

Review

Airborne Transmission of Avian Origin H9N2 Influenza A Viruses

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ABSTRACT: Influenza A viruses (IAV) are widespread viruses affecting avian and mammalian species worldwide. Outbreaks of IAV in poultry are usually associated with substantial morbidity and mortality, significantly affecting the poultry industry and food security. IAVs from avian species can be transmitted to mammals including humans and, thus, they are of inherent pandemic concern. Most of the efforts to understand the pathogenicity and transmission of avian origin IAVs have been focused on H5 and H7 subtypes due to their highly pathogenic phenotype in poultry. However, IAV of the H9 subtype that circulate endemically in poultry flocks in some regions of the world have also been associated with cases of zoonotic infections. As a result, the World Health Organization includes avian origin H9N2 IAV among the top in the list of IAVs of pandemic concern. In this review, we discuss the interspecies transmission of H9N2 between avian and mammalian species and the molecular factors that are thought relevant for this spillover. Additionally, we discuss factors that have been associated with the ability of these viruses to transmit through the respiratory route in mammalian species.

Keywords: H9N2; influenza; aerosol; interspecies; zoonotic; pandemic

INTRODUCTION

Influenza A viruses (IAV) are members of the family Orthomyxoviridae with a segmented RNA genome of negative polarity. IAV are divided into subtypes by the combination of the surface proteins, the hemagglutinin (HA, H1-H18) and the neuraminidase (NA, N1-N11) (Krammer et al. 2018). The natural hosts of IAV are wild aquatic birds, particularly waterfowl and seabirds, in which most of the IAV subtypes have been described (Carnaccini and Perez 2020, Peacock et al. 2019). From the wild bird reservoir, IAVs sporadically spill over to domestic poultry species causing disease outbreaks and become endemic. This has been the case for some H5Nx, H7Nx or H9N2 IAV viruses (Banet-Noach et al. 2007, Berhane et al. 2009, Choi et al. 2004, Monne et al. 2013, Morales et al. 2009, Sonnberg et al. 2012). IAVs of avian origin are classified in highly pathogenic avian influenza viruses (HPAIVs) and low pathogenic avian influenza viruses (LPAIVs) based on the pathotype in chickens and/or the presence of a polybasic cleavage site in the HA (Alexander 2003). The HA's polybasic cleavage site of HPAIVs allows for processing of the HA by endogenous cellular furin-like proteases, leading to systemic infections and increased pathogenicity. In contrast, LPAIVs contain no more than a tri-basic cleavage site in the HA, making them dependent on extracellular trypsin-like proteases for processing,

limiting infections to sites where such enzymes are abundant (e.g., respiratory and/or gastrointestinal systems) (Alexander 2003, Organization 2005, Service). Only viruses of the H5 or H7 subtype have been associated to the HPAI pathotype.

H9N2 AVIAN INFLUENZA VIRUSES

IAVs of the H9N2 subtype are widespread in different species of wild waterfowl, shorebirds, and poultry, such as chickens, turkeys, quails, among others (Hassan et al. 2020, Jackwood and Stallknecht 2007, Reid et al. 2016, Swieton et al. 2018, Swieton et al. 2020, Xu et al. 2007). H9N2 IAVs are the most prevalent LPAIVs, enzootic in poultry in parts of Asia, the Middle East, and Africa (reviewed in (Carnaccini and Perez 2020)). IAVs H9N2 were first isolated from turkeys in Wisconsin, USA in 1966 (Homme and Easterday 1970), with subsequent sporadic detections in poultry in the US. H9N2 viruses were first isolated from healthy ducks from farms and live poultry markets in Hong Kong between 1975-1985 (Markwell and Shortridge 1982, Shortridge 1992). In 1988, the first evidence of H9N2 infection in poultry in Asia was reported after a respiratory outbreak in quails (Perez et al. 2003). Currently, IAVs H9N2 are widespread in poultry species around the world with particularly high prevalence in Asia (Banet-Noach et al. 2007, Cameron et al. 2000, Guan et al. 2000).

H9N2 IAV infections are usually mild. However, significant economic losses are associated with H9N2 infections because of delayed growth and lower egg production. Studies have shown that H9N2 virus replication in the oviduct results in poor eggshell quality and deterioration of eggshell (Bonfante et al. 2018, Qi et al. 2016, Sid et al. 2017). H9N2 infections in poultry are also associated with secondary respiratory pathogens, such as infectious bronchitis virus and *Mycoplasma gallisepticum*, which can lead to high mortality (Arafat et al. 2020, Awuni et al. 2019, Bonfante et al. 2018, Chu et al. 2017, Jakhesara et al. 2014, Nili and Asasi 2002, Smietanka et al. 2014, Wang et al. 2015).

Phylogenetic analyses of the H9 HA have classified the H9 into two lineages: the American and Eurasian lineages, which are further divided into four sub-lineages (h9.1 to h9.4) (Carnaccini and Perez 2020). Strains in the h9.1 sub-lineage are present mostly in wild birds in America, those from the h9.2 sub-lineage circulate in Korean poultry and wild birds in Eurasia, whereas those from the h9.3 sub-lineage (BJ94-like strains) are present in poultry in China. Viruses from the h9.4 sub-lineage (G1-like) are endemic in poultry in the Middle East, India, Egypt and Africa (extensively reviewed in (Carnaccini and Perez 2020)). Antigenic characterization of the HA of H9 viruses showed that these viruses, like other IAVs, show an immunodominance mediated by the globular head of the HA. Interestingly, the HA of the H9 subtype lack the 130 lateral loop that forms the antigenic site A in other subtypes, such as H5 or H3 HAs (Li et al. 2020, Wiley et al. 1981). Such feature results in two antigenic sites that overlap, designated site I and site II (Kaverin et al. 2004). More recent work showed the presence of alternative non-overlapping antigenic sites designated H9-A and H9-B, where H9-A shares amino acids with site I and is immunodominant in comparison to H9-B (Peacock et al. 2016). Genetic and antigenic differences are observed within lineages circulating in specific regions, and antigenic drift has been observed in regions where these viruses are endemic, such as China and Egypt (Adel et al. 2017, Wei et al. 2016).

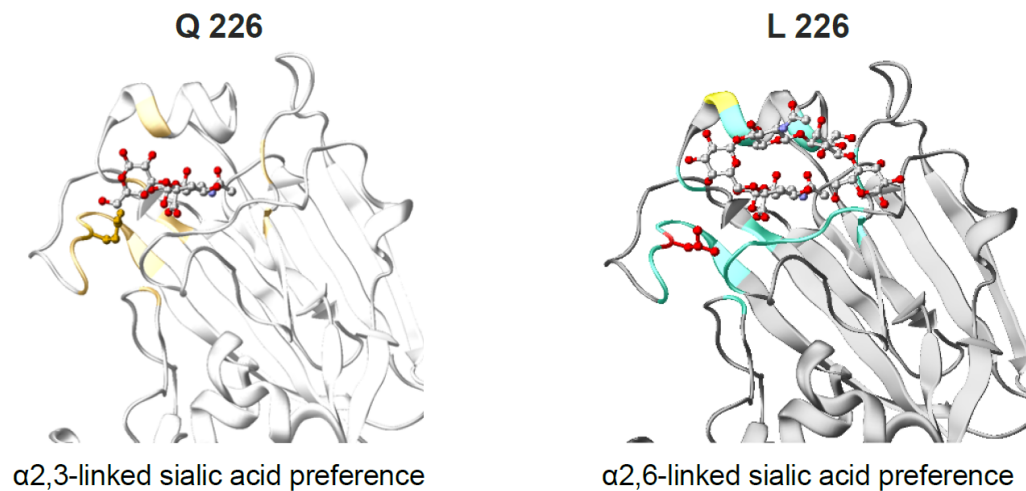
Of great significance, H9N2 IAV viruses have contributed the internal gene segments to more deadly zoonotic strains such as H5N1/N6, H7N9, and H10N8/N3 that have been implicated in human infections and loss of life (Guan et al. 1999, Lam et al. 2013, Pu et al. 2021, Wang et al. 2021). In addition, H9N2 IAVs are zoonotic viruses themselves and have also been reported in other mammalian species such as swine, dogs, horses, and mink. In humans, H9N2 IAV infections have presented with mild influenza-like symptoms such as respiratory symptoms, cough, fever, runny nose, sore throat, and headache (Butt et al. 2005, Cong et al. 2008, Jallow et al. 2020, Peiris et al. 1999, Sun et al. 2021). However, such mild infections could be the prelude to the selection of more virulent strains with the

capacity of transmit in humans more efficiently. Therefore, understanding the factors required for efficient transmission of H9N2 viruses in mammalian species is essential for adequate pandemic preparedness. In this review, we will discuss the transmission of H9N2 IAV in mammalian species providing an overview of the molecular features that may facilitate the respiratory transmission of these viruses, focusing mostly on the collective findings from our group.

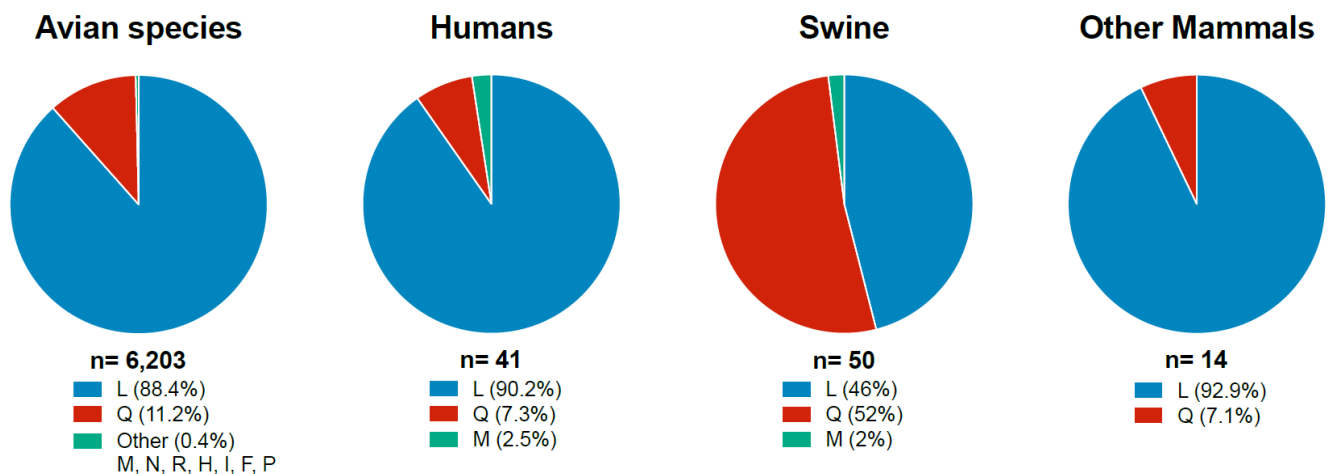
MOLECULAR MECHANISMS ASSOCIATED WITH INTERSPECIES TRANSMISSION OF IAVs

Several molecular signatures have been associated with host range restriction and species jump of IAV in mammalian species. Particularly, the segments encoding the HA and the polymerase complex play a major role in the host range and adaptation of IAVs (Van Hoeven et al. 2009). The HA is responsible for receptor-binding to the host cells and the fusion between the endosomal membranes and viral envelope (Das et al. 2018, Edinger et al. 2014). An important barrier in the avian to human transmission of IAV is the different binding specificities of IAV to terminal sialic acid present on the glycan receptors on the host cell surface. IAVs of avian origin bind preferentially to α 2,3-linked sialic acids (α 2,3SA) and those from human origin viruses bind preferentially to α 2,6-linked sialic acids (α 2,6SA) (Xiong et al. 2014). The α 2,3SA versus α 2,6SA preference is mediated by key residues located in the HA; specifically, positions 226 and 228 (H3-numbering used throughout the text) are critical for receptor specificity in H3 and H9 viruses (Fig 1A) (Obadan et al. 2019, Rogers et al. 1983, Wan and Perez 2007). H9N2 IAVs endemic in poultry have variations at position 226 with most isolates carrying leucine (L) and others glutamine (Q) (Fig 1B). At position 228, glycine (G) is present in almost all H9 isolates detected to date. Indeed, an analysis of more than 2,500 H9 isolates from avian and mammalian hosts showed a switch in the 226 position over the years, with the majority of H9N2 IAV isolates carrying Q226 before 2000 while newer H9N2 IAV isolates revealed high prevalence of 226L (Sun et al. 2020). Viruses with the Q226/G228 combination present dual binding or α 2,3SA avian-like preference. A single Q226L mutation produces a switch to α 2,6SA human-like preference (Vines et al. 1998, Wan et al. 2008). Consistent with these observations, most H9N2 IAVs identified in poultry farms and live bird markets exhibit binding to human-like receptors (Matrosovich et al. 2001, Zou et al. 2019). More importantly, most H9N2 viruses isolated from mammalian species, including human isolates, show the L226/G228 combination (Fig 1B). Interestingly when swine sequences are analyzed, there is an even distribution between isolates carrying Q226 or L226 (Fig 1B). In addition, other amino acid signatures can modulate and/or enhance binding of H9 HAs to terminal α 2,6SAs, such as the mutation from isoleucine (I) to threonine (T) at position 155 (Li et al. 2014) or the presence of a valine (V) at position 190 (Teng et al. 2016). The modulation of receptor binding preference by V190 is reminiscent of similar effects in HAs of the H1 subtype (Matrosovich et al. 2000). The presence of Q227 in combination with either aspartic acid (D) or glutamic acid (E) at position 190 favors binding α 2,6SAs receptors.

A. Position 226 binding preference



B. Number of variants in Position 226 in natural isolates



C. Transmission of field H9N2 viruses with L226 x Q226 in ferrets

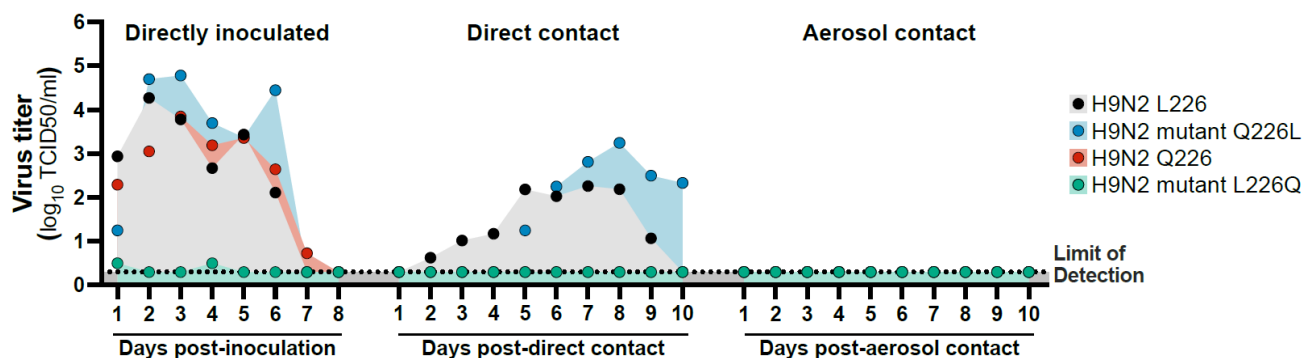


Figure 1. Impact of position 226 in the H9 of H9N2 IAV viruses. (A) The 3D molecular structure of the H9 HA glycoprotein globular head from A/Guinea Fowl/Hong Kong/WF10/99 (WF10) with glutamine (Q) or leucine (L) in position 226. The sialic acid binding is shown in each case. Structure constructed using the iTASSER structure prediction tool (Yang et al. 2015). (B) The different residues in position 226 of the H9 were analyzed from H9N2 IAV isolated for avian species, humans, swine, and other mammals (canine, equine, and mink). Full H9 sequences were downloaded from the global initiating on sharing all influenza data (GISAID). Sequence analyses were performed using Geneious Prime 2020.2.4

(<https://www.geneious.com>). (C) Summary of the replication, transmission by direct contact or airborne transmission in ferrets inoculated with H9N2 IAV viruses carrying L226 (H9N2 L226) or Q226 (H9N2 Q226) in the H9. Data for replication and transmission of mutant viruses with H9 Q226L (H9 mutant Q226L) or H9 L226Q (H9N2 mutant L226Q) are also shown. Results compiled from (Wan et al. 2008).

The viral polymerase subunits PB1, PB2, and PA, can also contribute to the adaptation of IAV of avian origin to mammalian hosts. Of interest, the change from E to lysine (K) in position 627 (E627K) in PB2 is a major determinant of host restriction. This has been the predominant host adaptation marker identified in human cases of H5N1 and H7N9 infections (Li et al. 2004, Wang et al. 2014). A higher frequency of PB2 K627 is observed in H9N2 viruses isolated from mammalian hosts in comparison to avian counterparts. Interestingly, more than 20% of H9N2 viruses from human cases possess the PB2 V627 signature, which was also observed in transmission experiments between avian and mammalian species (Luk et al. 2015). It is worth noting that position 627 modulates the optimal temperature for virus replication. The PB2 K627 is associated with increased polymerase activity and replication at 33-37°C, an attribute necessary for replication in the human respiratory tract. In contrast, the PB2 E627 mutation allows for optimal replication at 39-41°C, consistent with the body temperature of most bird species. Furthermore, the PB2 E627K have also been observed 3 days post-inoculation in mice when a duck-origin H9N2 IAV virus is previously serially passaged in chickens and quails, suggesting that the adaptation to land-based birds can also contribute to a faster acquisition of mutations that favor replication in mammals (Hossain et al. 2008), which suggest that PB2 E627K is a respiratory tract adaptation rather than a mammalian adaptation.

The PB2 A588V mutation is also potentially involved in mammalian adaptation. H9N2 IAVs carrying the PB2 V588 signature show enhanced virulence in mice (Xiao et al. 2016). For PB1, the I368V mutation detected in a ferret adapted H5N1 strain has shown increased frequency among recent H9N2 isolates, from 2.8% to 67% (Herfst et al. 2012). In PA, the K356R mutation increased viral replication in mice even without PB2 K627. More than 80% of avian H9N2 isolates collected after 2013 and half of human H9N2 isolates contain the PA R356 marker (Xu et al. 2016). A summary of molecular markers associated with transmission and adaptation of H9N2 IAV viruses is presented in Table 1.

Table 1. Molecular markers associated with adaptation and transmission of H9N2 IAV in mammalian host.

| Protein. | Marker | Effect | Host evaluated | Reference |
|----------|--------|--|--|--|
| PB2 | T58I | Observed in airborne transmission | Ferrets | (Kimble et al. 2011) |
| PB2 | D253N | Increase pathogenesis/Observed in airborne transmission | Mice and ferrets | (Kimble et al. 2014, Mok et al. 2011, Zhang et al. 2018) |
| PB2 | R340K | Increase transmission | Guinea pigs | (Lina et al. 2019) |
| PB2 | Q591K | Increase polymerase complex activity and replication | Mice | (Mok et al. 2011) |
| PB2 | E627K | Increase polymerase activity and viral replication in mammalian host | Human, mice, quail, guinea pigs and ferret | (Hossain et al. 2008, Li et al. 2004, Li et al. 2014, Liu et al. 2021, Wang et al. 2014) |

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| PB2 | A588V | Increase polymerase activity, transmission, and virulence | Mice and guinea pigs | (Lina et al. 2019, Xiao et al. 2016) |
| PB2 | D701N | Increase virulence and airborne transmission | Ferret | (Li et al. 2014) |
| PB2 | A707T | Observed in airborne transmission | Ferret | (Kimble et al. 2014) |
| PB1 | D120N | Observed in airborne transmission | Ferret | (Kimble et al. 2014) |
| PB1 | D439E | Observed in airborne transmission | Ferret | (Kimble et al. 2014) |
| PB1 | S261N | Reduced polymerase complex activity/ observed in airborne transmission | Ferret | (Chin et al. 2016, Kimble et al. 2011) |
| PB1 | I368V | Increase airborne transmission (H5 context) | Ferret | (Herfst et al. 2012) |
| PA | K356R | Increase polymerase activity and replication | Mice | (Xu et al. 2016) |
| PA | K26E | Increase replication/Observed in airborne transmission | Chickens, quail, and ferrets | (Clements et al. 2020, Kimble et al. 2014, Obadan et al. 2019) |
| HA1 | D225G | Increase transmission and replication | Pigs | (Mancera Gracia et al. 2017) |
| HA1 | Q226L | Increase $\alpha 2,6$ SA binding | Ferrets and quails | (Obadan et al. 2019, Wan et al. 2008) |
| HA1 | I155T | Increase $\alpha 2,6$ SA binding | Ferrets | (Li et al. 2014) |
| HA1 | A190V/ T190V | Increase replication | Mice | (Teng et al. 2016) |
| HA1 | V104A | Observed in airborne transmission | Ferrets | (Kimble et al. 2011) |
| HA1 | T189A | Increase airborne transmission | Ferrets, quails, and pigs | (Obadan et al. 2015, Sorrell et al. 2009) |
| HA1 | S263N | Observed in airborne transmission | Ferrets | (Kimble et al. 2014) |
| HA1 | S328C | Observed in airborne transmission | Ferrets | (Kimble et al. 2014) |
| HA2 | G192R | Increase airborne transmission | Ferrets, quails, and pigs | (Obadan et al. 2015, Sorrell et al. 2009) |
| NA | I28V | Increase airborne transmission | Ferrets, quails, and pigs | (Obadan et al. 2015) |
| NA | A30T | Observed in airborne transmission | Ferrets | (Kimble et al. 2011) |
| M2 | E95K | Observed in airborne transmission | Ferrets | (Kimble et al. 2014) |

| | | | | |
|----|-----|---|--------------|--|
| NS | D2N | Increase virulence and IFN-B antagonism/ observed in airborne transmission | Mice/ferrets | (Forbes et al. 2012, Kimble et al. 2014) |
|----|-----|---|--------------|--|

NATURAL INFECTION OF H9N2 IAV IN MAMMALS

According to the World Health Organization (WHO), 54 human cases of H9N2 IAV infection have been reported since December 2015, with 13 cases reported during 2021 from China (as of July 22 2021 (WHO 2021)). Additional cases have been reported in Bangladesh, India, Egypt, Oman, and Senegal (Lin et al. 2000, Pan et al. 2018, Pawar et al. 2012, Peacock et al. 2019, Peiris et al. 1999, Potdar et al. 2019). Most human infections with H9N2 viruses had confirmed contact with poultry and evidence of human-to-human transmission has not been reported. Serological investigations have shown H9N2 IAV antibodies in humans in Vietnam, Cambodia, Iran, Thailand, Pakistan, India, Egypt, and Hong Kong (Blair et al. 2013, Chaudhry et al. 2020, Heidari et al. 2016, Hoa et al. 2017, Ma et al. 2019, Quan et al. 2019, Tahir et al. 2020). In most cases, the presence of H9N2 IAV antibodies is associated with poultry workers; however, there are seropositive cases with no history of direct poultry exposure. H9N2 IAVs have also been isolated from pigs and serological surveys have shown presence of H9N2 IAV-specific antibodies in pig herds with prevalence as high as 15% (Cong et al. 2008, Ninomiya et al. 2002, Wang et al. 2016, Yuan et al. 2013). In addition, a serological survey showed that minks are also susceptible to infection with H9N2 IAV (31% of positive samples) and six different H9N2 isolates were isolated from tissues obtained from a mink farm. Similar procedures were performed in foxes and raccoon dogs (all species relevant in the fur industry), with a 59% and 41% of serologically positive samples respectively, but no virus strains were isolated (Yong-Feng et al. 2017)

EXPERIMENTAL INFECTIONS/TRANSMISSION OF H9N2 IAV IN MAMMALIAN MODELS

For an avian H9N2 virus to successfully transmit in mammals, the virus must evolve to become compatible with the new host environment, allowing effective replication and transmission(Kuiken et al. 2006). Reassortment between avian and mammalian influenza viruses has led to the emergence of pandemic viruses in the past. Transmission studies designed to evaluate the potential of IAV transmission in mammals is commonly performed in ferrets, which present similar characteristics for IAV infection as humans in terms of lung pathology, clinical signs, pathogenesis, and immunity (Maher and DeStefano 2004, Nguyen et al. 2021) . Wan and collaborators evaluated replication and transmission capabilities of different H9N2 IAV isolated from avian species between 1988-2003, using the ferret model, showing that all the isolates replicated in ferrets. However, just 2 of those isolates transmitted through direct contact (Fig 1C). The 2 isolates that transmitted in ferrets contained the L226 HA marker, supporting the advantage of L226 over Q226 for replication in mammals. Regarding the isolates that did not transmit, two had Q226 and one L226. The role of the L226 mutation on mammalian replication and contact transmission was confirmed when it was replaced by Q226 in a H9N2 IAV isolate (L226Q), resulting in complete loss in replication capability, even in the direct inoculated group (Fig 1C). The inverse experiment, Q226L mutation introduced in a virus naturally carrying Q that replicated poorly in ferret without transmission, resulted in enhanced replication and transmission through direct contact (Fig 1C) (Wan et al. 2008). To further understand the relevance of amino acid 226 on HA, the replication of H9N2 viruses containing either L226 or Q226 was tested in human airway epithelial cells grown in air-liquid interface (Wan and Perez 2007). Viruses with L226 grew with higher efficiency in comparison with viruses carrying Q226, and showed a different tropism by infecting non-ciliated cells similar to seasonal human H3N2 IAV (Wan and Perez 2007). In a separated study using ex

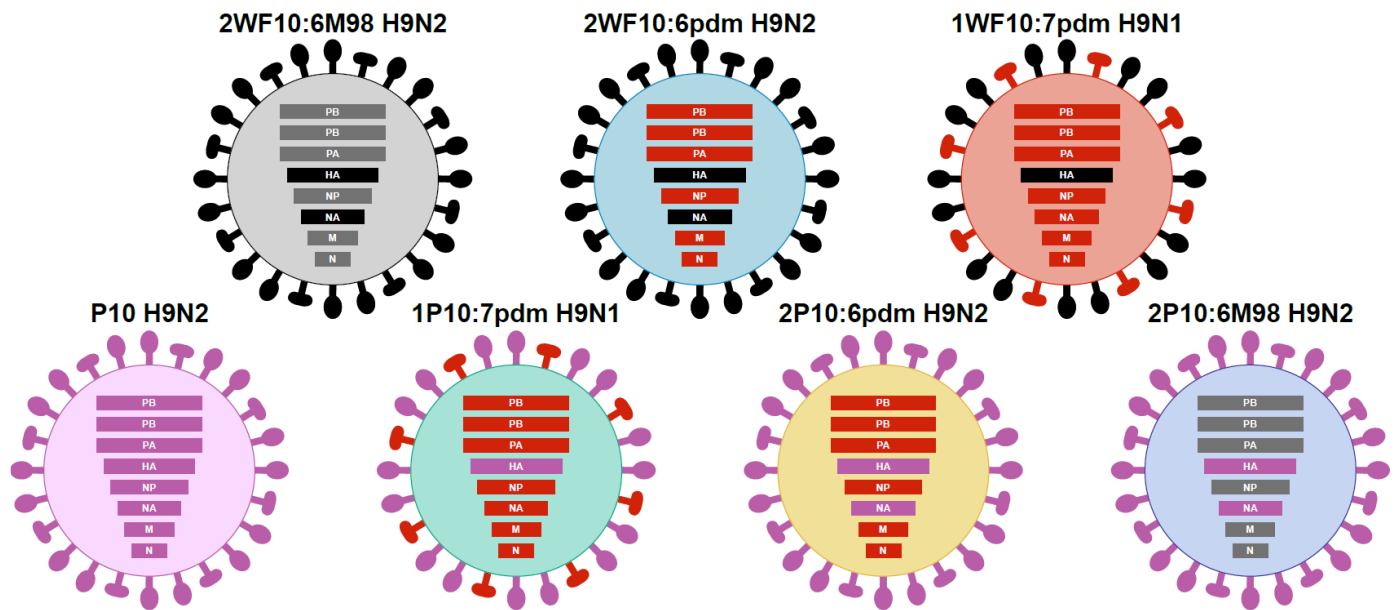
vivo human respiratory organ culture, H9N2 IAVs were shown to infect both the upper and lower human respiratory tract, with differences observed depending on strain (Chan et al. 2017).

More recent work demonstrated that although most H9N2 IAV isolates possess either Q226 or L226, this position is highly flexible and able to tolerate multiple amino acids (at least 10), some not previously detected in natural H9N2 isolates (Obadan et al. 2019). In vitro, most of these variants replicate to similar titers in comparison to viruses carrying either the Q226 or L226, despite their relative lower receptor binding avidity. Additionally, no impact on antigenicity or hemagglutination activity was observed, independent of the amino acid at position 226. Interestingly, viruses containing N226, M226, or I226 show increased breadth of receptor recognition, with dual binding to avian- and human-type receptors, a feature that might affect host range and potentially facilitate interspecies transmission. In contrast, viruses carrying Q226, C226, T226 or H226 show strict α 2,3 binding, demonstrating a residue-dependency in position 226 for receptor recognition (Obadan et al. 2019). In vivo competition studies in quails using varying mixtures of these variants demonstrated that the L226 provides a fitness advantage in vivo. A mixture of viruses without Q226 or L226 (var Δ LQ) was still able to replicate and transmit via direct contact in quails, granted with lower efficiency at 2dpc in comparison with mixtures containing L (var+L), Q (var+Q) or both (var+LQ). Interestingly, sequencing analysis from tracheal swabs showed that even in the var Δ LQ or var+Q groups, where viruses with L226 were not included in the mixture, L is still detected in the tracheal swabs collected at 3 dpi. Viruses carrying M226 or I226 were also readily detected, consistent with the detection of natural isolates containing such amino acid signatures. The predominant amino acid detected in contact quails was L226, even in groups in which quail were inoculated with the mixtures lacking L226 (var Δ LQ and var+Q), suggesting a strong advantage in transmission when L226 is present. These findings confirm that L226, Q226, and M226 confer fitness advantage to H9N2 viruses in poultry, explaining their predominance in natural isolates. Although the 226 position has great plasticity, most amino acids result in strains with preference for α 2,3-linked sialic acid and therefore, they are less likely to infect humans. This finding facilitates risk assessment for the zoonotic potential of H9N2 viruses (Obadan et al. 2019).

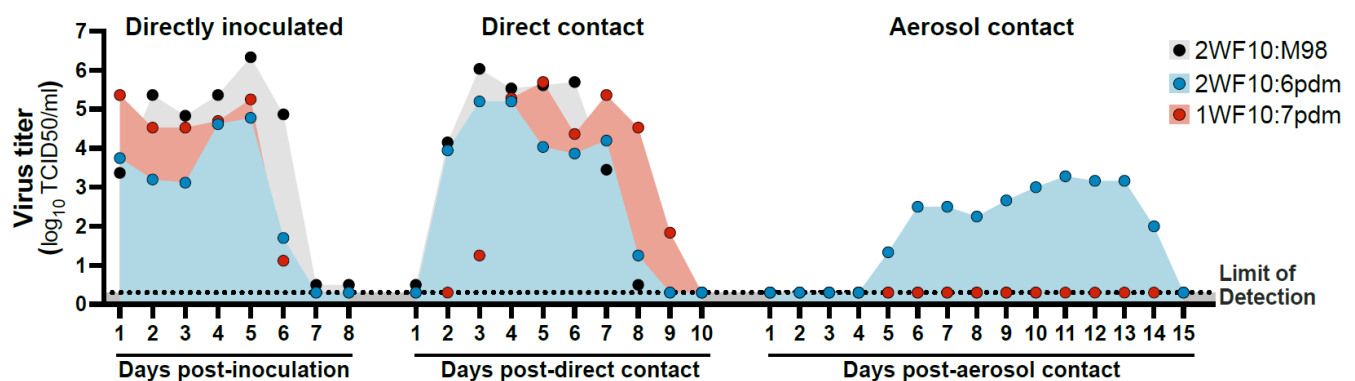
Despite direct-contact transmission in ferrets of the H9N2 IAV field isolates in the study described above, no airborne transmission was observed (in contrast to control ferret studies using human origin H1N1 or H3N2 IAVs) (Munster et al. 2009, Perez et al. 2009). Further, ferrets infected with a reassortant virus of the H9N2 subtype with the internal genes of a seasonal human H3N2 strain (2WF10:6M98; Fig 2A) showed clinical signs like those observed with the wild type seasonal H3N2 strain. However, direct contact (but not airborne) transmission of the 2WF10:6M98 was observed in ferrets (Fig 2B) (Wan et al. 2008). Interestingly, 10 serial passages of the 2WF10:6M98 in ferrets resulted in a virus (P10; Fig2A) that transmitted efficiently through direct contact and by respiratory droplets (Fig 2C). The P10 virus also showed an intermediate plaque size between the seasonal H3N2 and the H9N2 viruses, suggesting an intermediary replication fitness phenotype (Sorrell et al. 2009). Sequencing of the P10 virus revealed a T189A mutation in the HA1 portion, a G192R mutation in HA2 and a I28V mutation in NA (Table 1). These mutations were crucial for the respiratory transmission phenotype observed. A L374I mutation in PB2 was also detected; however, its contribution seems to be marginal for transmission (Sorrell et al. 2009). After the emergence of the pH1N1 virus in 2009, similar experiments were performed with a reassortant H9N2 virus with pH1N1 internal genes showing that the H9N2-pH1N1 (2WF10:pH1N1) or a H9N1-pH1N1 (1WF10:pH1N1; N1 and internal genes derived from pH1N1; Fig 2A) are able to transmit via direct contact without prior adaptation (Kimble et al. 2011). These results suggest that in the case of H9N2 IAV, just the introduction of internal genes from human-origin IAV H1N1 or H3N2 is enough to allow an efficient direct-contact transmission in mammals (Kimble et al. 2011, Wan et al. 2008). In addition, the 2WF10:6pH1N1 virus was also able to transmit by airborne route, although with delayed replication kinetics, whereas no evidence of airborne transmission

with 1WF10:7pH1N1 was observed, suggesting that compatibility and balance between the HA and NA is also important (Fig 2B). It is tempting to speculate that the pH1N1 backbone is more flexible to accept reassortment with diverse surface genes and does provide a replicative advantage in mammals, as has been observed in multiple examples of reassortant H1N1 and H3N2 viruses in swine (Everett et al. 2020, Powell et al. 2021, Ryt-Hansen et al. 2020, Zell et al. 2020). Sequencing information showed a S261N mutation in PB1 and V104A in HA in the viruses isolated from respiratory contact animals (Table 1); however, the role of those positions in airborne transmission remains unknown (Kimble et al. 2011). When pH1N1 internal genes are combined with the H9 HA (1P10:7pH1N1) or H9N2 HA/NA (2P10:6pH1N1) of the P10 ferret-adapted virus (Fig 2A), both viruses show efficient replication in vitro, generate moderate to severe lesions in the respiratory tract of ferrets and both are able to efficiently transmit via direct contact and respiratory droplets in the ferret system, (Kimble et al. 2011) (Fig 2C). Furthermore, these viruses efficiently transmitted between pigs by direct contact while only the 2P10:6pH1N1 transmitted in quails (Obadan et al. 2015).

A. Recombinant viruses tested for transmission in ferrets



B. Transmission of reassortant H9N2 viruses in ferrets



C. Transmission of ferret-adapted H9N2 viruses in ferrets

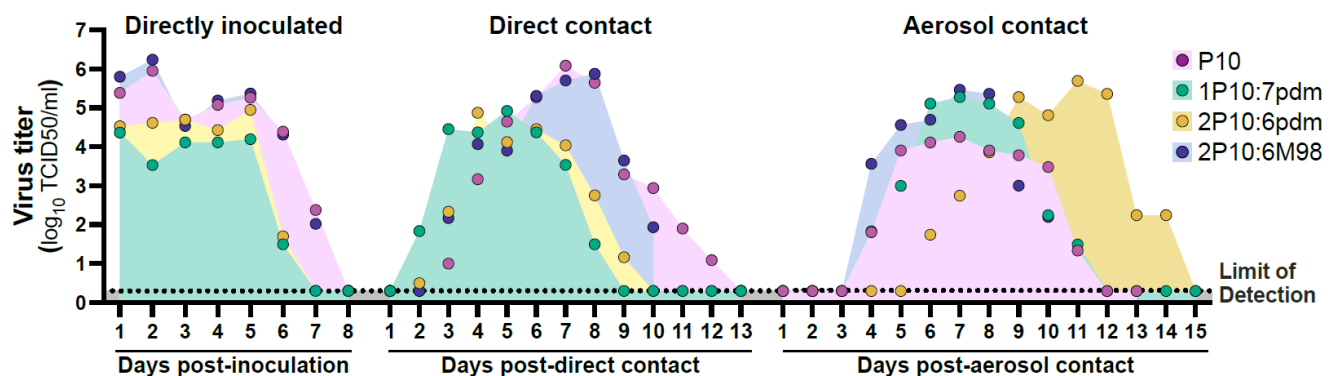


Figure 2. Transmission of reassortant and adapted H9 viruses in ferrets. (A) Schematic representation of the different reassortant viruses evaluated in ferrets. (B) Replication and transmission by direct contact or airborne in ferrets of viruses carrying the H9N2 subtype with internal genes of a seasonal H3N2 virus (2WF10:M98 H9N2) or pandemic H1N1 virus (2WF10:6pdm H9N2 or 1WF10:7pdm H9N1). (C) Replication and transmission by direct contact or airborne in ferrets of a virus carrying the H9N2 subtype with internal genes of a seasonal H3N2 virus adapted by serial passages in ferrets (P10). Viruses containing the HA (1P10:7pdm) or the HA/NA (2P10:6pdm) of the P10 virus and internal genes of a pandemic

H1N1 virus, or HA/NA of the P10 virus and internal genes of a seasonal H3N2 (2P10:6M98) were also evaluated. Results compiled from (Kimble et al. 2011, Sorrell et al. 2009, Wan et al. 2008).

To confirm the role of reassortment (and presence of mammalian-adapted gene segments) on the ability of H9 viruses to transmit in mammals, a transfection-based inoculation (TBI) study was performed in ferrets to select airborne transmissible H9 reassortant viruses under host selection pressure. In brief, ferrets were inoculated with cells previously transfected with 15 plasmids: 6 encoding the internal gene segments of the WF10 H9N2 virus, 7 gene segments (excluding the HA) of a prototypic pH1N1 virus and the surface gene segments of the P10 ferret-adapted virus. The resulting virus mixture was then serially passaged in ferrets, allowing for the selection of any possible H9 reassortant that was compatible/fit with respiratory droplet transmission in ferrets. The results show two different H9N1 viruses that were selected and able to transmit by the respiratory route in ferrets (Kimble et al. 2014). Both H9N1 viruses identified had a mixed population of internal genes from pH1N1 or H9N2, both containing PB2, NP and NA of from the pH1N1 strain and PA, HA and NS from the H9N2 strain. Both viruses differed in the PB1 and M gene segments where in one virus both were from pH1N1 strain, and the opposite was observed in the second virus. Sequencing analysis revealed mutations in PB2 (D253N), PA (K26E), HA1 (S263N) and NS (D2N) in one of the viruses (Table 1), some of which have been previously reported to have an impact replication and pathogenicity (Clements et al. 2020, Forbes et al. 2012, Mok et al. 2011, Ping et al. 2011, Zhang et al. 2018). The second virus showed mutations in PB2 (A707T), HA1 (S328C), M2 (E95K), and two mutations in PB1 (D120N and D439E) (Table 1) (Kimble et al. 2014). However, the mutations detected in the second virus have not been associated with any advantage in terms of replication or pathogenicity previously, highlighting that the molecular requirements for efficient transmission of H9N2 IAV viruses in mammalian species are far from understood and deserves further scrutiny. A summary of the different viruses discussed above, and the transmission phenotype is shown in Figure 3.




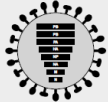
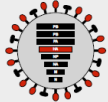
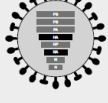
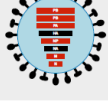
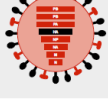
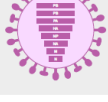
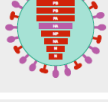
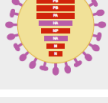
| VIRUS | DESCRIPTION | TRANSMISSION - DIRECT CONTACT OR AEROSOL | | | REFERENCE |
|--|--|---|--|---|-----------|
| | |  |  |  | |
| Avian-isolate 226L H9N2  | IAV H9N2 viruses isolated from avian species carrying 226L in the HA | Direct contact (66%) No Aerosol transmission | N/D | Direct contact depending on strain | 59 |
| Avian-isolate 226Q H9N2  | IAV H9N2 viruses isolated from avian species carrying 226Q in the HA | No transmission | N/D | Direct contact depending on strain | 59 |
| 2WF10:6M98 H9N2  | Recombinant H9N2 virus with internal genes from a seasonal IAV H3N2 | Direct contact No Aerosol transmission | N/D | N/D | 59, 94 |
| 2WF10:6pdm H9N2  | Recombinant H9N2 virus with internal genes from a pandemic IAV H1N1 | Direct contact Aerosol transmission | N/D | N/D | 95 |
| 1WF10:7pdm H9N1  | Recombinant H9N1 virus with internal genes and NA from a pandemic IAV H1N1 | Direct contact No Aerosol transmission | N/D | N/D | 95 |
| P10 H9N2  | Recombinant H9N2 virus with internal genes from a seasonal IAV H3N2 adapted by 10 serial passages in ferrets | Direct contact Aerosol transmission | N/D | N/D | 94 |
| 1P10:7pdm H9N1  | Recombinant H9N1 virus (HA from P10 virus) with internal genes and NA from a pandemic IAV H1N1 | Direct contact Aerosol transmission | Direct contact | No Direct contact | 95, 100 |
| 2P10:6pdm H9N2  | Recombinant H9N2 virus (HA and NA from P10 virus) with internal genes from a pandemic IAV H1N1 | Direct contact Aerosol transmission | Direct contact | Direct contact | 95, 100 |
| 2P10:6M98 H9N2  | Recombinant H9N2 virus (HA and NA from P10 virus) with internal genes from a seasonal IAV H3N2 | Direct contact Aerosol transmission | N/D | N/D | 94 |

Figure 3. Summary of H9 IAV reassortant viruses and their transmission ability in ferrets. Schematic representation of the different H9 viruses tested for transmission in ferrets by our group and described in this review, description of each virus, and type of transmission observed. Results compiled from (Kimble et al. 2011, Obadan et al. 2015, Sorrell et al. 2009, Wan et al. 2008).

Experimental infection/transmission with poultry adapted H9N2 viruses have been evaluated also in multiple alternative animal models such as guinea pigs, mice, and pigs, among others (Table 2) (Kimble et al. 2011, Lina et al. 2019, Sorrell et al. 2009, Wan et al. 2008, Wang et al. 2016, Yong-Feng et al. 2017). In one study, experimental infection of

pigs with H9N2 resulted in nasal shedding and seroconversion with 4 out of 6 H9N2 IAV isolates (Wang et al. 2016); however, transmission between pigs did not occur. When the internal gene segments were replaced with those from a pandemic H1N1 strain, increased virus replication and transmission between pigs was observed (Obadan et al. 2015, Qiao et al. 2012). Furthermore, when an H9N2 virus with the pH1N1 internal gene segments was serially passaged in pigs, the virus showed increased tropism capable of replicating in the entire respiratory tract as well as efficient pig-to-pig transmission (Mancera Gracia et al. 2017).

Some H9N2 IAV strains isolated from chicken houses were able to transmit experimentally between guinea pigs by direct contact but respiratory droplets transmission was not observed (Lv et al. 2012). Interestingly, direct contact transmission efficacy increased after nine serial passages of H9N2 IAV in guinea pigs, reaching 100% transmission after fifteen serial passages, consistent with the idea that molecular adaptative changes are required to result in a mammalian transmissible H9N2 virus. However, despite the improvement of direct contact transmission after fifteen passages, airborne transmission had only 16% efficacy in guinea pigs and no airborne transmission was observed in ferrets (Sang et al. 2015). Similarly, improvement in transmissibility is also achieved in guinea pigs when H9N2 IAV are serially passaged in mice prior to guinea pig infection. The mouse adapted H9N2 IAV has enhanced pathogenicity and is able to transmit through direct contact and respiratory droplets whereas the non-mouse adapted version does not transmit in guinea pigs (Lina et al. 2019). Reassortant viruses carrying unmodified H9N2 glycoproteins and internal genes derived from pH1N1 were also able to transmit by either direct contact or airborne in guinea pigs whereas a whole avian H9N2 virus did not (He et al. 2014), similarly to what was observed in pigs. The presence of pH1N1 PA gene alone seems to be sufficient to allow transmission between guinea pigs by direct contact but not respiratory droplets (Hao et al. 2019).

Further, H9N2 IAV isolated from live bird markets possessing L226/S228 in the HA plus K627 in the PB2 (all molecular markers of mammalian adaptation and/or respiratory tissue adaptation) can transmit via airborne between chickens and guinea pigs (Liu et al. 2021). Further, transmission studies in ferrets using H9N2 viruses have shown some natural avian-origin H9N2 IAV isolates are able to transmit via respiratory droplets in ferrets without further adaptation (Li et al. 2014, Wan et al. 2008, Zhang et al. 2021), highlighting the inherent risk for H9N2 IAV transmission in mammalian species and suggesting a strain-specific effect. Similarly, H9N2 viruses isolated from humans were able to transmit via direct contact and respiratory droplets in 1 out of 3 isolates evaluated. However, in that study, swine- and avian-origin H9N2 isolates were only able to transmit via direct contact (Group 2013). Direct-contact transmission of H9N2 IAV can also occur between minks, foxes, or raccoon dogs (Yong-Feng et al. 2017). Contact minks developed clinical signs consistent with H9N2 IAV infection whereas seroconversion, but no clinical signs, was observed in foxes and raccoon dogs (Yong-Feng et al. 2017). In another study, the airborne transmission of H9N2 IAV was evaluated in minks, but no positive results were obtained (Sun et al. 2021). H9N2 IAV infection was also reported in experimentally infected cats and dogs but transmission through direct contact was only observed among cats but not dogs (Zhang et al. 2013).

CONCLUSIONS

Although H9N2 IAV are LPAIV and cause mostly mild infections, these viruses still result in significant economic losses to the poultry industry. Furthermore, H9N2 IAV have been implicated as the donors of internal genes for prevalent HPAIV outbreaks that have resulted in human infections in some cases. Additionally, cases of H9N2 IAV have been reported in different mammalian species including humans, demonstrating their risk to public health and pandemic potential. Though most infections do not result in mammalian-to-mammalian transmission, different experiments have demonstrated that

transmission of H9N2 IAV in mammals is possible. Nevertheless, adaptation or reassortment with mammalian-adapted internal genes is required for an efficient transmission between mammals, particularly by the respiratory route, although airborne transmission of natural isolates has been observed. Therefore, continued surveillance and research to understand the evolution, pathogenicity, transmission, and antigenicity of H9N2 IAV is needed. Furthermore, understanding the molecular traits that facilitate transmission of H9N2 to and between mammals is crucial to evaluate their pandemic potential and to allow timely identification of viruses with increased potential for interspecies transmission.

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