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Review article

Avian Influenza: Molecular adaptations and the potential role of domestic animals as "mixing vessels"

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Abstract

Avian influenza is an infectious disease that mainly affects wild birds but can be transmitted to other animals, including mammals and, in rare cases, humans. This article specifically explores the role of domestic animals, such as dogs and cats, as "mixing vessels" in the adaptation process of avian influenza viruses (AIV). These animals are vulnerable to infection by various influenza strains due to the presence of both avian and human sialic acid receptors in their respiratory tracts. This dual susceptibility facilitates genetic reassortment between avian and human viruses. The recombination process can generate new viral variants, increasing the risk of interspecies transmission and, in extreme cases, of a pandemic. The review analyzes the molecular mechanisms that determine the transmission and adaptation of viruses to hosts and how these domestic animals may contribute to the emergence of new viral variants. The work highlights the importance of monitoring the spread of these viruses in domestic animals to better understand the risks of virus propagation and improve surveillance strategies.

Keywords: Avian Influenza, Interspecies transmission, Molecular adaptations, Zoonotic risk

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Introduction

The spread of avian influenza viruses (AIVs) represents a significant global public health challenge due to their adaptability to various species, including domestic and wild animals and, in some cases, humans (Tilocca et al., 2024). Although wild waterfowl are the principal natural reservoir of AIVs, these viruses have developed the ability to infect multiple domestic animals, including chickens, horses, swine, and dogs, and marine mammals such as dolphins, seals, and whales. The high adaptability of these viruses highlights the widespread risk of zoonotic transmission (Tiwari et al., 2024).

The tropism of AIVs is influenced by complex factors related to host physiology and environmental conditions. Viral characteristics that determine host specificity include the capacity to replicate efficiently and optimal interaction with the infected cell (Nomaguchi et al., 2012). The AIV genome, consisting of separate segments, allows for genetic reassortment, which enables rapid acquisition of entire genes, facilitating adaptation to new species. This can happen if a cell simultaneously hosts an avian virus and a flu virus already adapted to mammals, generating offspring with gene combinations capable of crossing interspecies barriers (Long et al., 2018).

This process, called 'antigenic shift,' can give rise to strains with antigens unknown to the human immune system, facilitating wider spread. The potential of AIV to mutate and adapt to

transmit between humans raises concerns about the threat of a new pandemic event. A pandemic can occur when an influenza virus accumulates enough mutations in people to transmit between people effectively. The timing of such pandemics is challenging to anticipate, as it depends on a complex interplay of ecological and virological factors (Tian et al., 2015).

The severity of AIV infection, with a mortality rate of over 50% of confirmed cases, is a serious warning for the scientific community and health authorities (Wiramus and Martin, 2013). In a context in which biological processes and environmental dynamics condition the virus' ability to spread and its epidemiological behavior, it becomes essential to thoroughly investigate its genetic peculiarities and the molecular connections between the pathogen and its hosts. Studying the proteins associated with the transition between species and the mutations that determine these processes is fundamental to deciphering the complexity of these infectious agents. This understanding, coupled with advanced epidemiological monitoring, is indispensable for predicting possible interspecies transmission and countering possible new pandemic events.

Avian influenza is a viral infection that affects wild and farmed birds but can sometimes be spread to other species, even humans (Blagodatski et al., 2021). It is caused by viruses belonging to type A of the Orthomyxoviridae family, known for their high capacity to vary and mutate. This highly adaptable pathogen consists of 100 nm to 30 µm viral particles. The structure is characterized by an envelope formed by a lipid bilayer derived from the host cell's membrane. This layer protects the internal virus components and facilitates its penetration into infected cells (Chlanda and Zimmerberg, 2016). AIVs are classified according to two proteins on their surface, neuraminidase (NA) and haemagglutinin (HA), which define the subtypes (Gaymard et al., 2016). Each variant has a different level of aggressiveness: the most lethal forms can cause severe outbreaks, leading to high mortality and heavy economic losses for the poultry industry (Grace et al., 2024). This virus shows remarkable resistance to low temperatures, allowing it to survive for long periods in various environments. However, it can be eliminated by heat treatment, common disinfectants, or exposure to sunlight. Transmission occurs through contact with infected birds, feces, or contaminated surfaces. AVIs fall into two main categories:

Highly pathogenic viruses (HPAI) are responsible for severe forms of the disease and are often fatal for birds. Low-pathogenic viruses (LPAI) cause mild or no symptoms and pose little risk to humans. The evolution of the AIV is a complex process resulting from the dynamic interaction between the virus, the hosts, and their immune defenses.

Genome organization and essential proteins

AIV has a negatively polarised genome segmented by single-stranded RNA. The AIV genome consists of eight RNA segments and codes for at minimum 17 proteins, including eight essential proteins. (AbuBakar et al., 2023); basic polymerase 2 (PB2), basic polymerase 1 (PB1), acid polymerase (PA), nucleoprotein (NP), matrix (M), non-structural protein (NS), haemagglutinin (HA), neuraminidase (NA).

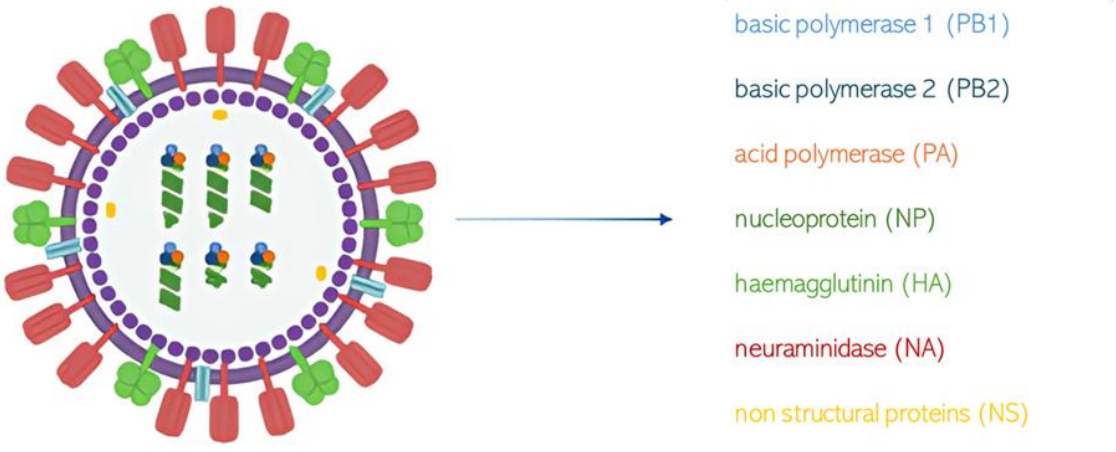

Figure 1: The figure shows the structure of AIV and the essential proteins responsible for viral replication and pathogenesis. Created with BioRender.com.

PB2 represents the first segment of the AIV genome and is transported into the host cell nucleus independently of the PA and PB1 subunits. It was observed in the mitochondria of infected cells, where it intervenes in regulating mitochondrial function during infection, exploiting the host cell's resources to ensure efficient infection (MacDonald et al., 2012). Furthermore, PB2 contains a specific domain folded independently with a 'cap' binding motif, facilitating its tearing' phenomenon through interaction with the 5' cap of the host mRNA. This interaction is crucial for synthesizing viral mRNAs inside the host nucleus (Guilligay et al., 2008).

The PB1 protein corresponds to the second segment of the genome; PB1-F2 protein expression can increase AIV pathogenicity by positively regulating polymerase activity and promoting apoptosis (Chakrabarti and Pasricha, 2013; Kash et al., 2006). It also translocates into the inner membrane space of host cell mitochondria, impairing the cells' own immune response (Yoshizumi et al., 2014).
The third genome segment codifies the PA protein, which, among its many functions, engages in cap binding, promoter binding, and endonuclease activity. Furthermore, it is hypothesized that the PA protein plays a crucial function in the adaptation and inter-host transmission of AIV (Dias et al., 2009; Taubenberger et al., 2005).

HA represents one of the two surface glycoproteins encoded by AIV, is initially produced as an inactive precursor (HA0) and then cleaved into two functional subunits (HA1; HA2), essential for the life cycle of the AIV (Hoffmann and Pöhlmann, 2018). HA1 binds to sialic acid (SA) receptors on the host cell surface, while HA2 facilitates fusion with cell membranes, allowing viral genetic material to enter. The virus' ability to bind to specific forms of sialic acid, such as α2,3 SA and α2,6 SA, determines its transmissibility between distinct species. AIVs prefer to bind to α2,3 SA, prevalent in the bird respiratory tract, whereas human influenza viruses bind to α2,6 SA, found in the human respiratory tract (Zuo et al., 2019). This difference partly explains the low transmission in humans. The low presence of α2,3 SA in humans and a more effective innate immune response hinder the diffusion among humans (De Graaf and Fouchier, 2014).

Highly conserved, NP plays a crucial role in viral genome replication and packaging by interacting with vRNA and the cap-binding domain of the PB2 protein (Szeto et al., 2020). They are associated with viral RNA within the virion, forming the ribonucleoprotein complex (RNP) (Noda and Kawaoka, 2010). Also associated with this complex are three polymerase proteins (PB1, PB2, and PA), which are essential for viral genome replication and transcription (Waters et al., 2021).

The sixth segment of the genome encodes for NA, a glycoprotein consisting of 469 amino acids. In addition to facilitating virus egress from infected cells, NA facilitates viral attachment and internalization by catalyzing the removal of non-functional receptors (Chauhan and Gordon, 2022). This process allows it to cross the mucus and reach functional receptors, such as α2,6 sialic type receptors, necessary to enter the host cell (McAuley et al., 2019). This protein may also possess a 'second binding site' for receptors, as suggested by specific mutations (e.g., D151G or G147R) linked to an increased ability for interaction (Liu et al., 2023).

The Matrix (M) gene encodes two distinct polypeptides: M1 and M2 (Chauhan and Gordon, 2022). Underneath the virion's lipid envelope is the M1 protein, which provides the necessary structural support. Also prominent among the internal proteins is M2, an ion channel crucial for the disassembly of the virus and its acidification inside the host cell, a passage crucial for virus activation once penetrated (Takeda et al., 2002). When endosomes reach the cell nucleus, their environment becomes more acidic as part of the host's normal intracellular degradation system. This lowering of pH activates the proton channel of the M2 protein, allowing protons to enter the virion (Aganovic, 2023).

The eighth and smallest segment of the genome encodes three proteins: NS1, NS2, and NS3. These proteins play key roles in the regulation of infection and virus replication. NS1 protein is found in the nucleus of the host cell but is also present in the cytoplasm and is crucial in evading immune defenses. It suppresses the production of interferon (IFN) and other host antiviral proteins, thus suppressing the immune response (Kim et al., 2021). NS2, codified by a mRNA spliced, is an essential structural protein of the virion and has multifunctional functions (Robb et al., 2009). Among its leading roles, it facilitates

the binding of M1 to the vRNP complex and promotes the export of viral genetic material from the nucleus to the cytoplasm, a key step in the virus' life cycle completion.

Evolutionary mechanisms

AIV evolution is a complex and intriguing process resulting from a continuous interaction among the virus, its numerous hosts, and their immune systems. Two main mechanisms, 'antigenic drift' and 'antigenic shift,' underlie the variations in the virus's antigenic characteristics and its ability to infect different species (Pavia et al., 2024).

Antigenic drift develops gradually by accumulating small mutations in viral genes encoding surface proteins, such as NA and HA. Such modifications can alter the antigenic structure, reducing the host immune system's ability to recognize it (Das et al., 2013). Consequently, individuals previously exposed to similar strains may no longer be protected from the newly emerging strains. This mechanism partially enables the virus to escape pre-existing immunity, facilitating its spread and contributing to the emergence of seasonal epidemics. Antigenic drift thus ensures a certain continuity in viral circulation, keeping the virus evolution active (Sandbulte et al., 2011).

Antigenic shift is a much more drastic and sudden phenomenon, which occurs when two or more influenza virus strains simultaneously infect the host. This scenario, common in some animal species exposed to different influenza viruses, leads to the reassortment of genetic material, which generates new strains with unique combinations of surface proteins (Shao et al., 2017). The combination of drift and antigenic shift allows the virus to adapt to new environments and hosts and increases spillover events.

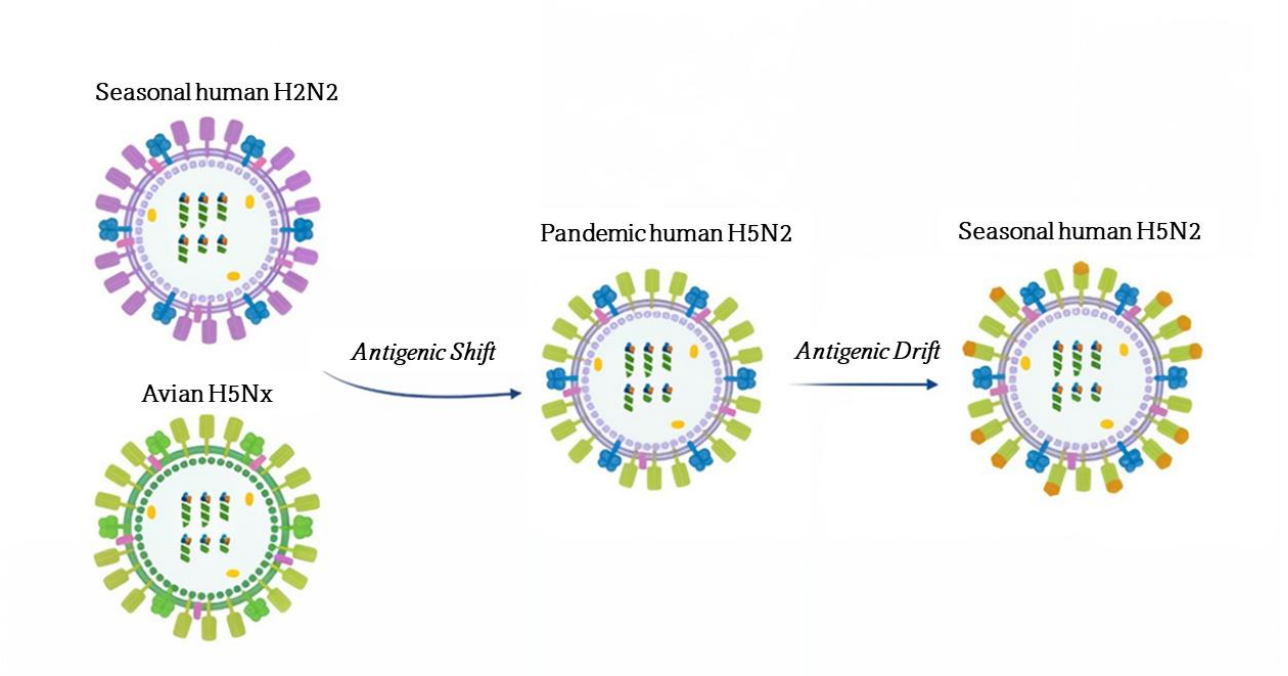

Figure 2: The image explains what happens to the virion during the mechanisms involved in gene reassortment. The term "antigenic shift" describes the genetic recombination that occurs during the reassortment of AIVs. Minor variations in the hemagglutinin and neuraminidase antigens on the virus surface due to mutations are known as "antigenic drift". The figure was created with BioRender.com.

Hosts and transmission

The natural hosts of AIVs are mainly wild aquatic birds of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, waders, and terns). The main transmission route is fecal-oral, and infection can occur mainly through direct or indirect contact with contaminated surface water. In most cases, they cause subclinical infections of the intestinal or respiratory tract (Abdelwhab and Mettenleiter, 2023). Most AIVs infecting aquatic birds cause asymptomatic infections with low pathogenicity, and these species are used as viral reservoirs. To date, this infection has been reported in more than 100 bird species belonging to over 25

families, and 133 HxNy subtypes have already been documented in aquatic birds (Bi et al., 2024). Besides aquatic birds, they have also been identified from all domestic and cage bird species, including pigeons, ostriches, chickens, turkeys, and zoo and pet birds (Alexander, 2000). The main transmission routes in poultry are represented by direct exposure to contaminated materials and contact with wild birds; it has also been observed that secondary spread can be strongly driven by the movement of vehicles and humans (Swayne and Glisson, 2013).

In poultry, the most frequently detected viruses belong to the H1 – H11 serotypes; this is demonstrated by the fact that many of these subtypes are enzootic in some countries such as South Africa (H6N2), China (H6N2; H7N9), Korea (H6N2), Asia (H5Nx), Africa (H5Nx) and Mexico (H7N3) (Swayne and Glisson, 2013; Xu et al., 2023). The occurrence of low-virulence strains, which sometimes go unnoticed, represents a risk for poultry production and human health, mainly if genetic combinations with other influenza viruses occur.

In humans, the virus is generally related to annual vaccination against seasonal influenza and previous exposures that confer protection against the most severe symptoms. The virus is mainly transmitted through direct contact between infected individuals, using the respiratory tract to disseminate. However, it is relevant to consider sporadic reports of human infections caused by AIVs of animal origin, which, although temporally and spatially restricted, could represent a serious threat to public health. The lack of pre-existing immunity against completely novel HA or NA antigens may facilitate more intense replication and wider virus dissemination in the population (de Vries et al., 2018). In this context, swine and AIVs stand out for their higher zoonotic risk, unlike those from cattle, horses, dogs, or bats, which present a significantly lower risk. The zoonotic risk of AIVs is certainly an event to be kept under close observation to avoid an uncontrolled diffusion and to predict any reassortments that may lead to the spread of new serotypes. However, it is of fundamental importance to evaluate and monitor any cases of human-to-human transmission, which, although rare, represent an epidemiological danger.

The presence of AIVs in swine has caused significant economic losses in farms in recent years, with serotypes H1N1, H1N2, and H3N2 being the most common in swine globally (Bourret, 2018; Hennig et al., 2022). Furthermore, it has been observed that the simultaneous detection of multiple subtypes is common in this species.

Numerous mammalian species can be affected by sporadic infections caused by different influenza viruses. These spillover events often go unnoticed but, in some cases, can be fatal, mainly if associated with bacterial or viral co-infections (Zhang et al., 2019). In rare cases, some AIVs have become endemic in livestock or companion animals after interspecific jumps (Parrish et al., 2015). An example is the equine influenza virus H3N8, which originated from an AIV transferred first to horses and then to dogs. Infected animals, including dogs, horses, and cattle, tend to develop a respiratory illness similar to influenza due to human or swine influenza viruses (Abdelwhab and Mettenleiter, 2023).

In a complex epidemiological context, some hosts, such as swine, can act as "mixing vessels," allowing co-infection of different AIVs. This process facilitates genetic reassortment, generating new viral genotypes. Cellular receptors, such as sialic acid, play a crucial role in determining the capacity of animal AIVs to infect human cells, influencing their susceptibility, virulence, and infectious capacity (Abdelwhab and Mettenleiter, 2023).

Sialic acid, linked to galactose at the α2 position, is the major receptor for influenza viruses. α2,6-SA, predominant in the human respiratory tract, is known as the "human receptor," while α2,3-SA, common in the gut of birds, is called the "avian receptor". Some animals, like swine, possess both receptors in varying proportions, making them vulnerable to human and avian AIV infection and, therefore, potential intermediates for genetic reassortment. (Nelli et al., 2010). The receptor pattern in the swine respiratory tract is comparable to that in humans. Although historically considered the main 'container' of reassortant viruses, its involvement in influenza pandemics is still supported by limited evidence (Lemon and Mahmoud, 2005).

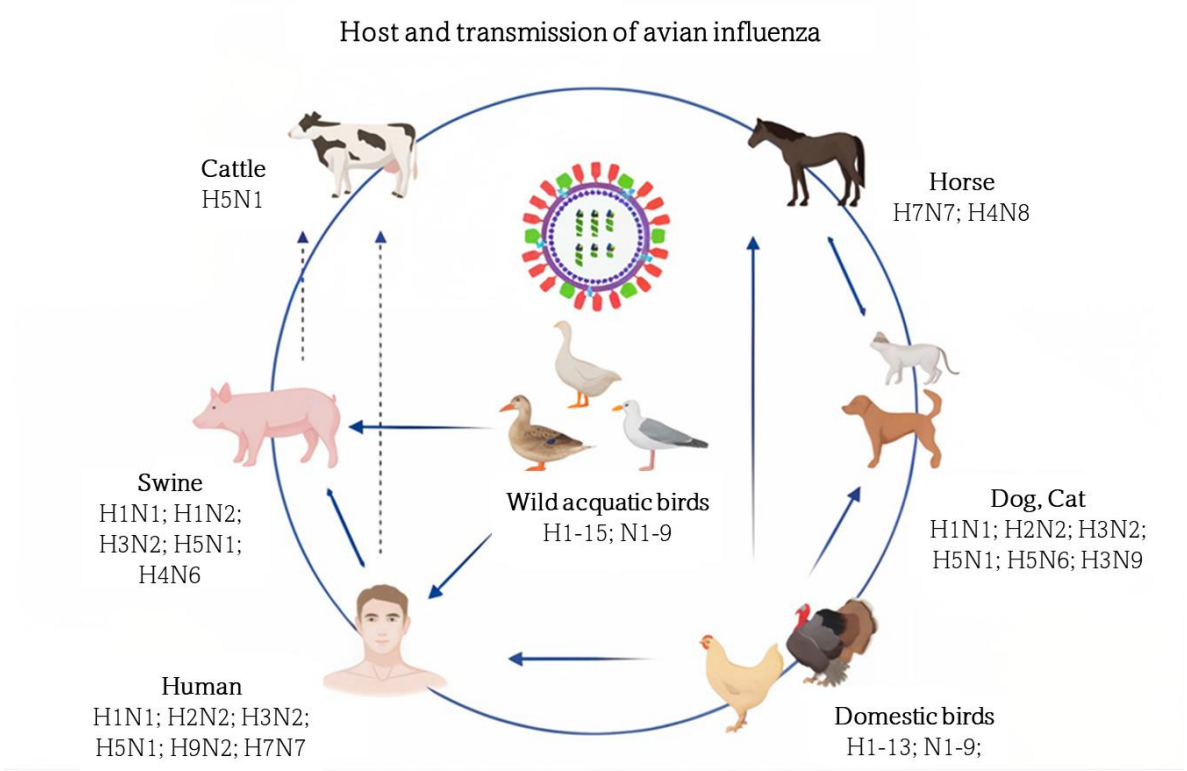

Figure 3: Hosts with documented AIVs infection and dynamics of interspecies transmission. Created with BioRender.com

Epidemiology and geographical distribution

The global distribution of AIVs is favored by several ecological, biological, and anthropogenic factors. From 2003 to 2023, the World Health Organization (WHO) has documented over 800 cases of AIV outbreaks in humans, mainly due to the H5N1 subtype, with a toll of approximately 400 deaths distributed in more than 20 countries (Charostad et al., 2023). Asia has always been the epicenter of avian influenza outbreaks, especially regarding the highly pathogenic H5N1 and H7N9 strains (Poovorawan et al., 2013). The first case of H5N1 was recorded in Hong Kong in 1997 and then spread to Vietnam, Thailand, Indonesia, and Cambogia, while the first H7N9 infection was detected in 2013 in China with over 1500 cases of the disease in humans and a mortality rate close to 40% (Gao et al., 2013). This is firmly attributable to the migratory routes of wild birds, as these act as natural reservoirs (Xie et al., 2023).

The first H5N1 outbreaks were notified in 2006 in northern Nigeria and later in other regions such as Egypt and South Africa. In 2019, WHO reported over 300 cases in Egypt, with a mortality rate above 33%. From 2019 to 2021, H5N8 epidemics were highlighted in chickens, turkeys, and ducks, and they became the predominant strain in poultry. The H5N8 strain was also notified in Zimbabwe in June 2017 and then spread rapidly, leaving only two of the nine provinces of South Africa unharmed. In early 2016, the low LPAI H9N2 strain was isolated in Morocco on chicken farms, showing a high mortality and morbidity rate (Boumart et al., 2018).

The first AIVs in Europe were recorded between 2005 and 2006, predominating the H5N1 strain (Rudisill et al., 2012). In 2014-2015, a new outbreak was attributed to the H5N8 strain. This epidemic continued until 2018, with over 1000 outbreaks in domestic birds and over 900 in wild birds in 30 European countries, including France and Germany (Paul et al., 2019). Between 2019 and 2020, 26 H5N8 outbreaks have been reported in Europe in regions such as Poland, Hungary, the Czech Republic, Germany, Slovakia and Romania. In Bulgaria, however, an outbreak was identified in poultry due to co-infection of the H5N2 and H5N8 strains (Adlhoch et al., 2020). In 2023, 88 new outbreaks in domestic birds and more than 170 in wild birds were reported in 23 European countries. In America, avian flu

outbreaks were recorded with lower incidence than in other countries. Since 2002, three pandemics have occurred in Chile (H7N3), the United States (H5N2), and Canada (H7N3), all of which were caused by rearrangement phenomena that allowed low pathogenic viruses to acquire highly pathogenic characteristics (Senne, 2007).

Key drivers associated with tropism

Adaptation of AIV to its host is a complex process involving several viral factors and host-specific characteristics. Among these, the hemagglutinin protein plays a crucial role as a mediator of the interaction between the human and virus cells. Initially produced in an inactive form (HA0), HA requires activation by host-specific proteases to function (Long et al., 2018). Proteases such as TMPRSS2, which is mainly localized in the respiratory tract, are crucial for this activation and allow the virus to enter the cell (Bertram et al., 2010). Other proteases, such as TMPRSS4 and TMPRSS13, help to activate HA in different tissue types and may extend viral tropism, influencing the efficiency of infection in different hosts (Limburg et al., 2019).

Another key factor is the host environment's pH, which is necessary for HA activation. Influenza viruses in humans are adapted to a lower activation pH (about 5.4), compatible with human respiratory tract conditions, while avian viruses require a higher pH (about 5.7) (Russell, 2021). This difference represents a natural barrier for AIV in humans. However, mutations that stabilize HA at pH values compatible with the human environment may increase the risk of cross-species spread (Guo et al., 2024).

Once inside the cell, the viral ribonucleoprotein complex, containing the viral genome, must be transported to the nucleus to initiate replication and transcription. This process is regulated by host proteins, such as HSP90 and importin α, which facilitate the correct transport and function of the viral complex (Dou et al., 2018; Zhang et al., 2011). These factors ensure the ideal conformation and correct localization of the viral components for efficient infection. The interaction between these mechanisms is strictly interconnected and determines the virus's ability to adapt to new hosts, particularly humans.

Mixing Vessels and viral receptors: Mechanisms involved in the evolution of influenza viruses

Hosts considered "mixing containers" are those that harbor simultaneous infections by multiple influenza viruses, enabling genetic reassortment and the emergence of new genotypes. These hosts often function as intermediaries in the influenza virus spread between different species, including mammals and, sometimes, humans (Ganti et al., 2021; Lee, 2024).

A crucial element in determining a host's susceptibility to a particular influenza virus is the virus's cellular receptors, which play a key part in virulence and infection. The primary receptor typically targeted by influenza viruses is sialic acid (SA), linked to galactose via an α (C2) bond (Zhao and Pu, 2022). The specificity of binding varies. Human influenza viruses prefer the α2,6 SA (linked to carbon 6 of galactose, known as α2,6-SA), whereas AIVs bind primarily to the α2,3 SA (linked to carbon 3, known as α2,3-SA) (Imai and Kawaoka, 2012).

It was traditionally believed that humans express only α2,6-linked sialic acid (SA) receptors in their respiratory tract, while birds primarily express α2,3-SA receptors in their intestinal tract. On the other hand, pigs were thought to possess both types of receptors, positioning them as ideal intermediaries for genetic reassortment between avian and human influenza viruses (Abdelwhab and Mettenleiter, 2023). Recent research has questioned this traditional view, showing that various mammalian and avian species possess both α2,6-SA and α2,3-SA receptors, though their prevalence and distribution vary across different tissues (Abdelwhab and Mettenleiter, 2023). This suggests that several species, not just pigs, could serve as mixing vessels for generating new variant viruses. Pigs are recognized as ideal hosts for the genetic reassortment of influenza viruses because of their unique ability to simultaneously host avian, human, and swine influenza viruses (Zhou et al., 1999). This role is linked to specific SA receptors in respiratory tracts. The α2,6-SA receptors were more abundant than avian receptors in the bronchi and trachea. In contrast, both receptors are present similarly in the lower respiratory tract, with a predominance of α2,3-SA (Nelli et al., 2010).

Although this characteristic makes pigs a crucial model for understanding reassortment processes, there is still a lack of concrete evidence demonstrating their significant involvement in

the creation of epidemic influenza viruses other than pdmH1N1 (Lemon and Mahmoud, 2005; Smith et al., 2009). This virus, resulting from a reassortment of swine, avian, and human strains, is the most well-known example of pigs acting as mixing vessels for influenza viruses. The receptor network is not limited to the respiratory tract (Nelli et al., 2010). Studies have revealed the presence of α2,3-SA and α2,6-SA receptors across multiple organs, including the spleen, heart, skeletal muscles, liver, and gastrointestinal tract. Moreover, the α2,6-SA receptor has been found on epithelial cells along the entire respiratory tract, while the α2,3-SA receptor is present in smaller quantities, predominantly in the bronchioles and alveoli. These data reinforce the idea that pigs, because of their anatomy and physiology, are fertile ground for genetic reassortment and the emergence of new influenza strains (Nelli et al., 2010; Trebbien et al., 2011).

Domestic animals as potential "mixing vessels"

Pet animals (dogs and cats)

Because of their frequent interaction with humans, dogs, and cats are vulnerable to influenza viruses that affect both animals and humans. A study by Ning et al. in 2012 aimed at understanding the *in-situ* tissue distribution of both sialic acid-linked influenza virus receptors in various organs of beagle dogs found that the animals had both α2,3-SA and α2,6-SA receptors in their respiratory tract. More specifically, the authors found the receptors in the trachea and nasal mucosa (Ning et al., 2012). The serotypes of influenza virus documented in recent years have been H3N2, H3N8, H6N1, H7N9, H10N8, H9N2, H5N1 and H5N2. It should also highlight the isolation of some seasonal and pandemic human influenza virus strains in pets (Klivleyeva et al., 2022; Lin et al., 2015; Wasik et al., 2021). In 2022, a human case in China was found to be infected with an AIV, H3N8. Concomitantly, viral RNA was isolated from an asymptomatic dog in the upper part of the pharynx (Bao et al., 2022). In addition, serological studies conducted in various regions of the world have shown that dogs develop antibodies against human influenza viruses, such as H1N1 and H3N2, and against several AIVs, including H5N1 (Klivleyeva et al., 2022; Kovalenko et al., 2021; Su et al., 2014; Wasik et al., 2021).

Several cases of isolation of these viruses in dogs suggest that these species can host and transmit subtypes of AIV, facilitating rearrangement among avian, swine, and human strains. A significant example of this phenomenon is the serotype H3N8 (CIV-H3N8) isolate in dogs. This virus, of equine origin, was first identified in Florida (USA) in 2004; since then, it has continued to spread among the canine population, becoming endemic (Borland et al., 2020). In 2006, the H3N2 subtype, another significant avian-origin influenza virus, emerged in dogs in China and later spread to Southeast Asia and North America (Chen et al., 2023). His genome was tightly linked to an avian virus, H3N2, implying that dogs can serve as a vehicle for passage to humans or other species. In addition, genetic reassortment events, including the integration of the H1N1pdm09 gene, were reported, further highlighting the dogs' role as potential "mixing vessels"(Borland et al., 2020). In South Korea, a previously unknown subtype was isolated in dogs in 2012. This virus is an example of how dogs can host and generate new influenza variants through genetic reassortment, resulting from reassortment between CIV-H3N2 and the human H1N1 influenza virus (Moon et al., 2015). Infections of H5N1 strains have been documented in dogs over the years (Borland et al., 2020; Szaluś-Jordanow et al., 2024). In parallel to canine influenza subtypes, dogs were also infected by various avian strains, including H5N1, a highly pathogenic virus (HPAIV) that has caused infections in dogs from contact with infected birds or consumption of raw poultry (Ly, 2023; Szaluś-Jordanow et al., 2024).

Although cases of H5N1 transmission to dogs are rare, the possibility of dogs acting as reservoirs for the spread of the virus remains a concern, especially in areas where the virus is endemic among avian populations. These cases highlight how dogs, because of the receptors for avian (α2,3) and human (α2,6) influenza viruses in their respiratory tract, can act as reservoirs for the diffusion of various influenza virus subtypes. The potential for co-infection and genetic reassortment in these hosts indicates that dogs could serve as essential intermediaries in transmitting the virus across species, potentially leading to the development of new influenza variants. Like dogs, cats are commonly found as pets worldwide and frequently interact with

humans. Their respiratory system includes receptors that can bind to influenza A viruses, particularly in the tracheal cells, alveolar epithelial cells, and pseudostratified columnar cells (Kristensen et al., 2024; Wang et al., 2013).

Over the years, AIVs were reported in cats occasionally. Still, it should be noted that these are species that have not received much attention in the epidemiological context of these viruses. It should be considered, however, that there have been some cases where cats have been susceptible to human pandemic and influenza viruses such as H9N2, H5N6, and H7N2 (Kristensen et al., 2024; Kroeze et al., 2012; Wang et al., 2013). In 2016, the transmission of AIV H7N2 from cats to humans was documented in two workers at an animal shelter in the United States (Belser et al., 2017). Infections with the H5N1 strain of avian influenza A have been reported multiple times in various regions worldwide, prompting concerns about their potential role in the transmission of the virus. The first documented cases occurred in Thailand in 2004 when three domestic cats in a household tested positive for H5N1 (Enserink and Kaiser, 2004). These cats were part of a flock that had contact with infected poultry, suggesting that transmission had occurred through raw meat from contaminated poultry consumption. This case was one of the first to demonstrate that domestic cats can also be infected with H5N1, although not all cats show severe clinical symptoms. In 2006, three cats in Germany were found dead and tested positive for H5N1(Klopfleisch et al., 2007). In Austria, cats were found in an animal shelter that, although not showing clinical symptoms, tested positive for the virus after encountering an infected swan (Leschnik et al., 2007). A recent study in 2024 documented the discovery of ten dead cats in South Dakota that tested positive for the avian influenza virus H5N1 (clade 2.3.4b). Analysis revealed widespread co-expression of α-2,6 and α-2,3 sialic acid receptors, suggesting a potential susceptibility to viral reassortment. Genomic sequencing showed that viruses isolated in cats were closely related to H5N1 sequences identified in cattle from the same region, highlighting a possible epidemiological link. In addition, the viral genomes had key mutations, such as T143A in the HA protein and F314L and L342Q in the PA protein, associated with increased polymerase activity and virulence (Chothe et al., 2024). These genetic changes suggest an adaptation of the virus to mammals, strengthening the hypothesis that cats may act as 'mixing vessels' for genetic reassortment between avian and mammalian viruses. The presence of both receptors in the tissues analyzed further confirms their potential role in this process (Chothe et al., 2024).

Chicken and turkeys

Although domestic poultry species such as chickens and turkeys have not always been considered possible 'mixing vessels' capable of generating gene reassortment in influenza type A viruses, studies in the scientific literature clearly show that such domestic animals have great potential. Indeed, it has been shown that both species display both human and avian SA receptors in variable distribution and organs. Wang et al. (2015) detected the presence of SA α2,3-gal and SA α2,6-gal receptors in the reproductive tract of hens, with a higher presence of the α-2,3-gal receptor in the isthmus and uterus. This was achieved by using the real-time RT-PCR assay based on SYBR green and exploiting the histochemical affinity of lectin. The authors concluded that the reproductive system of hens may constitute an ideal habitat for the multiplication of influenza viruses of avian and human origin (Wang et al., 2015). Again, exploiting the histochemical affinity of lectin, Kuchipudi et al. (2009) studied the distribution of receptors in chicken organs. Intestinal epithelial cells expressed only SAα2,3-Gal, while the SAα2,6-Ga receptor was more highly expressed in the trachea (Kuchipudi et al., 2009). Again, the authors found a co-presence of both receptors in the kidney cells, the endocardium, the meninges, and the muscle fibers of the analyzed chickens. This co-existence in the chickens could make them act as 'mixing vessels' should co-infection with avian and human viruses occur, thus generating gene reassortment (Kuchipudi et al., 2009). Most studies have found an increased presence of the α2,6-SA receptor in the trachea and lungs of chickens (Gambaryan et al., 2003, 2004; Kuchipudi et al., 2009; Yu et al., 2011). This has also been confirmed by a study published in the journal 'Science' in which, using a combined analysis of reversed-phase liquid chromatography (LC), electrospray ionization mass spectrometry (ESI)-mass spectrometry (MS), it was shown that chickens had more α2,6-SA than α2,3-SA receptors in the trachea and

lung (Suzuki et al., 2022). A detailed study on the presence of these receptors in different tissues of chickens and turkeys was conducted by Pillai et al. (2010). This study also confirmed the co-presence of the two receptors, showing their presence on the tracheal and bronchial epithelium of the two species from day one of age. The renal histological sections of the two species were also positive for both receptors, while their presence at the level of intestinal epithelial cells was only highlighted in the chicken (Pillai and Lee, 2010). Most turkeys express avian-type SA receptors, but studies have shown that human-type receptors increase with age (Costa et al., 2012; Kimble et al., 2010). Costa et al. (2012) demonstrated a simultaneous presence in the epithelial cells of turkeys' respiratory and intestinal tracts, suggesting that these organs in turkeys may offer a potentially suitable environment for genetic reassortment of the virus (Costa et al., 2012).

Swine

Swine is considered to be the host that shares most human and avian receptors. This has been amply demonstrated in the literature, but until now, this potential role of 'mixing vessels' has not produced any profound epidemiological implications (Scholtissek, 1990). The theory of the pig as the main 'mixing vessel' for AIVs was first proposed in 1985 by Scholtissek et al. due to the antigenic and genetic similarity between certain avian, swine, and human influenza virus subtypes (Scholtissek et al., 1985). The ability of swine to be infected with both avian and human viruses also points to their potential role in interspecies transmission. From an epidemiological point of view, it is crucial to emphasize that it has been demonstrated that pigs can transmit viruses produced through genetic reassortment to humans (Ma et al., 2008). The capacity of these animal species to carry out genetic reassortment was first demonstrated by Pieris et al. after they detected the co-presence of the avian and human H9N2 influenza virus in swine in China (Peiris et al., 2001). The pathogenicity of swine AIVs and their clinical manifestations are similar to human influenza viruses, suggesting that pigs could be an excellent model for assessing viral infection and host immunity with implications for human health (Mancera Gracia et al., 2020). One of the most interesting articles confirming the pig as a 'mixing vessel' hypothesis was conducted by Nelli and colleagues in 2010, who evaluated the relative expression and distribution of SAα2,3-Gal and SAα2,6-Gal receptors. Both SAα2,3-Gal and SAα2,6-Gal receptors were found in abundance in the main pig organs analyzed, including the trachea, lung, liver, kidney, spleen, heart, skeletal muscle, brain, small intestine, and colon (Nelli et al., 2010d). The distribution of these receptors in the respiratory system of pigs proved to be very similar to that observed in humans. Contrary to the limited data on the scarce presence of influenza receptors in the human intestine, an increasing presence of SAα2,3-Gal and SAα2,6-Gal receptors was detected in pigs along the entire intestinal tract, from the duodenum to the colon (Nelli et al., 2010d). Evidence of genetic reassortment in pigs has accumulated over the years, with several episodes documented in various parts of the world. A study conducted in Argentina in 2011 identified two independent reassortment events between the pandemic H1N1 influenza virus and a swine influenza virus (Pereda et al., 2011). In the US, in 2015, two human serotypes, H3N2 and H3N1, were identified in pigs that had undergone genetic reassortments. These strains had genes coding for the HA protein similar to those of the H3 strains responsible for human seasonal influenza (Rajão et al., 2015). Furthermore, a phylogenetic analysis conducted in China in 2016 on H1N1 viruses isolated in pigs revealed that they belonged to a new category of triple reassortment porcine influenza viruses. These strains included viral proteins derived from the 2009 H1N1 pandemic virus (Sun et al., 2016).

Conclusions

Avian influenza represents a growing global health risk; its capacity to become transmissible to humans and many animal species of veterinary interest has attributed it to the role of a possible infectious disease potentially responsible for one of the subsequent pandemic events. Its interspecies transmission dynamics are not yet fully known, especially the underlying molecular mechanisms and the possible epidemiological implications. Many studies have aimed to evaluate these mechanisms, and some cases of infections in atypical species have been documented in recent years, but the health repercussions and the strict epidemiological dynamics of the phenomenon are still not entirely clear. This review, through an in-depth analysis of the molecular and evolutionary mechanisms

that allow the virus to cross interspecific barriers, has highlighted how the role of domestic animals such as dogs and cats in gene reassignment is potentially strategic in understanding the transmission dynamics. These species are simultaneously present receptors for avian (α2,3-SA) and human (α2,6-SA) influenza viruses in their respiratory tract and can therefore be considered potential "mixing vessels". This phenomenon underlying viral evolution has historically been associated with pigs, but documented episodes of infection in dogs and cats underline the importance of including these species in virological surveillance programs. The proximity of domestic animals to humans represents a significant risk for interspecific transmission and the emergence of strains with pandemic potential. This work highlights the importance of adopting an integrated analytical approach based on the "One Health" paradigm, which recognizes the interconnection between human, animal, and environmental health. A key point that must be emphasized is that all the mechanisms underlying the evolution and transmission of viruses imply an in-depth knowledge of the complex molecular interactions that govern their behavior. To fully understand and monitor these mechanisms, it is necessary to implement epidemiological monitoring based on next-generation sequencing tools, through which unknown molecular adaptations and underestimated evolutionary dynamics can be predicted.

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