Airborne Transmission of Avian Origin H9N2 Influenza A Viruses in Mammals

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Abstract: Influenza A viruses (IAV) are widespread viruses affecting avian and mammalian species worldwide. 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, which circulate endemically in poultry flocks in some regions of the world, have also been associated with cases of zoonotic infections. In this review, we discuss the mammalian transmission of H9N2 and the molecular factors that are thought relevant for this spillover, focusing on the HA segment. Additionally, we discuss factors that have been associated with the ability of these viruses to transmit through the respiratory route in mammalian species. The summarized information shows that minimal amino acid changes in the HA and/or the combination of H9N2 surface genes with internal genes of human influenza viruses are enough for the generation of H9N2 viruses with the ability to transmit via aerosol.

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

1. 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) [1]. The natural hosts of IAV are wild aquatic birds, particularly waterfowl and seabirds, in which most of the IAV subtypes have been described [2,3]. IAVs sporadically spill over between the wild bird reservoir and domestic poultry species and may lead to disease outbreaks. This has been the case for some H5Nx, H7Nx or H9N2 IAV viruses [4–9]. IAVs of avian origin are classified into high pathogenicity avian influenza viruses (HPAIVs) and low pathogenicity avian influenza viruses (LPAIVs) based on the pathotype in chickens and/or the presence of a polybasic amino acid cleavage site composed of Arginine (R) or Lysine (K) [10]. The HA’s polybasic R/K 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, mostly limiting infections to sites where such enzymes are abundant (e.g., respiratory and/or gastrointestinal systems) [10–12]. However, LPAIVs have been shown to replicate in the oviducts and kidneys of some infected birds, suggesting the presence of proteases in other tissues that may be able to process monobasic cleavage sites [13–15]. Recent H9N2 isolates from Asia and Middle East have increasingly shown the presence of a tri-basic HA cleavage site, which was associated with increased pathogenicity and transmission in chickens in comparison with viruses with mono-basic cleavage sites [16]. However, these viruses with the tri-basic cleavage site did not demonstrate a HPAI phenotype but rather an intermediate step towards a gain in pathogenicity, and were still considered LPAIV. Only viruses of the H5 or H7 subtypes have been associated with the HPAI pathotype.
1.1. H9N2 Avian Influenza Viruses

IAVs of the H9N2 subtype are widespread in several species of wild waterfowl, shorebirds, and poultry, such as chickens, turkeys, and quails, among others [17–22]. H9N2 IAVs are the most prevalent LPAIVs, and are enzootic in poultry in parts of Asia, the Middle East, and Africa (reviewed in [3]). H9N2 IAVs were first isolated from turkeys in Wisconsin, USA in 1966 [23], 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 and 1985 [24,25]. In 1988, the first evidence of H9N2 infection in poultry in Asia was reported after a respiratory outbreak in quails [26]. Currently, H9N2 IAVs are widespread in poultry species around the world with particularly high prevalence in Asia [7,27,28]. Recently, H9N2 IAVs have also been detected in Sub-Saharan Africa, historically considered a cold spot for animal IAV [29].

H9N2 IAV infections can be mild. However, significant economic losses are associated with H9N2 IAV 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 [13,15,30]. H9N2 IAV infections in poultry are also associated with co-infections or secondary respiratory pathogens, such as infectious bronchitis virus and Mycoplasma gallisepticum, which can lead to high mortality [13,31–37].

Phylogenetic analyses of the H9 HA have classified the H9 into two lineages: the American and Eurasian lineages, which are further divided into four sublineages (h9.1 to h9.4) [3]. Strains in the h9.1 sublineage are present mostly in wild birds in America, those from the h9.2 sublineage circulate in Korean poultry and wild birds in Eurasia, whereas those from the h9.3 sublineage (BJ94-like strains) are present in poultry in China. Viruses from the h9.4 sublineage (G1-like) are endemic in poultry in the Middle East, India, Egypt and Africa (extensively reviewed in [3]). Antigenic characterization of the HA of H9 subtype IAVs showed that the HA, in particular the globular head, is the immunodominant component, similar to what is described for other IAVs subtypes. Interestingly, the HA of the H9 subtype lacks the 130 lateral loop that forms the antigenic site A, which is an important antigenic region in other subtypes, such as H5 or H3 HAs [38,39]. Such a feature results in two antigenic sites that overlap, designated site I and site II [40]. 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 [41]. 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 [42,43].

Of great significance, H9N2 IAVs have contributed the internal gene segments to more virulent zoonotic strains such as H5N1/N6, H7N9, and H10N8/N3 that have been implicated in human infections and loss of life [44–47]. 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, coughing, fever, nasal discharge, sore throat, and headache [48–52]. Only one fatality has been associated with H9N2 IAV infection in humans to date [53]. However, such mild infections could be the prelude to the selection of more virulent strains with the capacity to transmit in humans more efficiently because these mild infections can go unnoticed, allowing the virus to acquire mutations that could increase transmission and replication in humans. 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.
1.2. 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 (PB2, PB1 and PA proteins) play a major role in the host range and adaptation of IAVs [54–59]. The HA is responsible for receptor-binding to the host cells and the fusion between the endosomal membranes and viral envelope [60,61]. 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 α2,3-linked sialic acids (α2,3SA) which is the most abundant receptor in avian respiratory and intestinal tracts, and those from human origin bind preferentially to α2,6-linked sialic acids (α2,6SA), which is the most abundant receptor in the human respiratory tract [62]. Pigs express both α2,3SA and α2,6SA receptors in their respiratory tract, in a similar distribution to that found in humans, and have been pointed out as a potential intermediary host of influenza viruses [63,64]. The α2,3SA versus α2,6SA preference is mediated by key amino acid residues located in the HA; specifically, positions 226 and 228 (H3-numbering is used throughout the text) are critical for receptor specificity in H3 and H9 viruses (Figure 1A) [65–67]. H9N2 IAVs endemic in poultry have variations at position 226, with most isolates carrying leucine (L) and others glutamine (Q) (Figure 1B) [68]. At position 228, glycine (G) is present in almost all H9 isolates detected to date. Indeed, an analysis of more than 2500 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 the year 2000 while newer H9N2 IAV isolates revealed high prevalence of L226 [69]. Viruses with the Q226/G228 combination present dual binding or α2,3SA avian-like preference. A single Q226L mutation produces a switch to α2,6SA human-like preference [70,71]. Consistent with these observations, most H9N2 IAVs identified in poultry farms and live bird markets, which contain L226, exhibit binding to human-like receptors [72,73]. More importantly, most H9N2 viruses isolated from mammalian species, including human isolates, show the L226/G228 combination (Figure 1B). Interestingly, when swine sequences are analyzed, there is an even distribution between isolates carrying Q226 or L226 (Figure 1B).

In addition, other amino acid signatures can modulate and/or enhance binding of H9 HAs to terminal α2,6SAs, such as the mutation from isoleucine (I) to threonine (T) at position 155 [74] or the presence of a valine (V) at position 190 [75]. The modulation of receptor binding preference by V190 is reminiscent of similar effects in HAs of the H1 subtype [57]. The presence of Q227 in combination with either aspartic acid (D) or glutamic acid (E) at position 190 favors binding α2,6SAs receptors. Other changes such as A160D/N, Q156R, T205A, V245I, V216L, D208E, T212I, R1721 and S175N also enhance the human-like receptor binding in vitro [76,77]. The terminal sialic acid linkage is not the only factor that affects the binding of IAV HA to the host cell; other features such as the sialic acid structure (e.g., N-acetyleneuraminic acid versus N-glycolyneuraminic acid) or length may also play a role (reviewed in [55]). In addition to specific molecular markers, the pH of fusion may also be an important feature for the mammalian transmission of H9N2 IAVs, since most H9N2 isolates show a low pH of fusion similar to early pdmH1N1 [78,79].
A. Position 226 binding preference

\[ \text{Q 226} \]

\[ \text{L 226} \]

α2,3-linked sialic acid preference

α2,6-linked sialic acid preference

B. Number of variants in Position 226 in natural isolates

| Avian species | Humans | Swine | Other Mammals |
|---------------|--------|-------|---------------|
| n= 6,203      | n= 41  | n= 50 | n= 14         |
| L (88.4%)     | L (90.2%) | L (46%) | L (92.9%)     |
| Q (11.2%)     | Q (7.3%)  | Q (52%) | Q (7.1%)      |
| Other (0.4%)  | M (2.5%)  | M (2%)  |               |

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

Figure 1. Impact of position 226 in the H9 of H9N2 IAVs. (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 pockets are shown in each case (amino acid residues shown in yellow and cyan) with bound sialic acid, shown in gray and red. Structure constructed using the iTASSER structure prediction tool [80]. (B) The different residues in position 226 of the H9 were analyzed from H9N2 IAVs isolated for avian species, humans, swine, and other mammals (canine, equine, and mink). Full H9 sequences were downloaded from the Global Initiative on Sharing All Influenza Data (GISAID). Sequence analyses were performed using Geneious Prime 2020.2.4 (https://www.geneious.com, accessed on 19 May 2021). (C) Summary of the replication, transmission by direct contact or airborne transmission in ferrets inoculated with H9N2 IAVs 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 [71].
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 adaptation. This has been the predominant host adaptation marker identified in human cases of H5N1 and H7N9 infections [81,82]. A higher frequency of PB2 K627 is observed in H9N2 viruses isolated from mammalian hosts in comparison to their 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 [83]. 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 mutation has 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 [84], which suggests 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 [85]. 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% [86]. 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 [87]. NA cleavage activity and the interaction of viral ribonucleoproteins (vRNPs) with host restriction factors may also play a role in the interspecies transmission of IAV (extensively reviewed in [55]). 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 | [88] |
|         | D253N  | Increase pathogenesis/Observed in airborne transmission | Mice and ferrets | [89–91] |
|         | R340K  | Increase transmission | Guinea pigs | [92] |
|         | K526R  | Increase polymerase complex activity and replication | Mice | [93] |
|         | Q591K  | Increase polymerase complex activity and replication | Mice | [89] |
|         | E627K  | Increase polymerase activity and viral replication in mammalian host | Human, mice, quail, guinea pigs and ferret | [74,81,82,84,93,94] |
|         | A588V  | Increase virulence, transmission, and virulence | Mice and guinea pigs | [85,92,93] |
|         | D701N  | Increase virulence and airborne transmission | Ferret | [74] |
|         | A707T  | Observed in airborne transmission | Ferret | [90] |
| PB1     | D120N  | Observed in airborne transmission | Ferret | [90] |
|         | D439E  | Observed in airborne transmission | Ferret | [90] |
|         | S261N  | Reduced polymerase complex activity/observed in airborne transmission | Ferret | [88,95] |
|         | I368V  | Increase airborne transmission (H5 context) | Ferret | [86] |
| PA      | K356R  | Increase polymerase activity and replication | Mice, chickens, quail, and ferrets | [87] |
|         | K26E   | Increase replication/Observed in airborne transmission | Mice, chickens, quail, and ferrets | [67,90,96] |
Table 1. Cont.

| Protein | Marker | Effect                              | Host Evaluated          | Reference |
|---------|--------|-------------------------------------|-------------------------|-----------|
| HA1     | D225G  | Increase transmission and replication | Pigs                    | [97]      |
|         | Q226L  | Increase α2,6SA binding             | Ferrets and quails      | [67,71]  |
| I155T   |        | Increase α2,6SA binding             | Ferrets                 | [74]      |
| A190V/ T190V |        | Increase replication                | Mice                    | [75]      |
| V104A   |        | Observed in airborne transmission   | Ferrets                 | [88]      |
| T189A   |        | Observed in airborne transmission   | Ferrets, quails, and pigs | [98,99] |
| S263N   |        | Observed in airborne transmission   | Ferrets                 | [90]      |
| S328C   |        | Observed in airborne transmission   | Ferrets                 | [90]      |
| HA2     | G192R  | Increase airborne transmission      | Ferrets, quails, and pigs | [98,99] |
| NA      | I28V   | Increase airborne transmission      | Ferrets, quails, and pigs | [99]     |
|         | A30T   | Observed in airborne transmission   | Ferrets                 | [88]      |
| M2      | E95K   | Observed in airborne transmission   | Ferrets                 | [90]      |
| NS1/NS2 | D2N    | Increase virulence and IFN-B antagonism/ observed in airborne transmission | Mice/ferrets            | [90,100] |

1.3. Natural Infection of H9N2 IAV in Mammals

According to the World Health Organization (WHO), 58 human cases of H9N2 IAV infection have been reported since December 2015, with 17 of those cases reported during 2021 from the western Pacific region (as of 17 September 2021) [101]. Additional cases have been reported in Bangladesh, India, Egypt, Oman, and Senegal [2,48,102–105]. 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 anti-H9N2 IAV antibodies in humans in Vietnam, Cambodia, Iran, Thailand, Pakistan, India, Egypt, and Hong Kong [106–112]. In most cases, the presence of anti-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% [52,113–115]. 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 surveys were performed in foxes and raccoon dogs (all species relevant in the fur industry), with 59% and 41% of serologically positive samples, respectively, and H9N2 IAV was successfully isolated in one study [116,117].

1.4. 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 [62,118]. 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 are commonly performed in ferrets, which present similar characteristics for IAV infection as humans in terms of lung pathology, clinical signs, pathogenesis, and immunity [119,120]. Wan and collaborators evaluated the replication and transmission capabilities of different H9N2 IAV isolated from avian species between 1988 and 2003, using the ferret model [71]. Ferrets were intranasally inoculated and the following day, a naïve ferret was placed in the same cage to allow direct contact transmission. At the same time, another ferret was placed in the adjacent cage but within the same isolator allowing for aerosol transmission without direct contact transmission (similar parameters were used in all transmission experiments described in this review). Using this system, it was shown that all the isolates replicated in ferrets. However, just two of those isolates transmitted through direct contact (Figure 1C). The two 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 (Figure 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 (Figure 1C) [71]. 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, all of which contained the L226 amino acid residue [74,121], highlighting the inherent risk for H9N2 IAV transmission in mammalian species and suggesting a strain-specific effect. Another amino acid residue in the HA, T155, was pointed out as a necessary factor for H9N2 viruses to bind to human-type receptors [74]. Similarly, H9N2 viruses isolated from humans, as well as swine- and avian-origin H9N2 isolates, were able to transmit via direct contact in ferrets, but these included viruses containing both Q226 and L226 [122]. A more recent study showed that different H9N2 isolates from poultry and humans containing mostly L226 exhibited binding to human receptors and replicated efficiently in human bronchial epithelial cells, albeit with delayed kinetics at a lower temperature (33 °C). All human-origin viruses transmitted via direct contact; however, only the two more recent human isolates from the Y280 lineage (containing L226) showed the ability to transmit via aerosol transmission in ferrets [123]. 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 an air–liquid interface [66]. 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 [66]. 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 the strain [124].

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 [67]. 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 an 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 α2,3 binding, demonstrating a residue-dependency in position 226 for receptor recognition [67]. 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 (varΔLQ) was still able to replicate and transmit via direct contact in quails, granted with lower efficiency at 2 days post-contact (dpc) 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 varΔLQ 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 days post-inoculation (dpi). Viruses carrying M226 or I226 were also readily detected, consistent with the detection of natural isolates containing such amino acid signatures (Figure 1B). The predominant amino acid detected in contact quails was L226, even in groups in which quail were inoculated with the mixtures lacking L226 (varΔLQ and var + Q), suggesting a strong advantage in transmission when L226 is present. These findings confirm that L226, Q226, and M226 confer a 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 a preference for α2,3-linked sialic acid and therefore, they are less likely to
infect humans. This finding facilitates risk assessment for the zoonotic potential of H9N2 viruses [67].

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) [125,126]. Further, ferrets infected with a reassortant virus of the H9N2 subtype with the internal genes of a seasonal human H3N2 strain (2WF10:6M98; Figure 2A) showed clinical signs similar to those observed with the wild type seasonal H3N2 strain. However, direct contact (but not airborne) transmission of the 2WF10:6M98 was observed in ferrets (Figure 2B) [71]. Interestingly, 10 serial passages of the 2WF10:6M98 in ferrets resulted in a virus (P10; Figure 2A) that transmitted efficiently through direct contact and by respiratory droplets (Figure 2C). The P10 virus also showed an intermediate plaque size between the seasonal H3N2 and the H9N2 viruses, suggesting an intermediary replication fitness phenotype [98]. 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 [98]. 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; Figure 2A) are able to transmit via direct contact without prior adaptation [88]. 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 [71,88]. 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:pH1N1 was observed, suggesting that compatibility and balance between the HA and NA is also important (Figure 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 [127–130]. 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 [88]. When pH1N1 internal genes are combined with the H9 HA (1P10:7pH1N1) or H9N2 HA/NA (2P10:6pH1N1) of the P10 ferret-adapted virus (Figure 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 model [88] (Figure 2C). Furthermore, these viruses efficiently transmitted between pigs by direct contact [99]. Confirming these findings, experimental infection of pigs with field isolates of H9N2 IAV resulted in nasal shedding and seroconversion after infections with four out of six different H9N2 IAV isolates tested [113]; however, transmission between pigs did not occur. In one study, direct-contact transmission of a human-origin H9N2 isolate was confirmed in pigs, although with low efficiency [122]. When the internal gene segments were replaced with those from a pandemic H1N1 strain, increased virus replication and transmission between pigs was observed [131]. Furthermore, when an H9N2 IAV 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 direct contact transmission [97].
A. Reassortant viruses tested for transmission in ferrets

B. Transmission of reassortant H9N2/N1 viruses in ferrets

C. Transmission of ferret-adapted H9N2/N1 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 (2WF10:6M98 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 [71,88,98].
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 [90]. Both H9N1 viruses identified had a mixed population of internal genes from pH1N1 or H9N2, both containing PB2, NP and NA 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 NS1/NS2 (D2N) in one of the viruses (Table 1), some of which have been previously reported to have an impact in replication and pathogenicity [89,91,96,100,132]. The second virus showed mutations in PB2 (A707T), HA1 (S328C), M2 (E95K), and two mutations in PB1 (D120N and D439E) (Table 1) [90]. 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 IAVs in mammalian species are far from understood and deserve further scrutiny. A summary of the different viruses discussed above, and the transmission phenotypes is shown in Figure 3.

Experimental infection/transmission with poultry adapted H9N2 IAV have been evaluated also in multiple alternative animal models such as guinea pigs, mice, and others [71,88,92,98,113,116]. 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 [133]. 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 adaptive changes are required to result in a mammalian transmissible H9N2 IAV. 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 [134]. 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 [92]. Reassortant viruses carrying unmodified H9N2 IAV glycoproteins and internal genes derived from pH1N1 were also able to transmit by either direct contact or via air in guinea pigs whereas a whole avian H9N2 virus did not [135], 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 [136]. 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 air between chickens and guinea pigs [94]. Direct-contact transmission of H9N2 IAV can also occur between minks, foxes, or raccoon dogs [116]. Contact minks developed clinical signs consistent with H9N2 IAV infection whereas seroconversion, but no clinical signs were observed in foxes and raccoon dogs [116]. In another study, the airborne transmission of H9N2 IAV was evaluated in minks, but no positive results were obtained [51]. 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 [137].
Figure 3. Summary of H9 subtype IAV reassortant viruses and their transmission ability in ferrets. Schematic representation of the different H9 subtype IAVs 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 [71,88,98,99].

| VIRUS | DESCRIPTION | TRANSMISSION - DIRECT CONTACT OR AIRBORNE | REFERENCE |
|-------|-------------|------------------------------------------|-----------|
| Avian-isolate 226L H9N2 | H9N2 viruses isolated from avian species carrying 226L in the HA | Direct contact (66%) | No Aerosol transmission | N/D | Direct contact depending on strain | 71 |
| Avian-isolate 226Q H9N2 | H9N2 viruses isolated from avian species carrying 226Q in the HA | No transmission | N/D | Direct contact depending on strain | 71 |
| 2WF10-08MS H9N2 | Reassortant H9N2 virus with internal genes from a seasonal H3N2 IAV | Direct contact | No Aerosol transmission | N/D | N/D | 71, 98 |
| 2WF10-5pdm H9N2 | Reassortant H9N2 virus with internal genes from a pandemic H1N1 IAV | Direct contact | Aerosol transmission | N/D | N/D | 88 |
| 1WF10-7pdm H9N1 | Reassortant H9N1 virus with internal genes and NA from a pandemic H1N1 IAV | Direct contact | No Aerosol transmission | N/D | N/D | 88 |
| P10 H9N2 | Reassortant H9N2 virus with internal genes from a seasonal H3N2 IAV adapted by 10 serial passages in ferrets | Direct contact | Aerosol transmission | N/D | N/D | 98 |
| 1P10:7pdm H9N1 | Reassortant H9N1 virus (HA from P10 virus) with internal genes and NA from a pandemic H1N1 IAV | Direct contact | Aerosol transmission | Direct contact | No Direct contact | 88, 99 |
| 2P10:5pdm H9N2 | Reassortant H9N2 virus (HA and NA from P10 virus) with internal genes from a pandemic H1N1 IAV | Direct contact | Aerosol transmission | Direct contact | Direct contact | 88, 99 |
| 2P10:5M98 H9N2 | Reassortant H9N2 virus (HA and NA from P10 virus) with internal genes from a seasonal H3N2 IAV | Direct contact | Aerosol transmission | N/D | N/D | 98 |

2. 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 mammal-to-mammal 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 experimental airborne transmission of natural isolates has been observed. Therefore, continued surveillance and research is needed to understand the evolution, pathogenicity, transmission, and antigenicity of H9N2 IAV. 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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References
1. Krammer, F.; Smith, G.J.D.; Fouchier, R.A.M.; Peiris, M.; Kedzierska, K.; Doherty, P.C.; Palese, P.; Shaw, M.L.; Treanor, J.; Webster, R.G.; et al. Influenza. Nat. Rev. Dis. Primers 2018, 4, 3. [CrossRef]
2. Peacock, T.H.P.; James, J.; Sealy, J.E.; Iqbal, M. A Global Perspective on H9N2 Avian Influenza Virus. Viruses 2019, 11, 620. [CrossRef] [PubMed]
3. Carnaccini, S.; Perez, D.R. H9 Influenza Viruses: An Emerging Challenge. Cold Spring Harb. Perspect. Med. 2020, 10, a038588. [CrossRef] [PubMed]
4. Berhane, Y.; Hisanaga, T.; Kehler, H.; Neufeld, J.; Manning, L.; Argue, C.; Handel, K.; Hooper-McGrevy, K.; Jonas, M.; Robinson, J.; et al. Highly pathogenic avian influenza virus A (H7N3) in domestic poultry, Saskatchewan, Canada, 2007. Emerg. Infect. Dis. 2009, 15, 1492–1495. [CrossRef] [PubMed]
5. Choi, Y.K.; Ozaki, H.; Webby, R.J.; Webster, R.G.; Peiris, J.S.; Poon, L.; Butt, C.; Leung, Y.H.; Guan, Y. Continuing evolution of H9N2 influenza viruses in Southeastern China. J. Virol. 2004, 78, 8609–8614. [CrossRef]
6. Morales, A.C., Jr.; Hilt, D.A.; Williams, S.M.; Pantin-Jackwood, M.J.; Suarez, D.L.; Spackman, E.; Stallknecht, D.E.; Jackwood, M.W. Biologic characterization of H4, H6, and H9 type low pathogenicity avian influenza viruses from wild birds in chickens and turkeys. Avian Dis. 2009, 53, 552–562. [CrossRef] [PubMed]
7. Banet-Noach, C.; Perk, S.; Simanov, L.; Grebenyuk, N.; Rozenblut, E.; Pokamunski, S.; Pirak, M.; Tentler, Y.; Panshin, A. H9N2 influenza viruses from Israeli poultry: A five-year outbreak. Avian Dis. 2007, 51, 290–296. [CrossRef] [PubMed]
8. Monne, I.; Hussein, H.A.; Fusaro, A.; Valastro, V.; Hamoud, M.M.; Khalefa, R.A.; Dardir, S.N.; Radwan, M.I.; Capua, I.; Cattoli, G. H9N2 influenza A virus circulates in H5N1 endemically infected poultry population in Egypt. Influenza Respir. Viruses 2013, 7, 240–243. [CrossRef] [PubMed]
9. Sonnberg, S.; Phommachanh, P.; Naipospos, T.S.; McKenzie, J.; Chanthavisouk, C.; Pathammavong, S.; Darnell, D.; Meeduangchan, P.; Rubrum, A.M.; Souriya, M.; et al. Multiple introductions of avian influenza viruses (H5N1), Laos, 2009–2010. Emerg. Infect. Dis. 2012, 18, 1139–1143. [CrossRef]
10. Alexander, D.J. Should we change the definition of avian influenza for eradication purposes? Avian Dis. 2003, 47, 976–981. [CrossRef]
11. World Health Organization. Avian Influenza: Assessing the Pandemic Threat; World Health Organization: Geneva, Switzerland, 2005.
12. USDA. Animal and Plant Health Inspection Service: Avian Influenza (AI). Available online: https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/ai (accessed on 23 August 2021).
13. Bonfante, F.; Mazzetto, E.; Zanardello, C.; Fortin, A.; Gobbo, F.; Maniero, S.; Bigolaro, M.; Davidson, I.; Haddas, R.; Cattoli, G.; et al. A G1-lineage H9N2 virus with oviduct tropism causes chronic pathological changes in the infundibulum and a long-lasting drop in egg production. Vet. Res. 2018, 49, 83. [CrossRef] [PubMed]

14. Siemons, R.D.; Swayne, D.E. Replication of a waterfowl-origin influenza virus in the kidney and intestine of chickens. Avian Dis. 1990, 34, 277–284. [CrossRef] [PubMed]

15. Sid, H.; Hartmann, S.; Winter, C.; Rautenschlein, S. Interaction of Influenza A Viruses with Oviduct Explants of Different Avian Species. Front. Microbiol. 2017, 8, 1338. [CrossRef] [PubMed]

16. Zhang, J.; Ma, K.; Li, B.; Chen, Y.; Qiu, Z.; Xing, J.; Huang, J.; Hu, C.; Huang, Y.; Li, H.; et al. A risk marker of trisomic hemagglutinin cleavage site in influenza (H9N2) virus. Commun. Biol. 2021, 4, 71. [CrossRef] [PubMed]

17. Swieton, E.; Tarasiuk, K.; Olszewska-Tomczyk, M.; Iwan, E.; Smietanka, K. A Turkey-origin H9N2 Avian Influenza Virus Shows Low Pathogenicity but Different Within-Host Diversity in Experimentally Infected Turkeys, Quail and Ducks. Viruses 2020, 12, 319. [CrossRef] [PubMed]

18. Xu, K.M.; Li, K.S.; Smith, G.J.; Li, J.W.; Zhang, J.X.; Webster, R.G.; Peiris, J.S.; Chen, H.; Guan, Y. Evolution and molecular epidemiology of H9N2 influenza A viruses from quail in southern China, 2000 to 2005. J. Virol. 2007, 81, 2635–2645. [CrossRef] [PubMed]

19. Hassan, M.M.; El Zowalaty, M.E.; Islam, A.; Khan, S.A.; Rahman, M.K.; Jarhult, J.D.; Hoque, M.A. Prevalence and Diversity of Avian Influenza Virus Hemagglutinin Sero-Subtypes in Poultry and Wild Birds in Bangladesh. Vet. Sci. 2020, 7, 73. [CrossRef] [PubMed]

20. Jackwood, M.W.; Stallknecht, D.E. Molecular epidemiologic studies on North American H9 avian influenza virus isolates from waterfowl and shorebirds. Avian Dis. 2007, 51, 448–450. [CrossRef]

21. Swieton, E.; Joziak, M.; Minta, Z.; Smietanka, K. Genetic characterization of H9N2 avian influenza viruses isolated from poultry in Poland during 2013/2014. Virus Genes 2018, 54, 67–76. [CrossRef]

22. Reid, S.M.; Banks, J.; Ceeraz, V.; Seekings, A.; Howard, W.A.; Puranik, A.; Collins, S.; Manvell, R.; Irvine, R.M.; Brown, I.H. The Detection of a Low Pathogenicity Avian Influenza Virus Subtype H9 Infection in a Turkey Breeder Flock in the United Kingdom. Avian Dis. 2016, 60, 126–131. [CrossRef]

23. Homme, P.J.; Easterday, B.C. Avian influenza virus infections. IV. Response of pheasants, ducks, and geese to influenza A-turkey-Wisconsin-1966 virus. Avian Dis. 1970, 14, 285–290. [CrossRef] [PubMed]

24. Markwell, D.D.; Shortridge, K.F. Possible waterborne transmission and maintenance of influenza viruses in domestic ducks. Avian Pathol. 2019, 48, 470–476. [CrossRef] [PubMed]

25. Shortridge, K.F. Pandemic influenza: A zoonosis? Semin Respir. Infect. 1992, 7, 11–25.

26. Perez, D.R.; Lim, W.; Seiler, J.P.; Yi, G.; Peiris, M.; Shortridge, K.F.; Webster, R.G. Role of quail in the interspecies transmission of H9 influenza A viruses: Molecular changes on HA that correspond to adaptation from ducks to chickens. J. Virol. 2003, 77, 3148–3156. [CrossRef] [PubMed]

27. Guan, Y.; Shortridge, K.F.; Krauss, S.; Chin, P.S.; Dyrtling, K.C.; Ellis, T.M.; Webster, R.G.; Peiris, M. H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. J. Virol. 2000, 74, 9372–9380. [CrossRef]

28. Cameron, K.R.; Gregory, V.; Banks, J.; Brown, I.H.; Alexander, D.J.; Hay, A.J.; Lin, Y.P. H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. Virology 2000, 278, 36–41. [CrossRef]

29. Usman, R.; Akhter, M.; Ahmed, S.; Rehman, S.U.; Masood, A.; Muhammad, T.; Sharif, M. Detection of a Low Pathogenicity Avian Influenza Virus Subtype H9 Infection in a Turkey Breeder Flock in the United Kingdom. Avian Pathol. 2020, 49, 496–506. [CrossRef] [PubMed]
37. Chu, J.; Zhang, Q.; Zuo, Z.; El-Ashram, S.; Guo, Y.; Zhao, P.; Huang, S.; He, C.; Khan, A. Co-infection of Chlamydia psittaci with H9N2, ORT and Aspergillus fumigatus contributes to severe pneumonia and high mortality in SPF chickens. *Sci. Rep.* 2017, 7, 13997. [CrossRef] [PubMed]

38. Wiley, D.C.; Wilson, I.A.; Skehel, J.J. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* 1981, 289, 373–378. [CrossRef] [PubMed]

39. Li, J.; Gu, M.; Liu, K.; Gao, R.; Sun, W.; Liu, D.; Jiang, K.; Zhong, L.; Wang, X.; Hu, J.; et al. Amino acid substitutions in antigenic region B of hemagglutinin play a critical role in the antigenic drift of subclade 2.3.4.4 highly pathogenic H5NX influenza viruses. *Transbound. Emerg. Dis.* 2020, 67, 263–275. [CrossRef]

40. Kaverin, N.V.; Rudneva, I.A.; Lipatov, A.S.; Krauss, S.; Webster, R.G. Structural differences among hemagglutinins of influenza A virus subtypes are reflected in their antigenic architecture: Analysis of H9 escape mutants. *J. Virol.* 2004, 78, 240–249. [CrossRef]

41. Peacock, T.; Reddy, K.; James, J.; Adamiak, B.; Barclay, W.; Shelton, H.; Iqbal, M. Antigenic mapping of an H9N2 avian influenza virus reveals two discrete antigenic sites and a novel mechanism of immune escape. *Sci. Rep.* 2016, 6, 18745. [CrossRef]

42. Wei, Y.; Xu, G.; Zhang, G.; Wen, C.; Anwar, F.; Wang, S.; Lemmon, G.; Wang, J.; Carter, R.; Wang, M.; et al. Antigenic evolution of H9N2 chicken influenza viruses isolated in China during 2009–2013 and selection of a candidate vaccine strain with broad cross-reactivity. *Vet. Microbiol.* 2016, 182, 1–7. [CrossRef]

43. Adel, A.; Arafa, A.; Hussein, H.A.; El-Sanousi, A.A. Molecular and antigenic traits on hemagglutinin gene of avian influenza H9N2 viruses: Evidence of a new escape mutant in Egypt adapted in quails. *Res. Vet. Sci.* 2017, 112, 132–140. [CrossRef] [PubMed]

44. Lam, T.T.; Wang, J.; Shen, Y.; Zhou, B.; Duan, L.; Cheung, C.L.; Ma, C.; Lycett, S.J.; Leung, C.Y.; Chen, X.; et al. The genesis and source of the H7N9 influenza viruses causing human infections in China. *Nature* 2013, 502, 241–244. [CrossRef] [PubMed]

45. Guan, Y.; Shortridge, K.F.; Krauss, S.; Webster, R.G. Molecular characterization of H9N2 influenza viruses: Were they the donors of the “internal” genes of H5N1 viruses in Hong Kong? *Proc. Natl. Acad. Sci. USA* 1999, 96, 9363–9367. [CrossRef]

46. Van Hoeven, N.; Pappas, C.; Belser, J.A.; Maines, T.R.; Zeng, H.; Garcia-Sastre, A.; Sasisekharan, R.; Katz, J.M.; Tumpey, T.M.; World Health Organization. *Influenza at the Human-Animal Interface. Summary and Assessment, 20 July to 3 October 2016*.

47. Wang, Y.; Niu, S.; Zhang, B.; Yang, C.; Zhou, Z. The whole genome analysis for the first human infection with H10N3 influenza virus reveals two discrete antigenic sites and a novel mechanism of immune escape. *Cell* 2018, 174, 263–277. [CrossRef] [PubMed]

48. Butt, K.M.; Smith, G.J.; Chen, H.; Zhang, L.J.; Leung, Y.H.; Xu, K.M.; Lim, W.; Webster, R.G.; Yuen, K.Y.; Peiris, J.S.; et al. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J. Clin. Microbiol.* 2005, 43, 5760–5767. [CrossRef] [PubMed]

49. Jallow, M.M.; Fall, A.; Barry, M.A.; Diop, B.; Sy, S.; Goudiaby, D.; Fall, M.; Enouf, V.; Niang, M.N.; Dia, N. Genetic characterization of the first detected human case of low pathogenic avian influenza A/H9N2 in sub-Saharan Africa, Senegal. *Emerg. Microbes Infect.* 2020, 9, 1092–1095. [CrossRef] [PubMed]

50. Sun, H.; Li, F.; Liu, Q.; Du, J.; Liu, L.; Sun, H.; Li, C.; Liu, J.; Zhang, X.; Yang, J.; et al. Mink is a highly susceptible host species to circulating human avian influenza viruses. *Emerg. Microbes Infect.* 2021, 10, 472–480. [CrossRef]

51. Cong, Y.L.; Wang, C.F.; Yan, C.M.; Peng, J.S.; Jiang, Z.L.; Liu, J.H. Swine infection with H9N2 influenza virus in China in 2004. *Virus Genes* 2008, 36, 461–469. [CrossRef]

52. World Health Organization. *Influenza at the Human-Animal Interface. Summary and Assessment, 20 July to 3 October 2016; World Health Organization: Geneva, Switzerland, 2016.*

53. Van Hoeven, N.; Pappas, C.; Belser, J.A.; Maines, T.R.; Zeng, H.; Garcia-Sastre, A.; Sasisekharan, R.; Katz, J.M.; Tumpey, T.M. Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. *Proc. Natl. Acad. Sci. USA* 2009, 106, 3366–3371. [CrossRef] [PubMed]

54. Long, J.S.; Mistry, B.; Haslam, S.M.; Barclay, W.S. Host and viral determinants of influenza A virus species specificity. *Nat. Rev. Microbiol.* 2019, 17, 67–81. [CrossRef]

55. Rogers, G.N.; Paulson, J.C. Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species origin. *Virology* 1983, 127, 361–373. [CrossRef]

56. Matrosovich, M.; Tuzikov, A.; Bovin, N.; Gambaryan, A.; Klimov, A.; Castrucci, M.R.; Donatelli, I.; Kawaoka, Y. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J. Virol.* 2000, 74, 8052–8051. [CrossRef] [PubMed]

57. Hayashi, T.; Wills, S.; Bussey, K.A.; Takimoto, T. Identification of Influenza A Virus PB2 Residues Involved in Enhanced Polymerase Activity and Virus Growth in Mammalian Cells at Low Temperatures. *J. Virol.* 2015, 89, 8042–8049. [CrossRef]

58. Mehle, A.; Doudna, J.A. Adaptive strategies of the influenza virus polymerase for replication in humans. *Proc. Natl. Acad. Sci. USA* 2009, 106, 21312–21316. [CrossRef]

59. Edinger, T.O.; Pohl, M.O.; Stertz, S. Entry of influenza A virus: Host factors and antiviral targets. *J. Gen. Virol.* 2014, 95, 263–277. [CrossRef]

60. Das, D.K.; Govindan, R.; Nikic-Spiegel, I.; Krammer, F.; Lemke, E.A.; Munro, J.B. Direct Visualization of the Conformational Dynamics of Single Influenza Hemagglutinin Trimmers. *Cell* 2018, 174, 926–937 e912. [CrossRef]
62. Gambaryan, A.S.; Matrosovich, M.N. What adaptive changes in hemagglutinin and neuraminidase are necessary for emergence of pandemic influenza virus from its avian precursor? *Biochemistry* 2015, 80, 872–880. [CrossRef]

63. Xiong, X.; McCauley, J.W.; Steinhauser, D.A. Receptor binding properties of the influenza virus hemagglutinin as a determinant of host range. *Curr. Top. Microbiol. Immunol.* 2014, 385, 63–91. [CrossRef]

64. Rajao, D.S.; Vincent, A.L.; Perez, D.R. Adaptation of Human Influenza Viruses to Swine. *Front. Vet. Sci.* 2018, 5, 347. [CrossRef] [PubMed]

65. Rogers, G.N.; Paulson, J.C.; Daniels, R.S.; Skehel, J.J.; Wilson, I.A.; Wiley, D.C. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature* 1983, 304, 76–78. [CrossRef] [PubMed]

66. Wan, H.; Perez, D.R. Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. *J. Virol.* 2007, 81, 5181–5191. [CrossRef] [PubMed]

67. Obadan, A.O.; Santos, J.; Ferreri, L.; Thompson, A.J.; Carnaccini, S.; Geiger, G.; Gonzalez Reiche, A.S.; Rajao, D.S.; Paulson, J.C.; Perez, D.R. Flexibility In Vitro of Amino Acid 226 in the Receptor-Binding Site of an H9 Subtype Influenza A Virus and Its Effect In Vivo on Virus Replication, Tropism, and Transmission. *J. Virol.* 2019, 93. [CrossRef]

68. Bi, Y.; Li, J.; Li, S.; Fu, G.; Jin, T.; Zhang, C.; Yang, Y.; Ma, Z.; Tian, W.; Li, J.; et al. Dominant subtype switch in avian influenza viruses during 2016–2019 in China. *Nat. Commun.* 2020, 11, 5909. [CrossRef]

69. Sun, X.; Belser, J.A.; Maines, T.R. Adaptation of H9N2 Influenza Viruses to Mammalian Hosts: A Review of Molecular Markers. *Viruses* 2020, 12, 541. [CrossRef]

70. Vines, A.; Wells, K.; Matrosovich, M.; Castrucci, M.R.; Ito, T.; Kawaoka, Y. The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. *J. Virol.* 1998, 72, 7626–7631. [CrossRef] [PubMed]

71. Wan, H.; Sorrell, E.M.; Song, H.; Hossain, M.J.; Ramirez-Nieto, G.; Monne, I.; Stevens, J.; Cattoli, G.; Capua, I.; Chen, L.M.; et al. Replication and transmission of H9N2 influenza viruses in ferrets: Evaluation of pandemic potential. *PLoS ONE* 2008, 3, e2923. [CrossRef]

72. Zhang, Y.; Li, X.; Bo, H.; Wei, H.; Dong, L.; Yang, L.; Dong, J.; Liu, J.; Shu, Y.; et al. Molecular characterization and receptor binding specificity of H9N2 avian influenza viruses based on poultry-related environmental surveillance in China between 2013 and 2016. *Virology* 2019, 529, 135–143. [CrossRef] [PubMed]

73. Matrosovich, M.N.; Krauss, S.; Webster, R.G. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. *Virology* 2001, 281, 156–162. [CrossRef]

74. Li, X.; Shi, J.; Guo, J.; Deng, G.; Zhang, Q.; Wang, J.; He, X.; Wang, K.; Chen, J.; Li, Y.; et al. Genetics, Receptor Binding Property, and Transmissibility in Mammals of Naturally Isolated H9N2 Avian Influenza Viruses. *PLoS Pathog.* 2014, 10, e1004508. [CrossRef] [PubMed]

75. Teng, Q.; Xu, D.; Shen, W.; Liu, Q.; Rong, G.; Li, X.; Yan, L.; Yang, J.; Chen, H.; Yu, H.; et al. A Single Mutation at Position 190 in Hemagglutinin Enhances Binding Affinity for Human Type Sialic Acid Receptor and Replication of H9N2 Avian Influenza Virus in Mice. *J. Virol.* 2016, 90, 9806–9825. [CrossRef] [PubMed]

76. Liu, Y.; Li, S.; Sun, H.; Pan, L.; Cui, X.; Zhu, X.; Feng, Y.; Li, M.; Yu, Y.; Wu, M.; et al. Variation and Molecular Basis for Enhancement of Receptor Binding of H9N2 Avian Influenza Viruses in China Isolates. *Front. Microbiol.* 2020, 11, 60124. [CrossRef]

77. Peacock, T.P.; Sealy, J.E.; Harvey, W.T.; Benton, D.J.; Reeve, R.; Iqbal, M. Genetic determinants of receptor-binding preference and zoonotic potential of H9N2 avian influenza viruses. *J. Virol.* 2020, 95, e01651-20. [CrossRef]

78. Peacock, T.P.; Benton, D.J.; Sadeyen, J.R.; Chang, P.; Sealy, J.E.; Bryant, J.E.; Martin, S.R.; Shelton, H.; McCauley, J.W.; Barclay, W.S.; et al. Variability in H9N2 haemagglutinin receptor-binding preference and the pH of fusion. *Emerg. Microbes Infect.* 2017, 6, e11. [CrossRef] [PubMed]

79. Russell, C.J. Hemagglutinin Stability and Its Impact on Influenza A Virus Infectivity, Pathogenicity, and Transmissibility in Avians, Mice, Swine, Seals, Ferrets, and Humans. *Viruses* 2021, 13, 746. [CrossRef]

80. Yang, J.; Yan, R.; Roy, A.; Xu, D.; Poisson, J.; Zhang, Y. The I-TASSER Suite: Protein structure and function prediction. *Nat. Methods* 2015, 12, 7–8. [CrossRef]

81. Wang, D.; Yang, L.; Gao, R.; Zhang, X.; Tan, Y.; Wu, A.; Zhu, W.; Zhou, J.; Zou, S.; Li, X.; et al. Genetic tuning of the novel avian influenza A(H7N9) virus during interspecies transmission, China, 2013. *Eurosurveillance* 2014, 19, 20836. [CrossRef]

82. Li, K.S.; Guan, Y.; Wang, J.; Smith, G.J.; Xu, K.M.; Duan, L.; Rahardjo, A.P.; Puthavathana, P.; Buranathai, C.; Nguyen, T.D.; et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 2004, 430, 209–213. [CrossRef]

83. Luk, G.S.; Leung, C.Y.; Sia, S.F.; Choy, K.T.; Zhou, J.; Ho, C.C.; Cheung, P.P.; Lee, E.F.; Wai, C.K.; Li, P.C.; et al. Transmission of H7N9 Influenza Viruses with a Polymorphism at PB2 Residue 627 in Chickens and Ferrets. *J. Virol.* 2015, 89, 9939–9951. [CrossRef]

84. Hossain, M.J.; Hickman, D.; Perez, D.R. Evidence of expanded host range and mammalian-associated genetic changes in a duck H9N2 influenza virus following adaptation in quails and chickens. *PLoS ONE* 2008, 3, e3170. [CrossRef]

85. Xiao, C.; Ma, W.; Sun, N.; Huang, L.; Li, Y.; Zeng, Z.; Wen, Y.; Zhang, Z.; Li, H.; Li, Q.; et al. PB2-588 V promotes the mammalian adaptation of H10N8, H7N9 and H9N2 avian influenza viruses. *Sci. Rep.* 2016, 6, 19474. [CrossRef] [PubMed]

86. Herfst, S.; Schrauwen, E.J.; Linstert, M.; Chutinimitkul, S.; de Wit, E.; Munster, V.J.; Sorrell, E.M.; Bestebroer, T.M.; Burke, D.F.; Smith, D.J.; et al. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 2012, 336, 1534–1541. [CrossRef]
110. Quan, C.; Wang, Q.; Zhang, J.; Zhao, M.; Dai, Q.; Huang, T.; Zhang, Z.; Mao, S.; Nie, Y.; Liu, J.; et al. Avian Influenza A Viruses among Occupationally Exposed Populations, China, 2014–2016. *Emerg. Infect. Dis.* 2019, 25, 2215–2225. [CrossRef] [PubMed]

111. Ma, C.; Cui, S.; Sun, Y.; Zhao, J.; Zhang, D.; Zhang, L.; Zhang, Y.; Pan, Y.; Wu, S.; Duan, W.; et al. Avian influenza A (H9N2) virus infections among poultry workers, swine workers, and the general population in Beijing, China, 2013–2016: A serological cohort study. *Influenza Respir. Viruses* 2019, 13, 415–425. [CrossRef]

112. Heidari, A.; Mancin, M.; Nili, H.; Pourghanbari, G.H.; Lankarani, K.B.; Leardini, S.; Cattoli, G.; Monne, I.; Piccirillo, A. Serological evidence of H9N2 avian influenza virus exposure among poultry workers from Fars province of Iran. *Virol. J.* 2016, 13, 16. [CrossRef]

113. Wang, J.; Wu, M.; Hong, W.; Fan, X.; Chen, R.; Zheng, Z.; Zeng, Y.; Huang, R.; Zhang, Y.; Lam, T.T.; et al. Infectivity and Transmissibility of Avian H9N2 Influenza Viruses in Pigs. *J. Virol.* 2016, 90, 3506–3514. [CrossRef] [PubMed]

114. Yuan, Z.; Zhu, W.; Chen, Y.; Zhou, P.; Cao, Z.; Nie, J.; Zhang, C.; Ke, C.; Qi, W.; Su, S.; et al. Serological surveillance of H5 and H9 avian influenza A viral infections among pigs in Southern China. *Microb. Pathol.* 2013, 64, 39–42. [CrossRef] [PubMed]

115. Ninomiya, A.; Takada, A.; Okazaki, K.; Shortridge, K.F.; Kida, H. Seroepidemiological evidence of avian H4, H5, and H9 influenza A virus transmission to pigs in southeastern China. *Vet. Microbiol.* 2002, 88, 107–114. [CrossRef]

116. Bengtson, V.; Zhou, H.; Tang, J.; Sun, X.; Tappah, A.N.; Chen, X.; Guan, Y.; Liu, H.; Wang, Y.; et al. Intraweekly transmission of H9N2 influenza viruses in poultry, China. *Virology* 2010, 404, 11–21. [CrossRef]

117. Yuan, Z.; Zhu, W.; Chen, Y.; Zhou, P.; Cao, Z.; Nie, J.; Zhang, C.; Ke, C.; Qi, W.; Su, S.; et al. Serological surveillance of H5 and H9 avian influenza A viral infections among pigs in Southern China. *Microb. Pathol.* 2013, 64, 39–42. [CrossRef] [PubMed]

118. Perez, D.R.; Sorrell, E.; Angel, M.; Ye, J.; Hickman, D.; Pena, L.; Ramirez-Nieto, G.; Kimble, B.; Araya, Y. Fitness of Pandemic H1N1 and Seasonal Influenza A viruses during Co-infection: Evidence of competitive advantage of pandemic H1N1 influenza versus seasonal influenza. *PLoS Curr.* 2009, J, RRN1011. [CrossRef] [PubMed]

119. Perez, D.R.; Sorrell, E.; Angel, M.; Ye, J.; Hickman, D.; Pena, L.; Ramirez-Nieto, G.; Kimble, B.; Araya, Y. Fitness of Pandemic H1N1 and Seasonal Influenza A viruses during Co-infection: Evidence of competitive advantage of pandemic H1N1 influenza versus seasonal influenza. *PLoS Curr.* 2009, J, RRN1011. [CrossRef] [PubMed]

120. Belser, J.A.; Sun, X.; Brock, N.; Pappas, C.; Pulit-Penaloza, J.A.; Zeng, H.; Jang, Y.; Jones, J.; Carney, P.J.; Chang, J.; et al. Genetically and Antigenically Divergent Influenza A (H9N2) Viruses Exhibit Differential Replication and Transmission Phenotypes in Mammalian Models. *J. Virol.* 2020, 94, e00451-20. [CrossRef] [PubMed]

121. Zhang, X.; Li, Y.; Jin, S.; Wang, T.; Sun, W.; Zhang, Y.; Li, F.; Zhao, M.; Sun, L.; Hu, X.; et al. H9N2 influenza virus spillover into swine. *Eur. J. Virol.* 2020, 9, 60–66. [CrossRef]

122. SJCEIRS H9 Working Group. Assessing the fitness of distinct clades of influenza A (H9N2) viruses. *Emerg. Microbes Infect.* 2013, 2, e75. [CrossRef] [PubMed]

123. Belser, J.A.; Sun, X.; Brock, N.; Pappas, C.; Pulit-Penaloza, J.A.; Zeng, H.; Jang, Y.; Jones, J.; Carney, P.J.; Chang, J.; et al. Genetically and Antigenically Divergent Influenza A (H9N2) Viruses Exhibit Differential Replication and Transmission Phenotypes in Mammalian Models. *J. Virol.* 2020, 94, e00451-20. [CrossRef] [PubMed]

124. Chan, R.W.Y.; Chan, L.L.Y.; Mok, C.K.P.; Lai, J.; Tao, K.P.; Obadan, A.; Chan, M.C.W.; Perez, D.R.; Peiris, J.S.M.; Nicholls, J.M. Replication of H9 influenza viruses in the human ex vivo respiratory tract, and the influence of neuraminidase on virus release. *J. Virol.* 2020, 94, 6345–6354. [CrossRef]

125. Perez, D.R.; Sorrell, E.; Angel, M.; Ye, J.; Hickman, D.; Pena, L.; Ramirez-Nieto, G.; Kimble, B.; Araya, Y. Fitness of Pandemic H1N1 and Seasonal Influenza A viruses during Co-infection: Evidence of competitive advantage of pandemic H1N1 influenza versus seasonal influenza. *PLoS Curr.* 2009, J, RRN1011. [CrossRef] [PubMed]

126. Munster, V.J.; de Wit, E.; van den Brand, J.M.; Herfst, S.; Schrauwen, E.J.; Bestebroer, T.M.; van de Vijver, D.; Boucher, C.A.; Koopmans, M.; Rimmelzwaan, G.F.; et al. Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. *Science* 2009, 325, 481–483. [CrossRef]

127. Zell, R.; Groth, M.; Krumholz, A.; Lange, J.; Philippas, A.; Dürrwald, R. Novel reassortant swine H3N2 influenza A viruses in Germany. *Sci. Rep.* 2020, 10, 14296. [CrossRef] [PubMed]

128. Ryt-Hansen, P.; Krog, J.S.; Østergaard Breum, S.; Hjulsager, C.K.; Pedersen, A.G.; Trebbien, R.; Larsen, L.E. Co-circulation of multiple influenza A variants in swine inhabiting farm buildings. *bioRxiv* 2020, bioRxiv:2020.07.07.202866. [CrossRef] [PubMed]

129. Everett, H.E.; Nash, B.; Londt, B.Z.; Kelly, M.D.; Coward, V.; Nunez, A.; van Diemen, P.M.; Brown, I.H.; Brookes, S.M. Interspecies Transmission of Reassortant Swine Influenza A Virus Containing Genes from Swine Influenza A(H1N1)pdm09 and A(H1N2) Viruses. *Emerg. Infect. Dis.* 2020, 26, 273–281. [CrossRef]

130. Powell, J.D.; Abente, E.J.; Chang, J.; Souza, C.K.; Rajao, D.S.; Anderson, T.K.; Zeller, M.A.; Gauger, P.C.; Lewis, N.S.; Vincent, A.L. Characterization of contemporary 2010.1 H3N2 swine influenza A viruses circulating in United States pigs. *Virology* 2021, 553, 94–101. [CrossRef]

131. Qiao, C.; Liu, Q.; Bawa, B.; Shen, H.; Qi, W.; Chen, Y.; Mok, C.K.P.; Garcia-Sastre, A.; Richt, J.A.; Ma, W. Pathogenicity and transmissibility of reassortant H9 influenza viruses with genes from pandemic H1N1 virus. *J. Gen. Virol.* 2012, 93, 2367–2345. [CrossRef]

132. Ping, J.; Keleta, L.; Forbes, N.E.; Dankar, S.; Stecho, W.; Tyler, S.; Zhou, Y.; Babiuk, L.; Weingartl, H.; Halpin, R.A.; et al. Genomic and protein structural maps of adaptive evolution of human influenza A virus to increased virulence in the mouse. *PLoS ONE* 2011, 6, e21740. [CrossRef]

133. Lv, J.; Wei, B.; Yang, Y.; Yao, M.; Cai, Y.; Gao, Y.; Xia, X.; Zhao, X.; Liu, Z.; Li, X.; et al. Experimental transmission in guinea pigs of H9N2 avian influenza viruses from indoor air of chicken houses. *Virus Res.* 2012, 170, 102–108. [CrossRef]
134. Sang, X.; Wang, A.; Ding, J.; Kong, H.; Gao, X.; Li, L.; Chai, T.; Li, Y.; Zhang, K.; Wang, C.; et al. Adaptation of H9N2 AIV in guinea pigs enables efficient transmission by direct contact and inefficient transmission by respiratory droplets. Sci. Rep. 2015, 5, 15928. [CrossRef]

135. He, L.; Wu, Q.; Jiang, K.; Duan, Z.; Liu, J.; Xu, H.; Cui, Z.; Gu, M.; Wang, X.; Liu, X.; et al. Differences in transmissibility and pathogenicity of reassortants between H9N2 and 2009 pandemic H1N1 influenza A viruses from humans and swine. Arch. Virol 2014, 159, 1743–1754. [CrossRef] [PubMed]

136. Hao, M.; Han, S.; Meng, D.; Li, R.; Lin, J.; Wang, M.; Zhou, T.; Chai, T. The PA Subunit of the Influenza Virus Polymerase Complex Affects Replication and Airborne Transmission of the H9N2 Subtype Avian Influenza Virus. Viruses 2019, 11, 40. [CrossRef] [PubMed]

137. Zhang, K.; Zhang, Z.; Yu, Z.; Li, L.; Cheng, K.; Wang, T.; Huang, G.; Yang, S.; Zhao, Y.; Feng, N.; et al. Domestic cats and dogs are susceptible to H9N2 avian influenza virus. Virus Res. 2013, 175, 52–57. [CrossRef] [PubMed]