REVIEW

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Future VIROLOGY

Avian influenza A viruses: from zoonosis to pandemic

Mathilde Richard[1], Miranda de Graaf[1] & Sander Herfst*[,1]

ABSTRACT: Zoonotic influenza A viruses originating from the animal reservoir pose a threat for humans, as they have the ability to trigger pandemics upon adaptation to and invasion of an immunologically naive population. Of particular concern are the H5N1 viruses that continue to circulate in poultry in numerous countries in Europe, Asia and Africa, and the recently emerged H7N9 viruses in China, due to their relatively high number of human fatalities and pandemic potential. To start a pandemic, zoonotic influenza A viruses should not only acquire the ability to attach to, enter and replicate in the critical target cells in the respiratory tract of the new host, but also efficiently spread between humans by aerosol or respiratory droplet transmission. Here, we discuss the latest advances on the genetic and phenotypic determinants required for avian influenza A viruses to adapt to and transmit between mammals.

KEYWORDS
• airborne transmission
• avian influenza A virus
• HA stability • mammalian adaptation • pandemic
• receptor specificity
• zoonosis

Influenza A virus zoonoses are caused by strains originating from the animal reservoir, successfully crossing the species barrier and infecting humans. Influenza A pandemics are the result of zoonotic viruses gaining the capacity to adapt to humans (by mutation or reassortment) and subsequently circulate in the human population.

A nonhuman influenza A virus needs to cross at least two main barriers to become a pandemic virus. The first barrier is the animal-to-human transmission barrier that stands at the interface between the natural reservoir of influenza A viruses and humans. The natural reservoir of influenza A viruses is wild aquatic birds, in which the largest diversity of influenza A virus subtypes – 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes – has been detected [1]. However, the recent identification of two new HA and NA subtypes in bats (H17N10 and H18N11) raises the possibility that other animal species besides wild aquatic birds constitute the influenza A virus reservoir [2]. Cross-species transmission of influenza A viruses directly from wild aquatic bird to humans is rare, because of limited human exposure to wild birds. However, viruses that circulate in wild birds can infect intermediate species to which human exposure is more frequent, such as swine and poultry, before subsequent transmission to humans. Initially, swine were considered a necessary intermediate host to facilitate reassortment between avian, swine, and human influenza viruses and to allow the initial mammalian adaptation. However, the first documented cases of direct transmission of H5N1 viruses from poultry to humans in 1997 in Hong Kong revised the paradigm that only swine can serve as bridge species for the transmission of zoonotic viruses to humans. Moreover, this event highlighted poultry as an important intermediate host for the transmission of influenza A viruses from wild aquatic birds to humans [3].

Crossing the animal-to-human barrier is a required first step in the genesis of pandemic viruses. However, influenza A viruses originating from swine and poultry have successfully crossed the

[1]Department of Viroscience, Postgraduate School Molecular Medicine, Erasmus Medical Center, Rotterdam, The Netherlands
*Author for correspondence: Tel.: +31 10 7043161; Fax: +31 10 7044760; s.herfst@erasmusmc.nl

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species barrier and infected humans without becoming established in the human population. Upon crossing the animal-to-human transmission barrier, a zoonotic virus must subsequently overcome the human-to-human transmission barrier to become pandemic. To overcome this second barrier, the virus needs to adapt to its new host by mutation or reassortment. In the last century, only four influenza A viruses of subtypes H1, H2 and H3 gained the ability to be transmitted between humans. These viruses resulted in the Spanish H1N1 pandemic in 1918, the Asian H2N2 pandemic in 1957, the Hong Kong H3N2 pandemic in 1968 and the H1N1 pandemic in 2009 [4–6]. Whether only influenza A viruses of subtypes H1, H2 and H3 are able to transmit between humans is a highly debated topic in influenza research, but theoretically any subtype of influenza A virus could initiate a pandemic.

Currently, the influenza viruses of the H5 and H7 subtype are of great concern. The first documented transmission of avian H5N1 virus to humans was in 1997 in Hong Kong [3]. Since 2003, H5N1 viruses have caused severe outbreaks in poultry throughout Asia, Europe and Africa, and occasionally the virus is transmitted to humans, mostly upon contact with infected poultry. As of 24 January 2014, 650 laboratory-confirmed human cases of H5N1 infection have been reported to the WHO, of which 386 were fatal [7]. Although suspected cases of human-to-human transmission of H5N1 viruses have been described, no sustained transmission between humans has been reported.

In spring 2013, a novel avian-origin H7N9 virus was transmitted to humans in several provinces of China, resulting in 375 laboratory-confirmed human infections, including 115 fatalities (as of 28 February 2014) [8]. The detection of several human infections with H7N9 viruses in December 2013 raised concerns about the upcoming influenza season. This virus, which emerged upon multiple reassortments between avian viruses of diverse origins, has probably been circulating undetected in poultry for some time, as it causes subclinical infections in domestic birds [9]. Surprisingly, some H7N9 viruses already harbor mammalian adaption markers that have been associated with increased replication and transmission in mammals. However, apart from one case for which human-to-human transmission cannot be ruled out [10], no sustained transmission between humans has been reported. The possibility that H5N1 or H7N9 viruses further adapt to humans and subsequently acquire human-to-human transmissibility, either via reassortment with contemporary human viruses or mutation, underlines the pandemic potential of such zoonotic viruses.

Adaptation of influenza viruses from the animal reservoir to replicate and transmit in humans upon reassortment with human viruses, which led to the emergence of at least three of the last four pandemic viruses, has already been described extensively (reviewed in [11,12]). However, recent work on H5N1 viruses has demonstrated that avian viruses have the potential to infect and adapt to mammals without the need of reassortment with human viruses [13]. In this perspective, we focused this review on the current state of knowledge on the genetic and phenotypic changes that avian influenza viruses must undergo to facilitate crossing the species barrier and to confer human-to-human transmissibility.

Crossing the avian-to-human transmission barrier

Avian influenza viruses of subtypes H5, H6, H7, H9 and H10 have shown their ability to infect humans **(Table 1)**. The severity of the disease caused by these zoonotic viruses is diverse and is manifested by symptoms ranging from conjunctivitis to influenza-like symptoms, pneumonia associated with acute respiratory distress syndrome and encephalitis. Apart from H5N1 and H7N9 viruses, which stand out by the amount of fatalities they have caused, the H7N7 and H7N3 outbreaks, respectively in 2003 in The Netherlands and 2004 in Canada, are also distinguished by a large number of human cases. Transmission of avian influenza viruses of subtypes H5, H7 or H9 to humans is the result of frequent outbreaks of these viruses in poultry or their establishment in terrestrial birds, such as H9N2 viruses in Asia, H7N2 viruses in the USA or H5N1 viruses in a number of Asian and African countries [14]. By contrast, although poultry outbreaks of H10 influenza viruses are uncommon, human infections with influenza viruses of the H10 subtype have been reported [15]. Although some avian influenza virus subtypes have the ability to infect humans without prior adaptation, some markers of host adaptation have been defined. Although the exact requirements for avian influenza viruses

to cross the species barrier and infect humans remain largely unknown, it has become clear that the receptor-binding specificity, as it relates to host-specific receptor distribution throughout the respiratory tract and adaptation of the viral polymerase complex to allow efficient replication in humans, is among the key determinants of host adaptation.

• Receptor binding specificity

The influenza replication cycle is initiated when the HA surface glycoprotein binds to sialylated glycan receptors on susceptible host cells (reviewed in [32]). Hirst *et al.* were the first to demonstrate the ability of influenza viruses to agglutinate and elute from red blood cells [33]. Treatment of these cells with *Vibrio cholerae* neuraminidase (VCNA) revealed that this ability to agglutinate red blood cells was dependent on sialic acid (SA) [34]. Using red blood cells or other cells that only expressed a certain type of SA, it was discovered that the HA of human and animal influenza viruses display differences in the receptor specificity [35]. Human influenza viruses (H1, H2 and H3) attached to SAs that are linked to galactose in an α2,6 linkage (α2,6-SA), which are predominant on epithelial cells in the human upper respiratory tract (URT). By contrast, avian influenza viruses have a preference for SAs that are linked to the galactose in an α2,3 linkage (α2,3-SA), which are abundantly present on epithelial cells in the intestinal tract of birds and in the lower respiratory tract (LRT) of humans [36–39]. It has been previously assumed that avian viruses were not capable of efficiently infecting humans without

Table 1. Zoonotic avian influenza A viruses.

Subtype	Year (country)	Confirmed cases (fatalities), n	Illness	Identified human adaptation markers (segment)	Ref.
H5N1	1997/2003–present (Asia, Europe, Africa)	660 (391)	ILI, pneumonia, encephalitis	N224K (HA)[†] N158D (HA)[‡] T160A (HA)[‡] E627K[§] (PB2)	[7,16]
H6N1	2013 (Taiwan)	1 (0)	ILI	P186L (HA)[†]	[17]
H7N2	2003 (USA)	1 (0)	ILI[¶]	ND	[18]
H7N2	2007 (UK)	4 (0)	Conjunctivitis, ILI	ND	[19]
H7N3	2004 (Canada)	2 (0)	Conjunctivitis, ILI	ND	[20]
H7N3	2006 (UK)	1 (0)	Conjunctivitis	ND	[21]
H7N3	2012 (Mexico)	2 (0)	Conjunctivitis	ND	[22]
H7N7	1996 (UK)	1 (0)	Conjunctivitis	ND	[23]
H7N7	2003 (The Netherlands)	89 (1)	Conjunctivitis, ILI, pneumonia	E627K (PB2)[§]	[24]
H7N7	2013 (Italy)	3 (0)	Conjunctivitis	ND	[25]
H7N9	2013 (China, Taiwan, Hong Kong)	137 (45)	ILI	Q226L (HA)[†] E627K (PB2)[§]	[8]
H9N2	1999 (Hong Kong)	2 (0)	ILI	ND	[26]
	2003 (Hong Kong)	1 (0)	ILI	Q226L (HA)[†] G228S (HA)[†] T212A (HA)[‡]	[27]
	2008–2009 (Hong Kong)	2 (0)	ILI[¶]	ND	[28]
	2013 (Hong Kong)	2 (0)	ILI	ND	[29]
H10N7	2004 (Egypt)	2 (0)	ILI	ND	[30]
H10N7	2010 (Australia)	2 (0)	Conjunctivitis	ND	[15]
H10N8	2013 (China)	3 (2)	Pneumonia[¶]	ND	[31]

The first human cases of infection with H5N1 viruses were reported in 1997. Six years later (in 2003), H5N1 viruses re-emerged in humans.
[†]Substitution that confers an increase in binding to human-type α2,6-SA receptors on host cells.
[‡]Loss of a putative *N*-linked glycosylation site in the globular head of HA.
[§]Substitution that confers an increase in replication in mammalian cells.
[¶]Immunocompromised patients.
ILI: Influenza-like illness; ND: Not determined.

prior adaptation because of the scarcity of α2.3-SA along the URT of humans. As a result, preferred binding to α2.6-SA may facilitate cross-species transmission of zoonotic viruses. However, this distinction between human and avian receptor specificity is not absolute. Some avian isolates possess an α2.6-SA specificity or at least a dual specificity for both α2.6-SA and α2.3-SA, like H9N2 influenza viruses circulating in Asia in terrestrial poultry [40], H7 influenza viruses from the North American lineage [41], recent H5N1 influenza virus sublineages in Egypt [42] or the recently emerged H7N9 viruses [43,44]. On the other hand, most zoonotic influenza viruses have retained an α2.3-SA specificity. The fact that domesticated birds, such as chicken or quail, possess a mix α2.3-SA and α2.6-SA at the surface of both respiratory and intestinal epithelial cells [45], emphasizes their potential role as intermediate hosts for zoonotic influenza viruses to infect humans, and adaptation to poultry might play a role in evolution of receptor specificity of avian influenza viruses towards human specificity [46]. Moreover, early after their introduction in humans, H1, H2 and H3 influenza viruses not only gained binding to α2.6-SA but also substantially decreased their binding to α2.3-SA [47], suggesting that there might be, on top of a selection for increased affinity for α2.6-SA, a selection against α2.3-SA binding, which would be beneficial to overcome the α2.3-SA rich the mucus barrier in the human UTR.

Sialylated glycans exist in a large variety of different structures; branched or unbranched and with and without modifications, such as fucosylation or sulfation. Avian and mammalian influenza viruses, besides their main characteristic of binding respectively to α2.3-SA and α2.6-SA, bind to a large structurally diverse set of sialylated glycans [48,49]. On top of the nature of the linkage between SA and galactose, the overall structural conformation, influenced by the length of the sugar, may also play a role in receptor specificity [50]. It remains to be elucidated how structurally diverse the sialylated glycans in avian and human hosts are, how this diversity gives rise to host specificity and how this may influence viral tropism.

The HA protein binds to SA via the receptor-binding site (RBS), which forms a groove at the top of the protein. As a result, amino acid substitutions within and close to the RBS may modulate the receptor specificity and as a consequence influence virus cell and tissue tropism, and host range. To further elucidate the genetic and phenotypic requirements for avian influenza viruses to attach to respiratory tract cells in mammalian hosts, binding studies with mutated H5 and H9 viruses have been performed. Several amino acid substitutions, in or near the RBS, such as N186K, Q196R, N224K, Q226L, S227N or G228S (numbering throughout the manuscript is based on H3 HA) tune, alone or in combination, the receptor binding of avian viruses to increase binding to α2.6-SA and human URT tissues [37,40,46,51–54].

• Viral polymerase complex

In addition to the HA protein, the avian influenza virus RNA polymerase complex also requires adaptation for efficient replication in humans. Avian influenza viruses normally replicate at a temperature of approximately 41°C, that is, the temperature in the avian intestinal tract. However, key amino acid substitutions (E627K and D701N) increase *in vitro* and *in vivo* the viral replication at approximately 33°C, the accepted temperature for efficient replication in the mammalian URT [55,56]. The E627K substitution restores a defect in binding of E627-PB2 and NP leading to an impaired association of the newly formed viral ribonucleoproteins (vRNPs) during replication of avian viruses in mammalian cells [57]. D701N, located in the importin α-binding domain at the C-terminal of PB2, as well as the N319K substitution in NP, switch from importin-α3 to importin-α7 dependency for the nuclear transport of vRNP, resulting in increased transcription and replication in mammalian cells [58].

The mammalian adaptation marker D701N in PB2, as well as other human adaptation markers, emerged rapidly in poultry during a massive avian influenza H7N7 virus outbreak in The Netherlands [59]. Similar adaptation markers, such as E627K in PB2, emerged rapidly in the fatal case during this same H7N7 outbreak [60], and are also present in avian-origin H7N9 viruses isolated from humans and in H5N1 viruses belonging to the clade 2.2 [16]. Several other specific adaptive mutations located in other genes of the viral polymerase complex increase replication of zoonotic influenza viruses in mammalian hosts and are often associated with domains involved in functional protein interactions essential for viral replication (reviewed in [61]).

Crossing the human-to-human transmission barrier

• Airborne transmission of influenza viruses

Upon crossing the species barrier to humans, avian influenza viruses require additional genetic and phenotypic changes to become transmissible between humans. Although it is impossible to predict the subtype, origin and pathogenicity of the influenza virus that will cause the next pandemic, one thing is certain: the pandemic virus will be transmissible via the airborne route (via aerosols or respiratory droplets), as this is a property shared by all pandemic and epidemic influenza viruses. Although human-to-human transmission of influenza viruses can also occur through direct or indirect contact (via fomites) and despite the divergence in opinions on the importance of each mode of transmission (reviewed in [62,63]), the importance of the airborne route for influenza virus transmission is well supported experimentally [62,64], and efficient aerosolization of viruses is crucial for viruses with high transmission efficiency and pandemic potential.

There is no exact definition of particle size cutoff at which transmission changes from aerosols to (large) respiratory droplets. However, it is generally accepted that respiratory droplets, >5 μm, do not remain suspended in air and travel less than 1 m before settling on the mucosa of close contacts or environmental surfaces. By contrast, small aerosols, <5 μm, have a slow settling velocity, thus remain suspended in the air longer than the larger respiratory droplets and can travel further. The majority of aerosol particles expelled by breathing, coughing or sneezing of humans were measured at <1 μm [65,66].

Since the key factors that determine airborne transmission of influenza viruses among mammals have remained largely unknown, research on the routes of influenza virus transmission between animals and the determinants of airborne transmission has a high priority. Ferret and guinea pig models are frequently used to evaluate airborne transmission of influenza viruses between mammals. Ferrets have been used in influenza research since 1933 because they are susceptible to infection with human and avian influenza viruses [67]. After infection with influenza virus, ferrets develop respiratory disease and lung pathology similar to that observed in humans. Ferrets can also transmit human influenza viruses to other ferrets with or without direct contact. Furthermore, the distribution of α2,3-SA and α2,6-SA throughout the ferret and human respiratory tract is similar [68–70]. Although the experimental setup may vary between the different laboratories, the basic setup consists of an inoculated donor animal in a cage that is adjacent to a cage housing a naive recipient ferret. The two cages are separated from each other to prevent direct contact or fomite transmission, but to allow airflow from the donor to the recipient animal [71,72]. Using these ferret end guinea pig transmission models, pandemic and epidemic viruses isolated from humans are generally transmitted efficiently via the airborne route, whereas avian viruses are generally not airborne transmissible [73].

Studies on the airborne transmissibility of avian influenza viruses in mammalian models have helped to define some of the requirements for airborne transmission of influenza viruses.

• Attachment to the URT

All human influenza viruses have a binding preference for α2.6-SA receptors, or at least for both α2.6-SA and α2.3-SA receptors, and as a consequence attach preferentially to cells in the URT of humans [74]. Studies on human influenza viruses that caused the three pandemics of the 20th century (in 1918, 1957 and 1968), demonstrated that α2,6-SA preference was a critical determinant of host adaptation and subsequent airborne transmission between ferrets [75–77]. Retrospective analysis of pandemic H1N1 (1918), H2N2 and H3N2 viruses has revealed that only one or two mutations in the RBS are required to confer binding preference for α2,6-SA [47]. Two mutations, Q226L and G228S in the RBS, were sufficient for avian H2 and H3 viruses to switch from α2,3-SA to α2,6-SA specificity [47,78]. By contrast, human H1N1 viruses retained 226Q and 228G, but bound α2,6-SA nevertheless [79]. The α2,6-SA preference of H1N1 was found to be determined primarily by 190D and 225D instead [47,80]. Interestingly, for the 2009 pH1N1 virus, the substitution D225G was associated with increased disease severity [81], presumably because viruses with this substitution acquired dual receptor specificity for α2,3-SA and α2,6-SA [82]. Although a change in receptor specificity is a prerequisite for cross-species transmission and human adaptation of avian influenza viruses, recent studies on airborne transmission of human and avian (H5N1, H9N2 and H7N9) influenza viruses demonstrate that a change of receptor

specificity is necessary but not sufficient to result in airborne transmission between mammals [13,52,72,83]. Herfst *et al.* demonstrated that HPAI H5N1 virus A/Indonesia/5/05 (clade 2.1), harboring E627K in PB2 and Q226L and G228S in HA, was not transmitted via the airborne route between ferrets [13]. In a similar study using a reassortant virus harboring the HPAI H5 HA of A/Vietnam/1203/04 (clade 1) and the remaining seven gene segments from a 2009 pH1N1 virus, substitutions in the HA that changed the receptor binding preference (N224K and Q226L) yielded an airborne-transmissible H5, however additional substitutions in HA were required [83]. Recent publications on the novel avian-origin H7N9 virus demonstrate limited airborne transmission between ferrets [84–88]. Most human virus isolates harbor the Q226L substitution associated with binding to α2,6-SA in the URT of mammals. Hence, this amino acid substitution alone is not sufficient for sustained airborne transmission between mammals. Efficient transmission of avian viruses between humans thus require characteristics beyond the α2,6-SA linkage specificity.

In addition to substitutions in the RBS, changes in glycosylation patterns of HA can also affect the host range and transmissibility of influenza viruses. Loss of an *N*-linked glycosylation site near the RBS at position 158–160 was critical for H5N1 virus virulence in mice and direct contact transmission in guinea pigs [89]. The loss of this site in the HA of an H5N1 virus that had already acquired α2,6-SA receptor specificity, enhanced the binding affinity to α2,6-SA [90] and restored a decrease in replication of H5N1 viruses in ferrets caused by the α2,6-SA preference [83]. In the recently described airborne-transmissible H5N1 viruses, different substitutions led to the deletion of the same glycosylation site, suggesting that the loss of this glycosylation site was critical for airborne transmission between mammals [13,83].

• Increased viral replication in the URT

As described above, the viral RNA polymerase complex needs to adapt to allow efficient replication in the mammalian host. Amino acid substitutions in the polymerase complex that increase replication in the mammalian URT, such as E627K and D701N, also support transmission of multiple influenza subtypes in mammalian models [55,89,91] In the study of Herfst *et al.*, the airborne-transmissible H5N1 virus possessed the E627K amino acid substitution in PB2 [13]. However, it has yet to be elucidated if this residue was crucial for airborne transmission and whether it can be substituted by functionally equivalent mutations like D701N [55]. The airborne-transmissible H5N1 viruses of Herfst *et al.* possessed several additional substitutions in the polymerase genes, which may have contributed to the airborne phenotype as well.

• HA stability

Attachment of the influenza virus HA to SA receptors on the host cell surface is followed by receptor-mediated endocytosis. The influx of protons into the endosome leads to a low-pH-triggered conformational change of HA that mediates fusion of the viral and endosomal membranes and the subsequent release of the viral genome in the cytoplasm. The threshold pH of fusion for HAs of human influenza viruses is lower than that of avian influenza isolates, suggesting that the optimal fusion pH of a virus depends on the original host [92]. Lowering the optimal fusion pH for viruses with an avian H5 HA resulted in enhanced replication in the ferret URT and, in certain cases, in more efficient contact transmission [93,94]. Other investigators showed that introduction of HA mutations that lower the pH threshold of fusion (e.g., K58I with fusion at pH 5.4) in a chicken-origin H5N1 virus resulted in increased replication and virulence in mice [95]. Introduction of this K58I mutation in a human H5N1 isolate (A/Vietnam/1203/2004) resulted in increased replication in the URT of ferrets. However, in the absence of mutations that change the receptor binding preference to α2,6-SA, this virus was not transmissible to direct contact ferrets [94].

Although the differences in pH stability between avian and human influenza viruses seem plausible, the observed differences may merely be surrogate markers for other stability phenotypes, for example stability in aerosols, in mucus or upon exposure to air. HAs of H5N1 viruses, engineered to possess an α2.6-SA specificity, are less stable and more sensitive to acidic pH and high temperatures [83]. Such instability might lead to the inactivation of the HA in the environment, thereby abolishing transmission. Phenotypic analyses in the study of Imai *et al.* demonstrated the requirement of a T318I substitution in HA to lower the threshold pH of membrane fusion, thus compensating for a decrease in HA stability caused by the human receptor

binding mutations in the airborne-transmissible H5 [83]. In the Herfst *et al.* study, a H110Y substitution in HA was consistently detected in all airborne-transmissible H5N1 viruses. This residue lies at the trimer interface and might play a role in HA stability as well. The recent resolution of crystals of HAs of H5N1 airborne transmissible viruses gave some insight on the mechanism of HA stabilization by the T318I and H110Y substitutions. The former, close to the fusion peptide, would stabilize the positions of both the fusion peptide and helix A, and therefore counterbalance the destabilizing effects of human receptor binding substitutions and glycosylations sites in the membrane-distal locations, explaining the retention of the wild-type HA fusion pH [96]. The latter, on the other hand, would stabilize the HA trimer as a result from the formation of an hydrogen bond between Y110 and N413 of the adjacent monomer [97].

Altogether, these data suggest that in addition to a change in receptor binding preference, HA stability may be an important determinant of airborne transmission between mammals. Additional studies are needed to further elucidate the exact role of HA stability on virus transmission. The novel H7N9 viruses that were inefficiently transmitted via the airborne route between ferrets contain a relatively unstable HA, with an avian-like threshold pH of fusion of 5.6–5.8 [98]. Based on the recent knowledge from H5N1 viruses, increased airborne transmissibility of H7N9 between mammals may require a lower pH threshold for fusion [87].

• HA/NA balance

The functional balance between the HA and NA surface glycoproteins is crucial during influenza virus infection of host cells and subsequent release of newly formed virus particles after replication. An optimal interplay between the antagonistic receptor-binding (HA) and receptor-destroying (NA) activities is required [99]. Changes in either the receptor binding or cleavage activity can disturb this functional balance and may require restoration of this balance by compensatory mutations in HA, NA or both. It has also been hypothesized that a fine balance between HA and NA functions is necessary for progeny virions to be released as single particles, which might be beneficial for airborne transmission [100].

After the introduction of the N2 subtype in the human population in 1957, α2,6-SA specific NA activity of H3N2 influenza viruses slowly increased over time in order to match α2,6-SA HA specificity [101]. However, NAs of contemporary human viruses possess a conserved α2,3-SA specificity, which may be the result of the need to overcome α2,3-SA rich mucins in the URT. Another example of adjustments to maintain the HA:NA balance is the combination of NA stalk deletion, which decreases NA activity, and increased glycosylation in HA, which decreases receptor binding affinity, upon the introduction of influenza viruses in gallinaceous hosts [102].

To achieve efficient replication and transmission in mammals, it might be necessary to refine the balance between HA and NA, as a result of the α2,6-SA HA preference and the eventual loss of glycosylation sites. Some of the airborne-transmissible H5N1 viruses described to date that contain a mammalian adapted HA, also contain a mammalian NA (derived from human influenza viruses) [83,103–104]. Interestingly, the fully avian airborne H5N1 virus described by Herfst *et al.*, that harbors a mammalian adapted HA, did not require any adaptation of the NA protein to become airborne transmissible [13]. Although no adaptation was required for the H5N1 NA, it may be possible that the NAs of other avian influenza viruses need to coadapt to the receptor specificity of their HA for efficient virus attachment and entry, virus release and potentially transmissibility [101,105].

Future perspective

Avian influenza viruses need to overcome at least two barriers to achieve pandemic potential: the avian-to-human transmission barrier and the human-to-human transmission barrier. The latter appears to be the greatest obstacle for zoonotic viruses to spread and be maintained in the human population, and yet remains the least understood. Although progress has been made in our understanding of the airborne transmissibility of avian H5N1 viruses between mammals, the determinants of airborne transmissibility of influenza viruses remain elusive. However, the current state of knowledge suggests some minimal requirements for airborne transmission of influenza viruses: efficient attachment to relevant cells in the human airways facilitated by binding to the appropriate SA receptor and the loss of a potential *N*-glycosylation site; optimal stability of HA; increased viral replication through mammalian-adaptation substitutions in the polymerase complex; and virus shedding

as single particles rather than aggregates, which may be facilitated by a fine balance between the HA and NA activities.

Given that any zoonotic influenza virus of any subtype may represent a potential pandemic threat, it is imperative to define common biological properties of airborne-transmissible influenza viruses to allow a better assessment of the public health risk posed by the current and future zoonotic influenza viruses. Although the traits discovered by Imai *et al.* and Herfst *et al.* leading to airborne transmission of H5N1 viruses of different genetic clades are remarkably similar [13,83], it remains to be elucidated whether these findings can be extrapolated to other H5N1 virus clades or even to other influenza virus subtypes. Some of the substitutions identified in the laboratory-derived airborne transmissible H5N1 viruses were also reported for the recently emerged H7N9 viruses, which displayed limited airborne transmissibility ferrets (Q226L in HA, E627K in PB2), airborne H9N2 and H9N1 viruses (Q226L) [106,107] and the H2N2 and H3N2 pandemic viruses of the last century (Q226L/G228S) [76,77]. However, focusing on genetic markers rather than phenotypic markers might be misleading, since some substitutions may be dependent on the genetic backbone. It is therefore important to study the common phenotypic traits rather that the substitutions associated with the airborne-transmissible phenotype. The inclusion of simple phenotypic assays as described by Herfst *et al.* and Imai *et al.* (e.g., resialylated turkey red blood cell assays to study receptor binding specificity, fusion assays to study HA stability or minigenome assays to study polymerase complex activity) in ongoing surveillance studies could improve the screening and identification of circulating influenza viruses that might require immediate attention and appropriate control actions.

One of the newly identified requisites for airborne transmission highlighted by recent studies on H5N1 viruses is the necessity for HA to remain stable. Although the biological relevance of this phenotype has yet to be determined, it

EXECUTIVE SUMMARY

Zoonotic influenza A viruses

- Influenza A viruses of subtypes H5, H6, H7, H9 and H10 originating from the waterfowl reservoir have crossed the species barrier and infected humans on several occasions.
- Domesticated birds can serve as bridge species and promote direct adaptation of wild bird influenza viruses to humans.

Crossing the avian-to-human transmission barrier

- A switch in receptor binding specificity, from α2.3-linked sialic acid receptors to α2.6-linked sialic acid receptors, favors successful transmission of influenza A viruses from animal reservoir to humans.
- Efficient replication in mammalian hosts also requires adaptive changes in the viral polymerase complex.

Crossing the human-to-human transmission barrier

- Transmission via respiratory droplets and aerosols (airborne transmission) is the main route for efficient transmission between humans.
- A binding preference for α2.6-linked sialic acids that are predominantly present on cells in the upper airways is necessary but not sufficient for airborne transmission of influenza viruses between mammals.
- High level of replication in the upper airways, mediated by adaptive changes in the HA and viral polymerase complex, facilitates airborne transmission.
- Airborne transmission requires optimal stability of HA.
- A balance between HA and NA receptor specificities and activities may be necessary for efficient release and shedding of single viral particles.

Future perspective

- There is an urgent need to improve our knowledge of common phenotypic traits of airborne transmissibility of influenza A viruses in mammals to better assess the pandemic potential of any zoonotic viruses.

would be of interest to investigate in retrospect the HA stability and associated HA fusogenic properties of past pandemic viruses. Should HA stability be a common property shared by viruses with pandemic potential, it would be of interest to study putative stabilizing HA changes and their effect on airborne transmission in emerging influenza viruses. Of particular interest would be H7N9 viruses, whose limited airborne transmissibility in the ferret model and lack of sustained human-to-human transmission suggests that they still lack crucial determinants of airborne transmission.

While the minimal requirements for avian influenza viruses to cross the species barrier and to acquire airborne transmissibility are slowly being defined, there might be evolutionary constraints on the accumulation of genetic changes required to yield an airborne transmissible phenotype, influencing the likelihood of such an event [16]. Additionally, the potential of a zoonotic virus to achieve sustained human spread is not solely dictated by its ability to cross the species barrier and achieve efficient transmission between humans. Several factors such as the level of pre-existing (cross-)immunity in the human population, the length of the infectious period and the likelihood of virus transmission from infected to naive individuals, must not be overlooked should one aim to embrace the complexity of pandemic virus emergence.

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Papers of special note have been highlighted as:
• of interest; •• of considerable interest

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