infected with H5N1 showed a range of symptoms, including meningoencephalitis (in all of them), fibrinosuppurative alveolitis, and multiorgan acute necrotizing inflammation and other neurological manifestations. Virus antigens were detected in the neuropils and neurons, renal glomeruli, pulmonary alveolar septa, glandular bronchial cells, vascular walls of skeletal muscle, lymph nodes, tracheal vessels, spleen, pancreas, liver, and adrenal glands. The central nervous system and lungs were found to be the most affected organs, with a 100% and 73% incidence of affection, respectively (Lair et al., 2024). In 2022 and 2023, three seals in the coastal waters of The Netherlands and Germany were infected with H5N1, which histologically showed lymphohistiocytic and necrotizing encephalitis with mild gliosis and neuronal necroses, and viral signs were found in their brains, lungs, spleen, liver, kidney, throat and nose. One of the infected seals died, and the dead seal showed a multifocal non-suppurative interstitial pneumonia with lymphohistiocytic vasculitis in its lungs and a multifocal to coalescing lymphohistiocytic meningoencephalitis, lymphohistiocytic to necrotizing vasculitis, widespread single-cell necroses in the neuroparenchyma in the CNS. The primary mutation in the H5N1 genome in the brain of these seals was PB2-E627K (specifically, the PB2-E627K mutation was found in 40% of Dutch and 100% of German seals' brain viruses) (Mirolo et al., 2023). New England seals in 2022 were also infected by H5N1 and displayed respiratory symptoms and neurological signs. More mutations in experiments on these seals were reported, but two of the most common mutations were PB2-E627K and PB2-D701N (Puryear et al., 2023). Furthermore, cases of H5N1 infection with neurotropic manifestations in some of them were reported in sea lions, terns, and seals in South

American countries such as Chile, Peru, and Argentina in 2023. The Argentinian cases exhibited two mutations, PB2-Q591K and PB2-D701N (Rimondi et al., 2024). Subsequently, cases of H5N1 infections in sea lions and seals have been observed in Uruguay and Brazil, with a higher number of infections in South America compared to North America. According to Tomás et al. (2024), the virus in Uruguay is believed to have originated from the Chilean virus, as the genetic sequence of the Uruguayan virus closely resembles that of H5N1 in sea lions from Chile and Peru, as well as a human case in Chile with similar PB2 substitutions. Only one Uruguayan-infected sea lion and three turns showed neurological symptoms, although there are no clinical signs or records of dead instances (Tomás et al., 2024).

The most recent mammalian spillover is cattle infection with H5N1 in the United States in 2024. In March of 2024, the H5N1 outbreak in dairy cows was reported in the US. On March 25, the first H5N1 in dairy cattle and unpasteurized milk samples was reported in Texas (<https://www.who.int/emergencies/disease-outbreak-news/item/2024-DON512>). Until July 19, 2024, the H5N1 infection of 157 dairy cows in 13 states of the US was confirmed. Since April to July 2024, 13 human cases of H5N1 infection have been reported in the United States, which the origins of four of them were associated with sick dairy cows and the other nine were associated with sick poultry. (<https://www.cdc.gov/bird-flu/spotlights/h5n1-response-08162024.html>) The first human case of infection was infected by dairy cows in Texas, and the second human infection of the same origin in Michigan was reported on April 1 and May 22, 2024, respectively (Garg et al., 2024). The highest number of human cases are in Colorado until July 2024. (<https://www.cdc.gov/bird-flu/spotlights/h5n1-response-07192024.html>) This outbreak is

currently ongoing at the time of writing. Therefore, it is a moving target, and the information provided is subject to change as the outbreak continues. Dairy cows are the domestic ruminants that now are causing outbreaks in the US and causing concerns, although the first domestic ruminant infection in the US in 2024 was a juvenile goat whose brain and tissue samples were positive for H5N1 on a Minnesota farm on March 20 (AVMA, 2024). Subsequent brain and tissue samples of four other juvenile goats in Minnesota were positive for H5N1 later (Sah et al., 2024).

This new pandemic raised some health concerns because of 4 main observations, including cow-to-cow transmission, cow-to-human transmissions, cow-to-domestic cat transmission, and the potential for cattle to release the virus into their milk (Burrough et al., 2024). Cattle are mostly resistant to influenza A, B, and C virus infection and are mainly susceptible to influenza D virus, and their infection with H5N1, which is an influenza A virus, is concerning regarding their susceptibility (Oguzie et al., 2024). Moreover, the infection led to economic concerns due to reduced milk production. Infected cows' milk is thickened and creamy yellow and has higher amounts of aspartate aminotransferase, gamma-glutamyl transferase, creatinine kinase, and bilirubin (Burrough et al., 2024). More importantly, high levels of viral RNA were detected in the cattle's milk during the acute stage of their infection, and high concentrations of the H5N1 virus were observed in raw or unpasteurized milk of infected dairy cows in general (Nelli et al., 2024; Garg et al., 2024). The complete blood counts of infected cows exhibited various symptoms, such as variable anemia and leukocytopenia (Burrough et al., 2024). Some symptoms of sick cows were increased respiratory secretions, high temperatures, decreased appetite, lethargy, abnormal bowel movements, and reduced rumination

(Oguzie et al., 2024; Garg et al., 2024). In human cases, symptoms were limited to subconjunctival hemorrhage and thin, serous drainage in the eyes, without visual impairment, with only positive eye swabs (Uyeki et al., 2024; <https://www.cdc.gov/media/releases/2024/s0522-human-case-h5.html>). Infected

domestic cats consumed raw colostrum and milk from infected cows, and their symptoms and signs were ataxia, blindness, circling, copious oculonasal discharge, depressed mental state, stiff body movements, absence of menace reflexes and pupillary light responses with a weak blink response, mild and multifocal meningeal hemorrhages in the cerebrums. In addition, IAV immunoreactivity tests in cats' organs, including brain, lung, and heart, and segmental immunoreactivity within their retina were positive (Burrough et al., 2024).

The H5N1 subtype currently circulating between cattle in the US belongs to clade 2.3.4.4b, and it is considered to have originated from wild birds (Oguzie et al., 2024; Nelli et al., 2024). Dairy cows' respiratory tract expressed both SA α 2,3-gal and SA α 2,6-gal, with predominant receptors in their respiratory tracts and mammary glands being SA α 2,3-gal- β GalNAc (MAL-II), followed by less common receptors, which are α 2,6-linked SA (SNA) and SA α 2,3-gal- β GlcNAc (MAL-I). Therefore, distributions of their receptors can, to some extent, clarify the symptoms and signs of cattle (Nelli et al., 2024). During this particular outbreak, several new mutations were identified in this clade of H5N1 virus; most of them influence drug resistance, and one of them, PB2-M631L, affects mammalian adaption by increasing H5N1 replication in human cells (Oguzie et al., 2024).

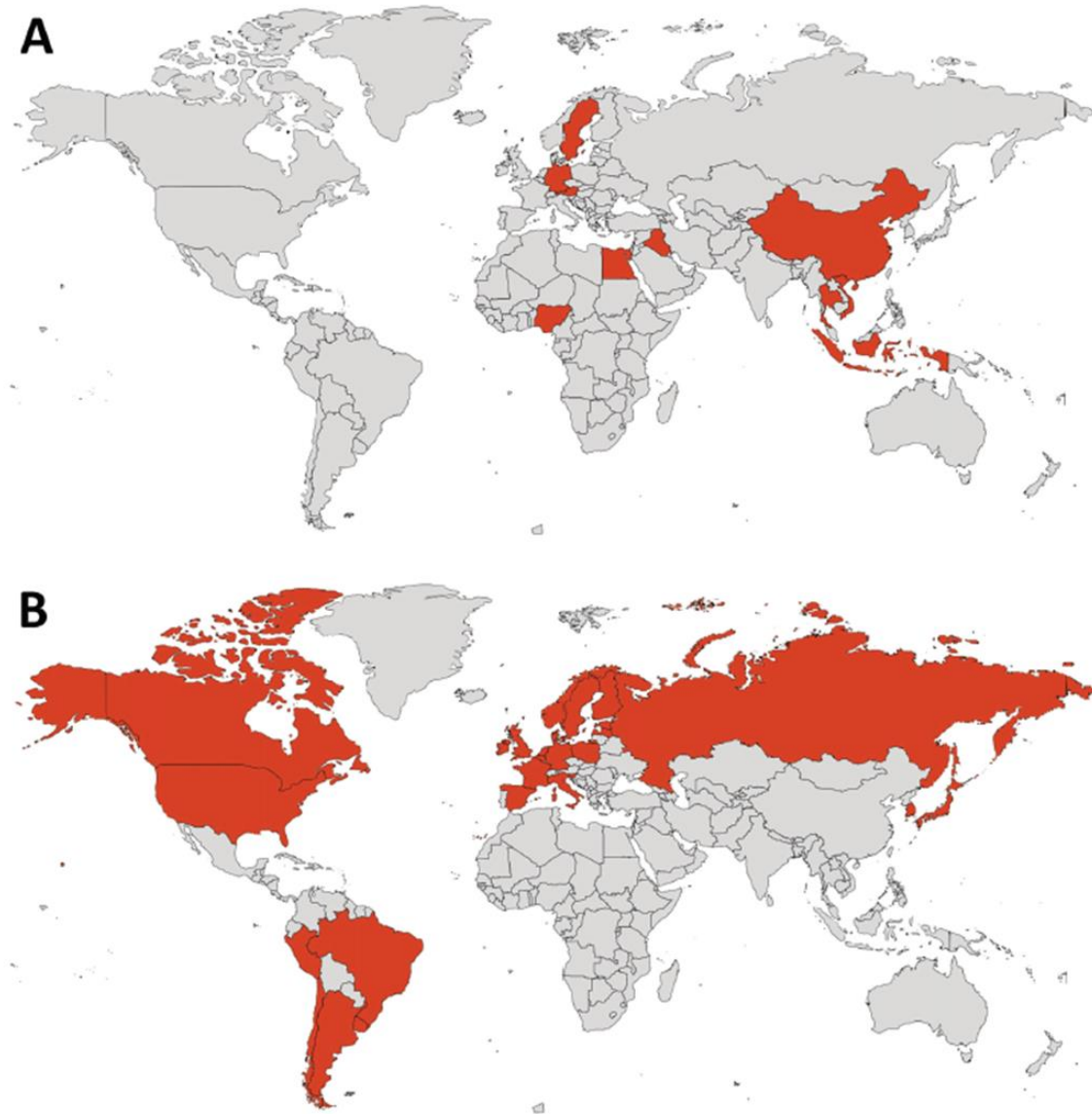


Figure 10. H5N1 global distribution. Map A shows the location of mammalian infections with H5N1 from 2003 to 2019, while Map B displays the location of these infections from 2020 to 2023. Therefore, the distribution of mammalian infections has increased globally during the last four years (Plaza et al., 2024).

Conclusion

The spread of H5N1 in mammals, along with common mutations in the H5N1 virus and the emergence of new mutations, underscores the need for further research to prevent potential human pandemics. Over the past decade, there has been a notable increase in H5N1 infections in mammals globally, with a substantial number of cases

occurring in the Americas for the first time. Hence, this large number of mammalian infections can heighten the probability of future human infections with H5N1. Moreover, specific mutations such as PB2-E627K, PB2-D701N, PB2-M535I, PB2-T271A, and PB2-Q591K are pivotal for viral adaptation to mammals and are frequently observed in various mammalian infections, particularly among foxes, cows, and marine mammals. Among these mutations, PB2-E627K stands out as the most significant. It is consistently present in new H5N1 virus infections and affects its efficient replication in mammals' upper respiratory systems.

Furthermore, mammal-to-mammal transmission and foodborne infections are also notable. Observation such as tiger-to-tiger transmission, cow-to-cow transmission, seal-to-seal transmission are becoming more common than before. Even though human-to-human transmission has not been independently confirmed, its possibility cannot be ruled out, while the mammal-to-mammal transmission rate has increased in the past few years. Additionally, cow-to-human transmission, the first observation of a mammal (instead of a bird) being a source of viruses for human infection, is another significant sign of AIV evolution that could bring new possibilities for human infection. Moreover, foodborne transmission can be considered as a reasonable new transmission pathway. There is evidence of foodborne transmission of H5N1 among various mammal species such as foxes, seals, sea lions, cats, tigers, etc. Therefore, observing H5N1 foodborne transmission in various mammals also indicates the possibility of H5N1 foodborne transmission to humans. This has led to concerns about the presence of H5N1 in cattle milk, which could have significant economic and public health implications.

More importantly, a significant number of cases have shown that the H5N1 avian influenza virus can invade the nervous system of mammals, notably observed in marine mammals in South America. The occurrence of systemic infections in birds and the demonstration of systemic infection in mammals by avian influenza viruses suggest the potential for future systemic infections in humans. This emphasizes the importance of further and more detailed research to understand the implications of human systemic infection with H5N1 and other avian influenza viruses, thereby raising awareness about the potential risks.

In summary, the sustained infection of H5N1 mutations in animals, along with foodborne transmission, animal-to-animal transmission, and new mutations, have been observed worldwide, prompting concerns about potential H5N1 human pandemics. While effective treatments and WHO approved vaccines are available against H5N1 in case of public health emergencies, a pandemic would still have its own health, biological, and economic consequences worldwide.

Future works

Due to the ongoing outbreak in the US and instances of H5N1 being found in cattle milk and goat infections, it is essential to conduct further research on mutations that may facilitate or enhance the release of the virus into the milk of cows and other mammals. Additionally, it is necessary to investigate the risks, potentialities, and mutations associated with systemic infections of H5N1 in humans due to observation of mammals' systemic infections with H5N1, specifically focusing on the possibility of brain infection in humans.

References:

AbuBakar, U., Amrani, L., Kamarulzaman, F. A., Karsani, S. A., Hassandarvish, P., & Khairat, J. E. (2023). Avian Influenza Virus Tropism in Humans. *Viruses*, 15(4), 833. <https://doi.org/10.3390/v15040833>

Alexander D. J. (2007). An overview of the epidemiology of avian influenza. *Vaccine*, 25(30), 5637–5644. <https://doi.org/10.1016/j.vaccine.2006.10.051>

Alkie, T. N., Cox, S., Embury-Hyatt, C., Stevens, B., Pople, N., Pybus, M. J., Xu, W., Hisanaga, T., Suderman, M., Koziuk, J., Kruczkiewicz, P., Nguyen, H. H., Fisher, M., Lung, O., Erdelyan, C. N. G., Hochman, O., Ojkic, D., Yason, C., Bravo-Araya, M., Bourque, L., ... Berhane, Y. (2023). Characterization of neurotropic HPAI H5N1 viruses with novel genome constellations and mammalian adaptive mutations in free-living mesocarnivores in Canada. *Emerging microbes & infections*, 12(1), 2186608. <https://doi.org/10.1080/22221751.2023.2186608>

American Veterinary Medical Association (AVMA). (2024, March 26). *Goat in Minnesota tests positive for HPAI*. <https://www.avma.org/news/goat-minnesota-tests-positive-hpai>

Amonsin, A., Payungporn, S., Theamboonlers, A., Thanawongnuwech, R., Suradhat, S., Pariyothorn, N., Tantilertcharoen, R., Damrongwantanapokin, S., Buranathai, C., Chaisingh, A., Songserm, T., & Poovorawan, Y. (2006). Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand. *Virology*, 344(2), 480–491. <https://doi.org/10.1016/j.virol.2005.08.032>

Bethmont, A., Bui, C. M., Gardner, L., Sarkar, S., Chughtai, A. A., & Macintyre, C. R. (2016). Quantified degree of poultry exposure differs for human cases of avian influenza H5N1 and H7N9. *Epidemiology and infection*, 144(12), 2633–2640. <https://doi.org/10.1017/S0950268816001035>

Bisset, A. T., & Hoyne, G. F. (2020). Evolution and Adaptation of the Avian H7N9 Virus into the Human Host. *Microorganisms*, 8(5), 778. <https://doi.org/10.3390/microorganisms8050778>

Biswas, S. K., Boutz, P. L., & Nayak, D. P. (1998). Influenza virus nucleoprotein interacts with influenza virus polymerase proteins. *Journal of virology*, 72(7), 5493–5501. <https://doi.org/10.1128/JVI.72.7.5493-5501.1998>

Biswas, S. K., & Nayak, D. P. (1996). Influenza virus polymerase basic protein 1 interacts with influenza virus polymerase basic protein 2 at multiple sites. *Journal of virology*, 70(10), 6716–6722. <https://doi.org/10.1128/JVI.70.10.6716-6722.1996>

Blagodatski, A., Trutneva, K., Glazova, O., Mityaeva, O., Shevkova, L., Kegeles, E., Onyanov, N., Fede, K., Maznina, A., Khavina, E., Yeo, S. J., Park, H., & Volchkov, P. (2021). Avian Influenza in Wild Birds and Poultry: Dissemination Pathways, Monitoring Methods, and Virus Ecology. *Pathogens (Basel, Switzerland)*, 10(5), 630. <https://doi.org/10.3390/pathogens10050630>

Bordes, L., Vreman, S., Heutink, R., Roose, M., Venema, S., Pritz-Verschuren, S. B. E., Rijks, J. M., Gonzales, J. L., Germeraad, E. A., Engelsma, M., & Beerens, N. (2023). Highly Pathogenic Avian Influenza H5N1 Virus Infections in Wild Red Foxes (*Vulpes*

vulpes) Show Neurotropism and Adaptive Virus Mutations. *Microbiology spectrum*, 11(1), e0286722. <https://doi.org/10.1128/spectrum.02867-22>

Broszeit, F., Tzarum, N., Zhu, X., Nemanichvili, N., Eggink, D., Leenders, T., Li, Z., Liu, L., Wolfert, M. A., Papanikolaou, A., Martínez-Romero, C., Gagarinov, I. A., Yu, W., García-Sastre, A., Wennekes, T., Okamatsu, M., Verheije, M. H., Wilson, I. A., Boons, G. J., & de Vries, R. P. (2019). N-Glycolylneuraminic Acid as a Receptor for Influenza A Viruses. *Cell reports*, 27(11), 3284–3294.e6. <https://doi.org/10.1016/j.celrep.2019.05.048>

Brown, J. D., Swayne, D. E., Cooper, R. J., Burns, R. E., & Stallknecht, D. E. (2007). Persistence of H5 and H7 avian influenza viruses in water. *Avian diseases*, 51(1 Suppl), 285–289. <https://doi.org/10.1637/7636-042806R.1>

Bui, C. M., Gardner, L., MacIntyre, R., & Sarkar, S. (2017). Influenza A H5N1 and H7N9 in China: A spatial risk analysis. *PloS one*, 12(4), e0174980. <https://doi.org/10.1371/journal.pone.0174980>

Bui, C., Bethmont, A., Chughtai, A. A., Gardner, L., Sarkar, S., Hassan, S., Seale, H., & MacIntyre, C. R. (2015). A Systematic Review of the Comparative Epidemiology of Avian and Human Influenza A H5N1 and H7N9 - Lessons and Unanswered Questions. *Transboundary and emerging diseases*, 63(6), 602–620. <https://doi.org/10.1111/tbed.12327>

Bui, C., Rahman, B., Heywood, A. E., & MacIntyre, C. R. (2016). A Meta-Analysis of the Prevalence of Influenza A H5N1 and H7N9 Infection in Birds. *Transboundary and emerging diseases*, 64(3), 967–977. <https://doi.org/10.1111/tbed.12466>

Burrough, E. R., Magstadt, D. R., Petersen, B., Timmermans, S. J., Gauger, P. C., Zhang, J., Siepker, C., Mainenti, M., Li, G., Thompson, A. C., Gorden, P. J., Plummer, P. J., & Main, R. (2024). Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus Infection in Domestic Dairy Cattle and Cats, United States, 2024. *Emerging infectious diseases*, 30(7), 1335–1343. <https://doi.org/10.3201/eid3007.240508>

Castillo, A., Fasce, R., Parra, B., Andrade, W., Covarrubias, P., Hueche, A., Campano, C., Tambley, C., Rojas, M., Araya, M., Hernández, F., Bustos, P., & Fernández, J. (2023). The first case of human infection with H5N1 avian Influenza A virus in Chile. *Journal of travel medicine*, 30(5), taad083. <https://doi.org/10.1093/jtm/taad083>

CDC Newsroom. (2024, May 22). *CDC Reports Second Human Case of H5 Bird Flu Tied to Dairy Cow Outbreak*. <https://www.cdc.gov/media/releases/2024/s0522-human-case-h5.html>

Centers for disease control and prevention. (2024, April 5). *Highly Pathogenic Avian Influenza A(H5N1) Virus: Identification of Human Infection and Recommendations for Investigations and Response*. <https://emergency.cdc.gov/han/2024/han00506.asp#:~:text=Since%201997%2C%20more%20than%20900,been%20reported%20worldwide%20since%202022>

Centers for disease control and prevention. (2024, February 1). *Reported Human Infections with Avian Influenza A Viruses*. <https://www.cdc.gov/flu/avianflu/reported-human-infections.htm>

Centers for disease control and prevention. (2024, June 3). *Current U.S. Bird Flu Situation in Humans*. <https://www.cdc.gov/flu/avianflu/inhumans.htm>

Centers for disease control and prevention. (2024, July 19). *CDC A(H5N1) Bird Flu Response Update, July 19, 2024*. <https://www.cdc.gov/bird-flu/spotlights/h5n1-response-07192024.html>

Centers for disease control and prevention. (2024, August 19). *CDC A(H5N1) Bird Flu Response Update August 16, 2024*. <https://www.cdc.gov/bird-flu/spotlights/h5n1-response-08162024.html>

Chang, P., Sadeyen, J. R., Bhat, S., Daines, R., Hussain, A., Yilmaz, H., & Iqbal, M. (2023). Risk assessment of the newly emerged H7N9 avian influenza viruses. *Emerging microbes & infections*, 12(1), 2172965. <https://doi.org/10.1080/22221751.2023.2172965>

Charostad, J., Rezaei Zadeh Rukerd, M., Mahmoudvand, S., Bashash, D., Hashemi, S. M. A., Nakhaie, M., & Zandi, K. (2023). A comprehensive review of highly pathogenic avian influenza (HPAI) H5N1: An imminent threat at doorstep. *Travel medicine and infectious disease*, 55, 102638. <https://doi.org/10.1016/j.tmaid.2023.102638>

Chaves, A. J., Busquets, N., Valle, R., Rivas, R., Vergara-Alert, J., Dolz, R., Ramis, A., Darji, A., & Majó, N. (2011). Neuropathogenesis of a highly pathogenic avian influenza virus (H7N1) in experimentally infected chickens. *Veterinary research*, 42(1), 106. <https://doi.org/10.1186/1297-9716-42-106>

Cruz, C. D., Icochea, M. E., Espejo, V., Troncos, G., Castro-Sanguinetti, G. R., Schilling, M. A., & Tinoco, Y. (2023). Highly Pathogenic Avian Influenza A(H5N1) from Wild Birds, Poultry, and Mammals, Peru. *Emerging infectious diseases*, 29(12), 2572–2576. <https://doi.org/10.3201/eid2912.230505>

Cui, P., Shi, J., Wang, C., Zhang, Y., Xing, X., Kong, H., Yan, C., Zeng, X., Liu, L., Tian, G., Li, C., Deng, G., & Chen, H. (2022). Global dissemination of H5N1 influenza viruses bearing the clade 2.3.4.4b HA gene and biologic analysis of the ones detected in China. *Emerging microbes & infections*, 11(1), 1693–1704.
<https://doi.org/10.1080/22221751.2022.2088407>

Durand, L. O., Glew, P., Gross, D., Kasper, M., Trock, S., Kim, I. K., Bresee, J. S., Donis, R., Uyeki, T. M., Widdowson, M. A., & Azziz-Baumgartner, E. (2015). Timing of influenza A(H5N1) in poultry and humans and seasonal influenza activity worldwide, 2004-2013. *Emerging infectious diseases*, 21(2), 202–208.
<https://doi.org/10.3201/eid2102.140877>

El Zowalaty, M. E., Bustin, S. A., Hussein, M. I., & Ashour, H. M. (2013). Avian influenza: virology, diagnosis and surveillance. *Future microbiology*, 8(9), 1209–1227.
<https://doi.org/10.2217/fmb.13.81>

Evseev, D., & Magor, K. E. (2021). Molecular Evolution of the Influenza A Virus Non-structural Protein 1 in Interspecies Transmission and Adaptation. *Frontiers in microbiology*, 12, 693204. <https://doi.org/10.3389/fmicb.2021.693204>

Freidl, G. S., Meijer, A., de Bruin, E., de Nardi, M., Munoz, O., Capua, I., Breed, A. C., Harris, K., Hill, A., Kosmider, R., Banks, J., von Dobschuetz, S., Stark, K., Wieland, B., Stevens, K., van der Werf, S., Enouf, V., van der Meulen, K., Van Reeth, K., Dauphin, G., ... FLURISK Consortium. (2014). Influenza at the animal-human interface: a review of the literature for virological evidence of human infection with swine or avian influenza viruses other than A(H5N1). *Euro surveillance: bulletin Europeen sur les maladies*

transmissibles = *European communicable disease bulletin*, 19(18), 20793.
<https://doi.org/10.2807/1560-7917.es2014.19.18.20793>

Friedman, N., Drori, Y., Pando, R., Glatman-Freedman, A., Sefty, H., Bassal, R., Stein, Y., Shohat, T., Mendelson, E., Hindiye, M., & Mandelboim, M. (2017). A(H1N1)pdm09 influenza infection: vaccine inefficiency. *Oncotarget*, 8(20), 32856–32863. <https://doi.org/10.18632/oncotarget.16459>

Gambaryan, A., Tuzikov, A., Pazynina, G., Bovin, N., Balish, A., & Klimov, A. (2006). Evolution of the receptor binding phenotype of influenza A (H5) viruses. *Virology*, 344(2), 432–438. <https://doi.org/10.1016/j.virol.2005.08.035>

Gamblin, S. J., Vachieri, S. G., Xiong, X., Zhang, J., Martin, S. R., & Skehel, J. J. (2021). Hemagglutinin Structure and Activities. *Cold Spring Harbor perspectives in medicine*, 11(10), a038638. <https://doi.org/10.1101/cshperspect.a038638>

Garg, S., Reed, C., Davis, C. T., Uyeki, T. M., Behraves, C. B., Kniss, K., Budd, A., Biggerstaff, M., Adjemian, J., Barnes, J. R., Kirby, M. K., Basler, C., Szablewski, C. M., Richmond-Crum, M., Burns, E., Limbago, B., Daskalakis, D. C., Armstrong, K., Boucher, D., Shimabukuro, T. T., ... Dugan, V. (2024). Outbreak of Highly Pathogenic Avian Influenza A(H5N1) Viruses in U.S. Dairy Cattle and Detection of Two Human Cases - United States, 2024. *MMWR. Morbidity and mortality weekly report*, 73(21), 501–505. <https://doi.org/10.15585/mmwr.mm7321e1>

Godoy, M., Oca, M. M., Caro, D., Pontigo, J. P., Kibenge, M., & Kibenge, F. (2023). Evolution and Current Status of Influenza A Virus in Chile: A Review. *Pathogens (Basel, Switzerland)*, 12(10), 1252. <https://doi.org/10.3390/pathogens12101252>

Guan, Y., Smith, G. J., Webby, R., & Webster, R. G. (2009). Molecular epidemiology of H5N1 avian influenza. *Revue scientifique et technique (International Office of Epizootics)*, 28(1), 39–47. <https://doi.org/10.20506/rst.28.1.1868>

Hao, W., Wang, L., & Li, S. (2020). Roles of the Non-Structural Proteins of Influenza A Virus. *Pathogens (Basel, Switzerland)*, 9(10), 812. <https://doi.org/10.3390/pathogens9100812>

Herfst, S., Schrauwen, E. J., Linster, M., Chutinimitkul, S., de Wit, E., Munster, V. J., Sorrell, E. M., Bestebroer, T. M., Burke, D. F., Smith, D. J., Rimmelzwaan, G. F., Osterhaus, A. D., & Fouchier, R. A. (2012). Airborne transmission of influenza A/H5N1 virus between ferrets. *Science (New York, N.Y.)*, 336(6088), 1534–1541. <https://doi.org/10.1126/science.1213362>

Hilsch, M., Goldenbogen, B., Sieben, C., Höfer, C. T., Rabe, J. P., Klipp, E., Herrmann, A., & Chiantia, S. (2014). Influenza A matrix protein M1 multimerizes upon binding to lipid membranes. *Biophysical journal*, 107(4), 912–923. <https://doi.org/10.1016/j.bpj.2014.06.042>

Husain, M. (2014). Avian influenza A (H7N9) virus infection in humans: epidemiology, evolution, and pathogenesis. *Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases*, 28, 304–312. <https://doi.org/10.1016/j.meegid.2014.10.016>

Isin, B., Doruker, P., & Bahar, I. (2002). Functional motions of influenza virus hemagglutinin: a structure-based analytical approach. *Biophysical journal*, 82(2), 569–581. [https://doi.org/10.1016/S0006-3495\(02\)75422-2](https://doi.org/10.1016/S0006-3495(02)75422-2)

Jonges, M., Welkers, M. R., Jeeninga, R. E., Meijer, A., Schneeberger, P., Fouchier, R. A., de Jong, M. D., & Koopmans, M. (2014). Emergence of the virulence-associated PB2 E627K substitution in a fatal human case of highly pathogenic avian influenza virus A(H7N7) infection as determined by Illumina ultra-deep sequencing. *Journal of virology*, 88(3), 1694–1702. <https://doi.org/10.1128/JVI.02044-13>

Kandeil, A., Patton, C., Jones, J. C., Jeevan, T., Harrington, W. N., Trifkovic, S., Seiler, J. P., Fabrizio, T., Woodard, K., Turner, J. C., Crumpton, J. C., Miller, L., Rubrum, A., DeBeauchamp, J., Russell, C. J., Govorkova, E. A., Vogel, P., Kim-Torchetti, M., Berhane, Y., Stallknecht, D., ... Webby, R. J. (2023). Rapid evolution of A(H5N1) influenza viruses after intercontinental spread to North America. *Nature communications*, 14(1), 3082. <https://doi.org/10.1038/s41467-023-38415-7>

Kayali, G., Kandeil, A., El-Shesheny, R., Kayed, A. S., Maatouq, A. M., Cai, Z., McKenzie, P. P., Webby, R. J., El Refaey, S., Kandeel, A., & Ali, M. A. (2016). Avian Influenza A(H5N1) Virus in Egypt. *Emerging infectious diseases*, 22(3), 379–388. <https://doi.org/10.3201/eid2203.150593>

Kayed, A. E., Kutkat, O., Kandeil, A., Moatasim, Y., El Taweel, A., El Sayes, M., El-Shesheny, R., Aboulhoda, B. E., Abdeltawab, N. F., Kayali, G., Ali, M. A., & Ramadan, M. A. (2023). Comparative pathogenic potential of avian influenza H7N3 viruses isolated from wild birds in Egypt and their sensitivity to commercial antiviral drugs. *Archives of virology*, 168(3), 82. <https://doi.org/10.1007/s00705-022-05646-w>

Kutter, J. S., Linster, M., de Meulder, D., Bestebroer, T. M., Lexmond, P., Rosu, M. E., Richard, M., de Vries, R. P., Fouchier, R. A. M., & Herfst, S. (2023). Continued adaptation of A/H2N2 viruses during pandemic circulation in humans. *The Journal of general virology*, 104(8), 001881. <https://doi.org/10.1099/jgv.0.001881>

Lai, S., Qin, Y., Cowling, B. J., Ren, X., Wardrop, N. A., Gilbert, M., Tsang, T. K., Wu, P., Feng, L., Jiang, H., Peng, Z., Zheng, J., Liao, Q., Li, S., Horby, P. W., Farrar, J. J., Gao, G. F., Tatem, A. J., & Yu, H. (2016). Global epidemiology of avian influenza A H5N1 virus infection in humans, 1997-2015: a systematic review of individual case data. *The Lancet. Infectious diseases*, 16(7), e108–e118. [https://doi.org/10.1016/S1473-3099\(16\)00153-5](https://doi.org/10.1016/S1473-3099(16)00153-5)

Lair, S., Quesnel, L., Signore, A. V., Delnatte, P., Embury-Hyatt, C., Nadeau, M. S., Lung, O., Ferrell, S. T., Michaud, R., & Berhane, Y. (2024). Outbreak of Highly Pathogenic Avian Influenza A(H5N1) Virus in Seals, St. Lawrence Estuary, Quebec, Canada. *Emerging infectious diseases*, 30(6), 1133–1143. <https://doi.org/10.3201/eid3006.231033>

Lamb, R. A., & Choppin, P. W. (1983). The gene structure and replication of influenza virus. *Annual review of biochemistry*, 52, 467–506. <https://doi.org/10.1146/annurev.bi.52.070183.002343>

Lamb, R. A. (2008). Influenza. *Encyclopedia of Virology*, 95-104. <https://doi.org/10.1016/b978-012374410-4.00654-3>

Lazniewski, M., Dawson, W. K., Szczepinska, T., & Plewczynski, D. (2018). The structural variability of the influenza A hemagglutinin receptor-binding site. *Briefings in functional genomics*, 17(6), 415–427. <https://doi.org/10.1093/bfpg/elx042>

Le, Q. M., Sakai-Tagawa, Y., Ozawa, M., Ito, M., & Kawaoka, Y. (2009). Selection of H5N1 influenza virus PB2 during replication in humans. *Journal of virology*, 83(10), 5278–5281. <https://doi.org/10.1128/JVI.00063-09>

Lee, R. T., Gunalan, V., Van, T. D., Le, L. T., Eisenhaber, F., & Maurer-Stroh, S. (2013). A new piece in the puzzle of the novel avian-origin influenza A (H7N9) virus. *Biology direct*, 8, 26. <https://doi.org/10.1186/1745-6150-8-26>

Lagan, P., McKenna, R., Baleed, S., Hanna, B., Barley, J., McConnell, S., Georgaki, A., Sironen, T., Kauppinen, A., Gadd, T., Lindh, E., Ikonen, N., McMenamy, M. J., & Lemon, K. (2023). Highly pathogenic avian influenza A(H5N1) virus infection in foxes with PB2-M535I identified as a novel mammalian adaptation, Northern Ireland, July 2023. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin*, 28(42), 2300526. <https://doi.org/10.2807/1560-7917.ES.2023.28.42.2300526>

Li, B., Xiang, G., He, J., Li, H., Xu, C., Yu, A., Zhao, Z., Wang, X., Zhang, L., Zhang, H., Zhang, H., Xie, M., Wang, P., & Yu, D. (2021). H7N9 influenza virus surveillance in Gansu, China in 2017. *Virus research*, 296, 198335. <https://doi.org/10.1016/j.virusres.2021.198335>

Liu, L., Chen, G., Huang, S., & Wen, F. (2023). Receptor Binding Properties of Neuraminidase for influenza A virus: An Overview of Recent Research Advances. *Virulence*, 14(1), 2235459. <https://doi.org/10.1080/21505594.2023.2235459>

Liu, Q., Zhou, B., Ma, W., Bawa, B., Ma, J., Wang, W., Lang, Y., Lyoo, Y., Halpin, R. A., Lin, X., Stockwell, T. B., Webby, R., Wentworth, D. E., & Richt, J. A. (2014). Analysis of recombinant H7N9 wild-type and mutant viruses in pigs shows that the Q226L mutation in HA is important for transmission. *Journal of virology*, 88(14), 8153–8165. <https://doi.org/10.1128/JVI.00894-14>

Lu, S., Li, T., Xi, X., Chen, Q., Liu, X., Zhang, B., Ou, J., Liu, J., Wang, Q., Zhu, B., Liu, X., Bai, C., Qu, J., Lu, H., Zhang, Z., & Song, Y. (2014). Prognosis of 18 H7N9 avian influenza patients in Shanghai. *PloS one*, 9(4), e88728. <https://doi.org/10.1371/journal.pone.0088728>

Malik Peiris J. S. (2009). Avian influenza viruses in humans. *Revue scientifique et technique (International Office of Epizootics)*, 28(1), 161–173. <https://doi.org/10.20506/rst.28.1.1871>

Martínez-Sobrido, L., Peersen, O., & Nogales, A. (2018). Temperature Sensitive Mutations in Influenza A Viral Ribonucleoprotein Complex Responsible for the Attenuation of the Live Attenuated Influenza Vaccine. *Viruses*, 10(10), 560. <https://doi.org/10.3390/v10100560>

Mathur, M. B., Patel, R. B., Gould, M., Uyeki, T. M., Bhattacharya, J., Xiao, Y., Gillaspie, Y., Chae, C., & Khazeni, N. (2014). Seasonal patterns in human A (H5N1) virus

infection: analysis of global cases. *PloS one*, 9(9), e106171.
<https://doi.org/10.1371/journal.pone.0106171>

Miroló, M., Pohlmann, A., Ahrens, A. K., Kühl, B., Rubio-García, A., Kramer, K., Meinfelder, U., Rosenberger, T., Morito, H. L., Beer, M., Ludlow, M., Wohlsein, P., Baumgärtner, W., Harder, T., & Osterhaus, A. (2023). Highly pathogenic avian influenza A virus (HPAIV) H5N1 infection in two European grey seals (*Halichoerus grypus*) with encephalitis. *Emerging microbes & infections*, 12(2), e2257810.
<https://doi.org/10.1080/22221751.2023.2257810>

Mukaigawa, J., & Nayak, D. P. (1991). Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. *Journal of virology*, 65(1), 245–253. <https://doi.org/10.1128/JVI.65.1.245-253.1991>

Nelli, R. K., Harm, T. A., Siepker, C., Groeltz-Thrush, J. M., Jones, B., Twu, N. C., Nenninger, A. S., Magstadt, D. R., Burrough, E. R., Piñeyro, P. E., Mainenti, M., Carnaccini, S., Plummer, P. J., & Bell, T. M. (2024). Sialic Acid Receptor Specificity in Mammary Gland of Dairy Cattle Infected with Highly Pathogenic Avian Influenza A(H5N1) Virus. *Emerging infectious diseases*, 30(7), 1361–1373.
<https://doi.org/10.3201/eid3007.240689>

Noisumdaeng, P., Phadungsombat, J., Weerated, S., Wiriyarat, W., & Puthavathana, P. (2022). Genetic evolution of hemagglutinin and neuraminidase genes of H5N1 highly pathogenic avian influenza viruses in Thailand. *PeerJ*, 10, e14419.
<https://doi.org/10.7717/peerj.14419>

Oguzie, J. U., Marushchak, L. V., Shittu, I., Lednicky, J. A., Miller, A. L., Hao, H., Nelson, M. I., & Gray, G. C. (2024). Avian Influenza A(H5N1) Virus among Dairy Cattle, Texas, USA. *Emerging infectious diseases*, 30(7), 1425–1429. <https://doi.org/10.3201/eid3007.240717>

Pan American Health Organization. (2024, March 20). *Outbreaks of avian influenza caused by influenza A(H5N1) in the Region of the Americas*. <https://www.paho.org/en/documents/epidemiological-update-outbreaks-avian-influenza-caused-influenza-ah5n1-region-americas-1>

Paterson, D., & Fodor, E. (2012). Emerging roles for the influenza A virus nuclear export protein (NEP). *PLoS pathogens*, 8(12), e1003019. <https://doi.org/10.1371/journal.ppat.1003019>

Pena, L., Vincent, A. L., Loving, C. L., Henningson, J. N., Lager, K. M., Li, W., & Perez, D. R. (2012). Strain-dependent effects of PB1-F2 of triple-reassortant H3N2 influenza viruses in swine. *The Journal of general virology*, 93(Pt 10), 2204–2214. <https://doi.org/10.1099/vir.0.045005-0>

Perales, B., Sanz-Ezquerro, J. J., Gastaminza, P., Ortega, J., Santarén, J. F., Ortín, J., & Nieto, A. (2000). The replication activity of influenza virus polymerase is linked to the capacity of the PA subunit to induce proteolysis. *Journal of virology*, 74(3), 1307–1312. <https://doi.org/10.1128/jvi.74.3.1307-1312.2000>

Perdue, M. L., & Suarez, D. L. (2000). Structural features of the avian influenza virus hemagglutinin that influence virulence. *Veterinary microbiology*, 74(1-2), 77–86. [https://doi.org/10.1016/s0378-1135\(00\)00168-1](https://doi.org/10.1016/s0378-1135(00)00168-1)

Petrich, A., Dunsing, V., Bobone, S., & Chiantia, S. (2021). Influenza A M2 recruits M1 to the plasma membrane: A fluorescence fluctuation microscopy study. *Biophysical journal*, 120(24), 5478–5490. <https://doi.org/10.1016/j.bpj.2021.11.023>

Peukes, J., Xiong, X., Erlendsson, S., Qu, K., Wan, W., Calder, L. J., Schraidt, O., Kummer, S., Freund, S. M. V., Kräusslich, H. G., & Briggs, J. A. G. (2020). The native structure of the assembled matrix protein 1 of influenza A virus. *Nature*, 587(7834), 495–498. <https://doi.org/10.1038/s41586-020-2696-8>

Plaza, P. I., Gamarra-Toledo, V., Euguí, J. R., & Lambertucci, S. A. (2024). Recent Changes in Patterns of Mammal Infection with Highly Pathogenic Avian Influenza A(H5N1) Virus Worldwide. *Emerging infectious diseases*, 30(3), 444–452. <https://doi.org/10.3201/eid3003.231098>

Possas, C., Marques, E. T., Oliveira, A., Schumacher, S., Mendes, F., Siqueira, M. M., Antunes, A., & Homma, A. (2023). Highly Pathogenic Avian Influenza Vaccines: Challenges for Innovation, Technological Development and Pandemic Preparedness. <https://doi.org/10.1101/2023.05.31.23290790>

Puryear, W., Sawatzki, K., Hill, N., Foss, A., Stone, J. J., Doughty, L., Walk, D., Gilbert, K., Murray, M., Cox, E., Patel, P., Mertz, Z., Ellis, S., Taylor, J., Fauquier, D., Smith, A., DiGiovanni, R. A., Jr, van de Guchte, A., Gonzalez-Reiche, A. S., Khalil, Z., ... Runstadler, J. (2023). Highly Pathogenic Avian Influenza A(H5N1) Virus Outbreak in New England Seals, United States. *Emerging infectious diseases*, 29(4), 786–791. <https://doi.org/10.3201/eid2904.221538>

Refaey, S., Azziz-Baumgartner, E., Amin, M. M., Fahim, M., Roguski, K., Elaziz, H. A., Iuliano, A. D., Salah, N., Uyeki, T. M., Lindstrom, S., Davis, C. T., Eid, A., Genedy, M., & Kandeel, A. (2015). Increased Number of Human Cases of Influenza Virus A(H5N1) Infection, Egypt, 2014-15. *Emerging infectious diseases*, 21(12), 2171–2173. <https://doi.org/10.3201/eid2112.150885>

Rimondi, A., Vanstreels, R. E. T., Olivera, V., Donini, A., Lauriente, M. M., & Uhart, M. M. (2024). Highly Pathogenic Avian Influenza A(H5N1) Viruses from Multispecies Outbreak, Argentina, August 2023. *Emerging infectious diseases*, 30(4), 812–814. <https://doi.org/10.3201/eid3004.231725>

Roche, B., Lebarbenchon, C., Gauthier-Clerc, M., Chang, C. M., Thomas, F., Renaud, F., van der Werf, S., & Guégan, J. F. (2009). Water-borne transmission drives avian influenza dynamics in wild birds: the case of the 2005-2006 epidemics in the Camargue area. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*, 9(5), 800–805. <https://doi.org/10.1016/j.meegid.2009.04.009>

Saelens, X. (2019). The Role of Matrix Protein 2 Ectodomain in the Development of Universal Influenza Vaccines. *The Journal of infectious diseases*, 219(Suppl_1), S68–S74. <https://doi.org/10.1093/infdis/jiz003>

Sah, R., Srivastava, S., Kumar, S., Mehta, R., Donovan, S., Sierra-Carrero, L., Luna, C., Woc-Colburn, L., Cardona-Ospina, J. A., Hineostroza-Jordan, M., Wessolossky, M., & Rodriguez-Morales, A. J. (2024). Concerns on H5N1 avian influenza given the

outbreak in U.S. dairy cattle. *Lancet regional health. Americas*, 35, 100785.
<https://doi.org/10.1016/j.lana.2024.100785>

Sha, J., Chen, X., Ren, Y., Chen, H., Wu, Z., Ying, D., Zhang, Z., & Liu, S. (2016). Differences in the epidemiology and virology of mild, severe and fatal human infections with avian influenza A (H7N9) virus. *Archives of virology*, 161(5), 1239–1259.
<https://doi.org/10.1007/s00705-016-2781-3>

Shi, J., Zeng, X., Cui, P., Yan, C., & Chen, H. (2023). Alarming situation of emerging H5 and H7 avian influenza and effective control strategies. *Emerging microbes & infections*, 12(1), 2155072. <https://doi.org/10.1080/22221751.2022.2155072>

Shtyrya, Y. A., Mochalova, L. V., & Bovin, N. V. (2009). Influenza virus neuraminidase: structure and function. *Acta naturae*, 1(2), 26–32. <https://doi.org/10.32607/20758251-2009-1-2-26-32>

Skeik, N., & Jabr, F. I. (2008). Influenza viruses and the evolution of avian influenza virus H5N1. *International journal of infectious diseases: IJID: official publication of the International Society for Infectious Diseases*, 12(3), 233–238.
<https://doi.org/10.1016/j.ijid.2007.07.002>

Spackman, E. (2020). A Brief Introduction to Avian Influenza Virus. *Methods in molecular biology (Clifton, N.J.)*, 2123, 83–92. https://doi.org/10.1007/978-1-0716-0346-8_7

Sreenivasan, C. C., Thomas, M., Kaushik, R. S., Wang, D., & Li, F. (2019). Influenza A in Bovine Species: A Narrative Literature Review. *Viruses*, 11(6), 561.
<https://doi.org/10.3390/v11060561>

Stegeman, A., Bouma, A., Elbers, A. R., de Jong, M. C., Nodelijk, G., de Klerk, F., Koch, G., & van Boven, M. (2004). Avian influenza A virus (H7N7) epidemic in The Netherlands in 2003: course of the epidemic and effectiveness of control measures. *The Journal of infectious diseases*, 190(12), 2088–2095. <https://doi.org/10.1086/425583>

Su, S., Bi, Y., Wong, G., Gray, G. C., Gao, G. F., & Li, S. (2015). Epidemiology, Evolution, and Recent Outbreaks of Avian Influenza Virus in China. *Journal of virology*, 89(17), 8671–8676. <https://doi.org/10.1128/JVI.01034-15>

Su, S., Gu, M., Liu, D., Cui, J., Gao, G. F., Zhou, J., & Liu, X. (2017). Epidemiology, Evolution, and Pathogenesis of H7N9 Influenza Viruses in Five Epidemic Waves since 2013 in China. *Trends in microbiology*, 25(9), 713–728. <https://doi.org/10.1016/j.tim.2017.06.008>

Takashita, E. (2021). Influenza Polymerase Inhibitors: Mechanisms of Action and Resistance. *Cold Spring Harbor perspectives in medicine*, 11(5), a038687. <https://doi.org/10.1101/cshperspect.a038687>

Tan, M., Zeng, X., Xie, Y., Li, X., Liu, J., Yang, J., Yang, L., & Wang, D. (2023). Reported human infections of H9N2 avian influenza virus in China in 2021. *Frontiers in public health*, 11, 1255969. <https://doi.org/10.3389/fpubh.2023.1255969>

Tian, H., Zhou, S., Dong, L., Van Boeckel, T. P., Cui, Y., Newman, S. H., Takekawa, J. Y., Prosser, D. J., Xiao, X., Wu, Y., Cazelles, B., Huang, S., Yang, R., Grenfell, B. T., & Xu, B. (2015). Avian influenza H5N1 viral and bird migration networks in

Asia. *Proceedings of the National Academy of Sciences of the United States of America*, 112(1), 172–177. <https://doi.org/10.1073/pnas.1405216112>

Tomás, G., Marandino, A., Panzera, Y., Rodríguez, S., Wallau, G. L., Dezordi, F. Z., Pérez, R., Bassetti, L., Negro, R., Williman, J., Uriarte, V., Grazioli, F., Leizagoyen, C., Riverón, S., Coronel, J., Bello, S., Páez, E., Lima, M., Méndez, V., & Pérez, R. (2024). Highly pathogenic avian influenza H5N1 virus infections in pinnipeds and seabirds in Uruguay: Implications for bird-mammal transmission in South America. *Virus evolution*, 10(1), veae031. <https://doi.org/10.1093/ve/veae031>

Uchida, Y., Kanehira, K., Takemae, N., Hikono, H., & Saito, T. (2017). Susceptibility of chickens, quail, and pigeons to an H7N9 human influenza virus and subsequent egg-passaged strains. *Archives of virology*, 162(1), 103–116. <https://doi.org/10.1007/s00705-016-3090-6>

Ungchusak, K., Auewarakul, P., Dowell, S. F., Kitphati, R., Auwanit, W., Puthavathana, P., Uprasertkul, M., Boonnak, K., Pittayawonganon, C., Cox, N. J., Zaki, S. R., Thawatsupha, P., Chittaganpitch, M., Khontong, R., Simmerman, J. M., & Chunsutthiwat, S. (2005). Probable person-to-person transmission of avian influenza A (H5N1). *The New England journal of medicine*, 352(4), 333–340. <https://doi.org/10.1056/NEJMoa044021>

Uyeki, T. M. (2008). Global epidemiology of human infections with highly pathogenic avian influenza A (H5N1) viruses. *Respirology (Carlton, Vic.)*, 13 Suppl 1, S2–S9. <https://doi.org/10.1111/j.1440-1843.2008.01246.x>

Uyeki, T. M., Milton, S., Abdul Hamid, C., Reinoso Webb, C., Presley, S. M., Shetty, V., Rollo, S. N., Martinez, D. L., Rai, S., Gonzales, E. R., Kniss, K. L., Jang, Y., Frederick, J. C., De La Cruz, J. A., Liddell, J., Di, H., Kirby, M. K., Barnes, J. R., & Davis, C. T. (2024). Highly Pathogenic Avian Influenza A(H5N1) Virus Infection in a Dairy Farm Worker. *The New England journal of medicine*, 390(21), 2028–2029. <https://doi.org/10.1056/NEJMc2405371>

Van Kerkhove, M. D. (2013). Brief literature review for the WHO global influenza research agenda--highly pathogenic avian influenza H5N1 risk in humans. *Influenza and other respiratory viruses*, 7 Suppl 2(Suppl 2), 26–33. <https://doi.org/10.1111/irv.12077>

Varga, Z. T., Grant, A., Manicassamy, B., & Palese, P. (2012). Influenza virus protein PB1-F2 inhibits the induction of type I interferon by binding to MAVS and decreasing mitochondrial membrane potential. *Journal of virology*, 86(16), 8359–8366. <https://doi.org/10.1128/JVI.01122-12>

Verhagen, J. H., Fouchier, R. A. M., & Lewis, N. (2021). Highly Pathogenic Avian Influenza Viruses at the Wild-Domestic Bird Interface in Europe: Future Directions for Research and Surveillance. *Viruses*, 13(2), 212. <https://doi.org/10.3390/v13020212>

Wang, C., Luo, J., Wang, J., Su, W., Gao, S., Zhang, M., Xie, L., Ding, H., Liu, S., Liu, X., Chen, Y., Jia, Y., & He, H. (2014). Novel human H7N9 influenza virus in China. *Integrative zoology*, 9(3), 372–375. <https://doi.org/10.1111/1749-4877.12047>

Wang, S., Li, N., Jin, S., Zhang, R., & Xu, T. (2021). Polymerase acidic subunit of H9N2 polymerase complex induces cell apoptosis by binding to PDCD 7 in A549 cells. *Virology journal*, 18(1), 75. <https://doi.org/10.1186/s12985-021-01547-7>

World Health Organization. (2021, February 26). *Human infection with avian influenza A (H5N8) - Russian Federation*. <https://www.who.int/emergencies/disease-outbreak-news/item/2021-DON313>

World Health Organization. (2023, October 3). *Influenza (Avian and other zoonotic)*. [https://www.who.int/news-room/fact-sheets/detail/influenza-\(avian-and-other-zoonotic\)](https://www.who.int/news-room/fact-sheets/detail/influenza-(avian-and-other-zoonotic))

World health organization. (2024, April 9). *Avian Influenza A(H5N1) - United States of America*. <https://www.who.int/emergencies/disease-outbreak-news/item/2024-DON512>

Wu, X. X., Zhao, L. Z., Tang, S. J., Weng, T. H., Wu, W. G., Yao, S. H., Wu, H. B., Cheng, L. F., Wang, J., Hu, F. Y., Wu, N. P., Yao, H. P., Zhang, F. C., & Li, L. J. (2020). Novel pathogenic characteristics of highly pathogenic avian influenza virus H7N9: viraemia and extrapulmonary infection. *Emerging microbes & infections*, 9(1), 962–975. <https://doi.org/10.1080/22221751.2020.1754135>

Yan, S., & Wu, G. (2011). Possible reason for cross-species and cross-subtype reassortment in polymerase basic protein 2 from influenza A virus. *Protein and peptide letters*, 18(5), 434–439. <https://doi.org/10.2174/092986611794927956>

Yee, K. S., Carpenter, T. E., & Cardona, C. J. (2009). Epidemiology of H5N1 avian influenza. *Comparative immunology, microbiology and infectious diseases*, 32(4), 325–340. <https://doi.org/10.1016/j.cimid.2008.01.005>

Yin, Y., Liu, Y., Fen, J., Liu, K., Qin, T., Chen, S., Peng, D., & Liu, X. (2023). Characterization of an H7N9 Influenza Virus Isolated from Camels in Inner Mongolia,

China. *Microbiology spectrum*, 11(2), e0179822. <https://doi.org/10.1128/spectrum.01798-22>

Zhu, W., Li, X., Dong, J., Bo, H., Liu, J., Yang, J., Zhang, Y., Wei, H., Huang, W., Zhao, X., Chen, T., Yang, J., Li, Z., Zeng, X., Li, C., Tang, J., Xin, L., Gao, R., Liu, L., Tan, M., ... Wang, D. (2022). Epidemiologic, Clinical, and Genetic Characteristics of Human Infections with Influenza A(H5N6) Viruses, China. *Emerging infectious diseases*, 28(7), 1332–1344. <https://doi.org/10.3201/eid2807.212482>