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REVIEW

Determinants of virulence of influenza A virus

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Abstract Influenza A viruses cause yearly seasonal epidemics and occasional global pandemics in humans. In the last century, four human influenza A virus pandemics have occurred. Occasionally, influenza A viruses that circulate in other species cross the species barrier and infect humans. Virus reassortment (i.e. mixing of gene segments of multiple viruses) and the accumulation of mutations contribute to the emergence of new influenza A virus variants. Fortunately, most of these variants do not have the ability to spread among humans and subsequently cause a pandemic. In this review, we focus on the threat of animal influenza A viruses which have shown the ability to infect humans. In addition, genetic factors which could alter the virulence of influenza A viruses are discussed. The identification and characterisation of these factors may provide insights into genetic traits which change virulence and help us to understand which genetic determinants are of importance for the pandemic potential of animal influenza A viruses.

Influenza A virus

Influenza A virus is a single-stranded negative-sense segmented RNA virus, with a genome consisting of eight gene segments, that can encode up to 16 proteins [1–5] (Fig. 1a). Influenza A viruses are divided into subtypes based on the genetic and antigenic properties of their envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, 17 HA and 10 NA subtypes have been identified. The HA glycoprotein of influenza A viruses is initially synthesised as a single polypeptide precursor (HA0), which needs to be cleaved into HA1 and HA2 subunits by cellular proteases to become biologically active [6]. In the initial stages of virus replication, the HA glycoprotein binds to specific sialic acid (SA) receptors on the surface of susceptible cells (Fig. 1b). Human influenza viruses preferentially bind to α2,6-linked SA receptors, which are mainly expressed on epithelial cells in the human upper respiratory tract (URT), whereas avian influenza viruses bind to α2,3-linked SA receptors, which are abundantly present on epithelial cells in the intestine of birds and in the lower respiratory tract (LRT) of humans [7]. After binding to these SA receptors, virus particles enter the cell through receptor-mediated endocytosis. Subsequently, a low-pH-triggered conformational change of HA mediates fusion of the viral membrane with the endosomal membrane. The viral ribonucleoproteins (vRNPs) (Fig. 1c) are released into the cytoplasm and are translocated to the nucleus, where transcription and replication takes place by the viral RNA-dependent RNA polymerase complex, consisting of the three polymerase subunits, polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA). M1 and NEP subsequently mediate transport of the newly synthesised vRNPs to the cytoplasm. At the plasma membrane, new virus particles are assembled and released via budding. Efficient release of the viral particles is facilitated by the NA surface glycoprotein, which cleaves off SA residues from the cell surface, thereby allowing the new virus particle to detach from the cell.

The influenza A virus genome encodes additional proteins that are either not directly involved in virus replication or for which the function has not yet been elucidated. The polymerase subunit PB1 can encode two more proteins, the PB1-F2 and PB1 N40 proteins. Similarly, the influenza virus PA gene contains a second open reading frame that is accessed via ribosomal frameshifting, which encodes the PA-X protein.

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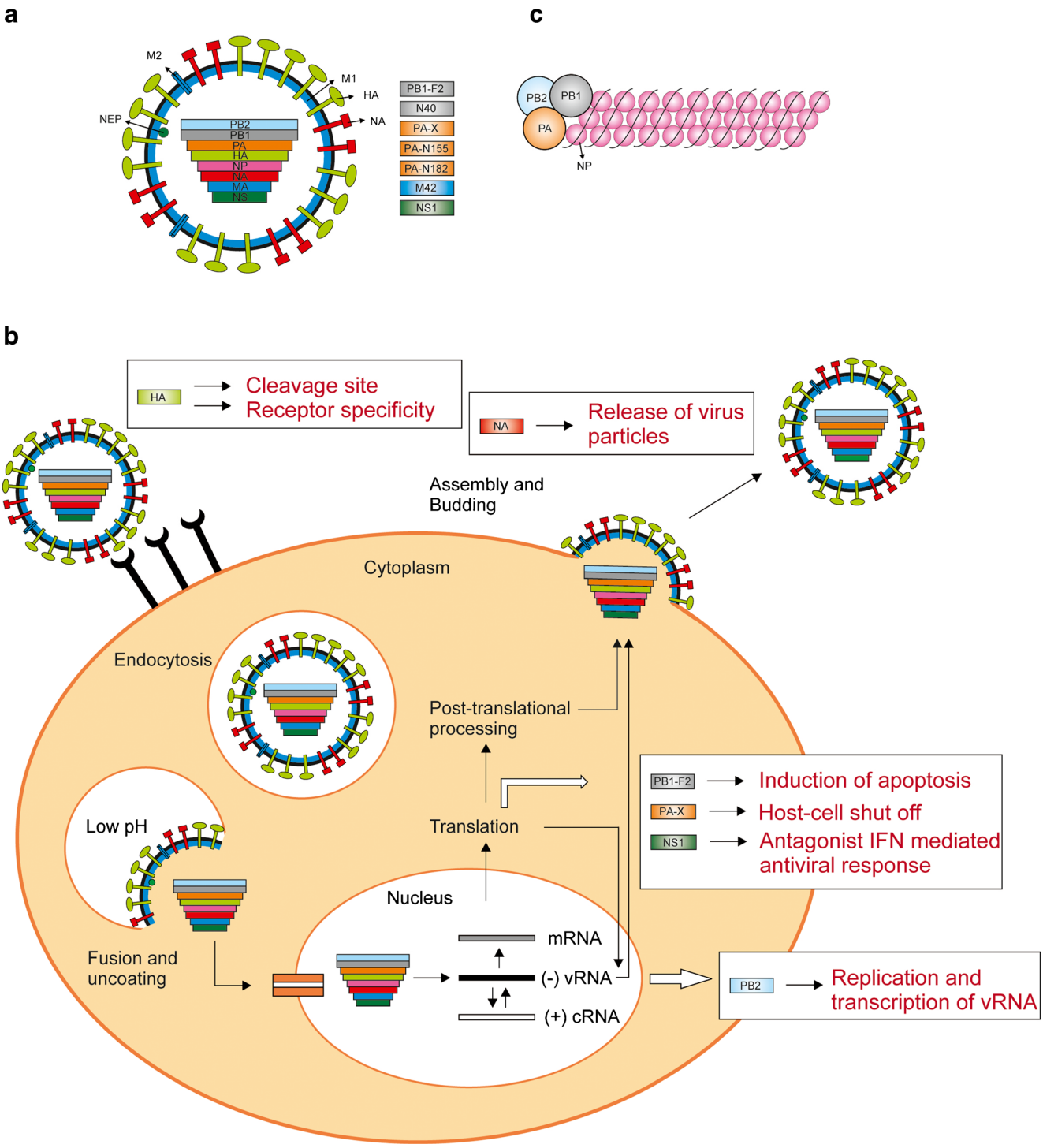

The NS1 protein functions as an antagonist to block the type 1 interferon (IFN)-mediated host antiviral response [8]. M42 has only been discovered recently and encodes a novel M2-like protein with a variant extracellular domain [2]. Currently, the identification of novel influenza virus proteins is receiving considerable interest and influenza segment PA has now been shown to encode as many as four proteins, PA, PA-X, PA-N155 and PA-N182 [5].

Influenza epidemics and pandemics

Influenza A viruses are the cause of recurrent epidemics and occasional pandemics. The annual epidemics result in about three to five million cases of severe illness, and about 250,000–500,000 deaths worldwide. Infection with influenza A virus results in protective immunity against the viral surface glycoproteins HA and NA. However, the accumulation of

◀ **Fig. 1** Influenza A virus particle and replication cycle. **a** Schematic representation of influenza A virus particle and gene segments. The influenza genome consists of eight single-stranded RNAs. The non-structural proteins and/or newly identified proteins with unknown function are depicted in the *rectangles*. **b** Schematic representation of an influenza A virus replication cycle. The viral HA binds to the appropriate host receptor and the virus enters via receptor-mediated endocytosis. A low-pH-triggered conformational change of HA mediates fusion between the viral and endosomal membranes, and the RNPs are released into the cytoplasm. The RNPs translocate to the nucleus, where the vRNA transcription and replication takes place by the RNA-dependent RNA polymerases (PB1, PA and PB2). The mRNA is transported out of the nucleus and translated into the viral proteins. Viral proteins that are needed for replication and transcription are translocated back into the nucleus. Newly synthesised vRNA, along with the polymerase proteins and NP, forms the vRNPs. M1 and NEP subsequently mediate transport of the newly synthesised vRNPs to the cytoplasm. At the plasma membrane, new virus particles are assembled and released via budding. Release from the host cell is mediated by NA, which cleaves off SA from cellular and viral glycoproteins so that the new virus particles can detach from the cell. Several influenza A proteins have been shown to be important determinants of virulence (indicated with *rectangles*). The HA glycoprotein (*light green*) is important as a tropism factor, for the cleavage of HA by cellular proteases and is a prerequisite for starting viral infection. When some HAs are cleavable by ubiquitous proteases, this can result in systemic virus replication in some hosts (e.g. poultry). HA is also responsible for attachment to the different host cell receptors. The PB2 polymerase protein (*light blue*) has the ability to increase replication levels of vRNA in the nucleus. The PB1-F2 protein (*grey*) contributes to viral pathogenicity by inducing apoptosis of infected cells. The PA-X protein (*orange*) regulates the host-cell shutoff. The NS1 protein (*dark green*) can counteract the innate immune response of the host. The NA protein (*red*) acts as a virulence factor by allowing efficient release of the virus particle from the cell. **c** Schematic illustration of the vRNP structure. The viral RNA is encapsidated by NP, and this structure is bound to the polymerase complex (PB2, PB1 and PA)

point mutations in HA and, to a lesser extent, NA allows the virus to escape the host immunity. This phenomenon, known as antigenic drift, explains the occurrence of seasonal influenza epidemics. As a result of this antigenic drift, the vaccine composition has to be updated almost annually [9].

Antigenic shift refers to the introduction of a new influenza A virus subtype in humans. Antigenic shift can be caused by the direct introduction of a new influenza A virus from the animal reservoir or by reassortment, i.e. the mixing of genes from two (or more) influenza A viruses, between animal and human influenza A viruses. While influenza viruses are continuously changing by antigenic drift, antigenic shift happens only occasionally.

Influenza viruses with pandemic potential may arise in pigs upon reassortment, as pig cells express both human and avian influenza receptors, therewith providing an opportunity for the replication of avian and human influenza viruses in the same cell [10]. In the human population, co-infections with different influenza strains have also been observed [11]. In 2001, an H1N2 virus was identified which was the result of reassortment between contemporary circulating H1N1 and H3N2 viruses. Fortunately, this virus did not persist in the human population [12]. Reassortment is also an important factor to increase the genetic variation of influenza viruses, as multiple reassortment events have been shown to occur between different lineages of the H3N2 virus [13].

In the last century, four human influenza A virus pandemics have emerged, at least three of which resulted from reassortment of human and animal influenza A viruses [14–16] (Fig. 2). Future influenza pandemics are inevitable, but it will be difficult to predict which influenza virus subtype will cause a pandemic, when it will cause a pandemic and what the severity of the pandemic will be.

Pandemics threats

Historically, influenza viruses of three HA subtypes (H1, H2 and H3) have acquired the ability to be transmitted efficiently between humans. Currently, influenza viruses of the H1 and H3 subtype co-circulate in humans; however, influenza viruses of the H2, H5, H6, H7 and H9 subtypes are also considered to represent a pandemic threat.

In 1997, a large outbreak of highly pathogenic avian influenza (HPAI) H5N1 virus in poultry in Hong Kong resulted in the first documented cases of the direct transmission of HPAI H5N1 virus from poultry to humans, with a fatal outcome in 6 out of 18 cases [17]. As a result, this outbreak warranted the mass culling of 1.5 million chickens. After 2004, HPAI H5N1 viruses have spread throughout Asia, Europe and Africa since, causing severe disease outbreaks in poultry. Furthermore, HPAI H5N1 viruses have been isolated from mammals on numerous occasions. Since 2003, over 600 cases of human HPAI H5N1 infections have been reported, more than half of which were fatal [18]. Although rare cases of H5N1 virus transmission between humans have been reported, sustained human-to-human transmission of HPAI H5N1 virus has not yet been detected [19]. It is this absence of efficient human-to-human transmission that has prevented an H5N1 pandemic from occurring. With the sporadic introduction of HPAI H5N1 virus in the human population, it is feared that these avian H5N1 viruses may mutate or reassort with contemporary human influenza viruses, possibly resulting in adaptation to humans. Fortunately, at present, reassortment of HPAI H5 virus with contemporary human influenza viruses has not been detected in nature. However, co-infections of avian and human influenza viruses in humans or pigs may provide new opportunities for reassortment [20]. Due to the enzootic nature of HPAI H5N1 in poultry, the brood host range (over 20 different mammalian species) and the accumulation of mammalian adaptation mutations, this virus is currently considered to represent a significant pandemic threat to humans.

Several outbreaks of HPAI H7 viruses in poultry have resulted in transmission to humans. In 2003, a large outbreak

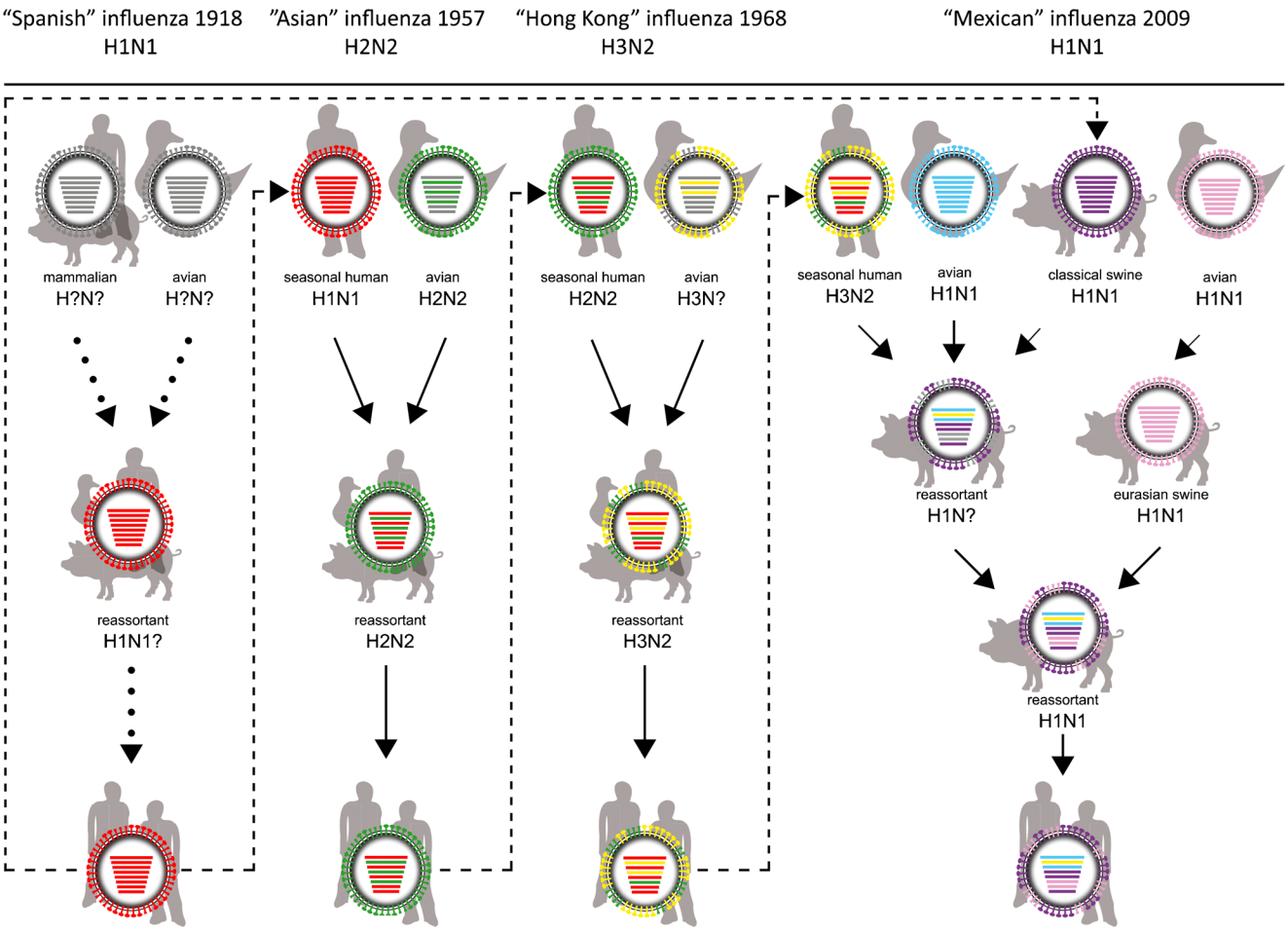

Fig. 2 Reassortment and adaptation events of pandemic influenza A viruses. For the 1918 H1N1 "Spanish influenza" pandemic, evidence for two scenarios has been presented: (1) the virus emerged upon reassortment between avian and mammalian influenza viruses and (2) the gradual adaptation of avian influenza genes to the human host. The 1918 H1N1 virus caused seasonal epidemics until 1957, when the H2N2 virus emerged upon reassortment of the seasonal H1N1 with an avian H2N2 virus, thereby introducing the avian HA, NA and PB1 genes. The H2N2 virus circulated in humans until 1968, when reassortment of the H2N2 with an avian H3 virus resulted in the exchange of the H3 HA and PB1 genes and the generation of the H3N2 "Hong Kong" influenza. The pH1N1 virus consists of the NA and M genes of the Eurasian swine lineage, and the other genes of a "triple reassortant" swine influenza virus that had previously acquired its genes upon reassortment between human, avian and (classical) swine viruses. The *grey colour* in virus particles indicates uncertainty of viral gene segment origin or lack of data. The *dotted arrows* indicate uncertain scenarios and the *solid arrows* indicate events that are supported by scientific evidence. The *dashed arrows* represent pandemic viruses circulating in subsequent influenza seasons. Partially adapted from [101]

of an HPAI H7N7 virus in poultry in the Netherlands resulted in 89 cases of human infections, one of which was fatal [21]. HPAI H7N7 virus displayed an unusual tissue tropism; the virus targeted the conjunctiva, resulting in conjunctivitis, a symptom rarely reported for other influenza virus subtypes [22]. Recently, a novel reassortant influenza H7N9 virus of avian origin emerged in China. This LPAI H7N9 virus is associated with severe and fatal respiratory disease. As of July 2013, 135 confirmed cases of human infection with H7N9 virus have been reported, 44 of which have resulted in deaths [23]. The emergence of this new H7N9 virus in humans emphasises the pandemic potential of influenza A viruses of the H7 subtype.

Since the mid-1990s, H9N2 viruses have become endemic in poultry populations throughout Eurasia. The first human case of infection with an avian H9N2 virus was documented in 1999 in Hong Kong [24] and, sporadically, human infections have been described since [25]. Furthermore, this subtype has been isolated from pigs and numerous reassortment events between H9N2 virus and other influenza virus subtypes (i.e. H7N9, H5N1 and H6N2) [26] have been reported. H9N2 viruses with either avian and human or human receptor specificity [27] are now prevalent in many Eurasian countries, thereby increasing the possibility of this virus to infect humans.

This year, the first human infected case of avian H6N1 was documented [28]. Avian influenza A viruses of the H6 subtype were found to replicate in mice and ferrets without prior adaptation [29]. Given the first human case, the high prevalence and frequent reassortment of H6 viruses in birds,

concerns have risen about the possible emergence of a pandemic H6 virus [30, 31].

Influenza viruses of the H2 subtype have not circulated in humans since 1968 and, therefore, a large proportion of the current world population is likely to be susceptible to infection with H2 viruses if they would re-emerge. As H2 viruses continue to circulate in swine and several avian species [32], they again pose a potential pandemic threat.

The pH1N1 virus in 2009 illustrated that a new pandemic does not necessarily require the introduction of a virus with an HA subtype that is new to the human population. A novel influenza A (H3N2) variant virus (H3N2v) containing seven gene segments of swine influenza origin and the pH1N1 M segment was isolated from 12 humans in 2011 [33]. Although there is a low level of cross-reactive antibodies with a human H3N2 virus that circulated in the 1990s [34], H3N2v is antigenically different from the currently circulating seasonal H3N2 viruses and can, thus, potentially infect a large proportion of the human population.

Our understanding of avian influenza virus infections of humans is still rather limited. The H7N9 outbreak that started in May 2013 highlights the continuous threat of avian influenza viruses and underlines the unpredictability of which viruses are likely to cross the species barrier.

Insight into mechanisms by which influenza viruses cross the species barrier is, therefore, crucial to interpret surveillance data and to allow the early detection of influenza viruses with pandemic potential. Since the pH1N1 virus emerged after extensive reassortment in swine, it is important to continue surveillance activities not only in birds, but also in swine to monitor novel swine influenza viruses which may have the ability to infect humans. Recently, reassortment studies demonstrated that pH1N1 virus was found to preferentially incorporate the NA and PB1 gene segments of a seasonal H3N2 virus [35, 36]. In addition, a recent study showed that co-circulating H1N1, H1N2 and H3N2 viruses can reassort rapidly in swine [37]. Thus, it is possible that additional reassortment events occur between currently circulating swine or human influenza viruses.

Influenza A virus virulence factors

HA

Amino acid substitutions in HA that affect the receptor binding preference can influence the cellular host range and tissue tropism, which may alter virulence (Table 1). Specific amino acid residues in HA determine the receptor binding specificity of human and avian influenza viruses, and these specific residues differ among virus subtypes. The binding patterns of pandemic influenza A viruses of the H1 subtype are determined by amino acids at positions 190 and 225 in HA (H3 numbering) [38, 39]. For influenza viruses of the H2 and H3 subtypes, positions 226 and 228 are important for receptor binding specificity [40]. Since the HA proteins of the 1918, 1957 and 1968 pandemic strains were derived from avian influenza viruses, adaptation of avian HA proteins to the human receptor is considered to be a prerequisite for efficient human-to-human transmission [40]. Numerous studies have described amino acid substitutions in HA of HPAI H5N1 viruses that change and/or increase binding to human α2,6-linked SA receptors that are present in the human URT [41, 42]. The majority of these amino acid substitutions are located in or near the receptor binding site of HA. However, a potential N-glycosylation motif at amino acid positions 154–156 of

Table 1 Important determinants of viral pathogenicity

Protein	Function	Position	References
HA	Alter cellular host range and tissue tropism	Receptor binding site	[40, 102]
	Determines by which cellular proteases HA is cleaved	Cleavage site	[6]
	Potential N-glycosylation motif which affects binding	154–156	[43, 44]
PB2	Replication advantage in mammalian species	627	[53]
		701	[54, 57]
		590/591	[59]
PA-PB1-NP-NEP	Increased polymerase activity in mammalian cells	N/A	[60]
PB1-F2	Induction of apoptosis; antagonize the IFN response	66	[61, 65]
PA-X	Regulates host-cell shutoff	N/A	[4]
NS-1	Evasion of host immune response	C-term	[72, 73]
		92	[71]
NA	Release of virus particles	N/A	[75, 103]
	Additional mechanisms that promote cleavage of HA	unknown	[76, 77]
	Adaptation and increased virulence upon deletion	Stalk region	[79, 80]

HA, which is proximal but not immediately adjacent to the receptor binding site, may also affect binding preference and virulence [43, 44].

One of the best-known virulence determinants of HPAI viruses is the multibasic cleavage site (MBCS) in HA. The sequence of this cleavage site determines on which cellular proteases HA0 is cleaved and, consequently, determines viral tropism and virulence (Fig. 3). The cleavage of HA by cellular proteases is a crucial step in the replication cycle; virus particles with uncleaved HAs are not infectious. The HA of low pathogenic avian influenza (LPAI) viruses and human influenza viruses harbours a monobasic cleavage site that is cleaved by trypsin-like proteases. These trypsin-like proteases are only present in the respiratory tract of humans and the respiratory and/or intestinal tract of birds, thereby restricting virus replication to these tissues [45]. Cleavage of the human influenza virus HA is thought to occur by trypsin-like serine proteases of the type II transmembrane serine protease (TTSP) family that are present at the plasma membrane or by extracellular proteases present in the respiratory tract s[6].

Influenza viruses of the H5 and H7 subtypes may become highly pathogenic after circulation in poultry. The switch from a low pathogenic to a high pathogenic phenotype of these H5 and H7 influenza viruses is caused by the introduction of basic amino acid residues into the HA0 cleavage site. This HA0 can subsequently be cleaved by ubiquitously expressed proprotein convertases of the subtilisin family, like furin or PC5/6, thereby facilitating systemic replication in chickens [6]. Recent studies demonstrated that non-H5 and -H7 subtypes could support a highly pathogenic phenotype, which demonstrates that the introduction of an MBCS in a non-H5 or -H7 avian influenza virus is not deleterious [46, 47].

In mammals, the association between the presence of an MBCS and systemic spread is less obvious. In the ferret model, incorporation of an MBCS in the HA of a human influenza H3N2 virus did not result in increased virulence or a change in tissue tropism. These findings suggest that, in addition to an MBCS, other factors are involved in causing systemic spread in ferrets. The presence of an MBCS in H5 HA also does not always result in systemic spread in mammals, since the inoculation of cynomolgus macaques with HPAI H5N1 resulted in respiratory tract infection only [48]. However, in mice, deletion of the MBCS of an HPAI H5N1 virus results in a virus that only causes respiratory tract infection, in contrast to the systemic spread of the wild-type HPAI H5N1 virus, indicating that the MBCS is a major virulence factor in mice [49]. Although the extra-respiratory tract infection of HPAI H5N1 virus in mammals is likely caused by multiple factors [50, 51], the MBCS has been shown to be essential [52].

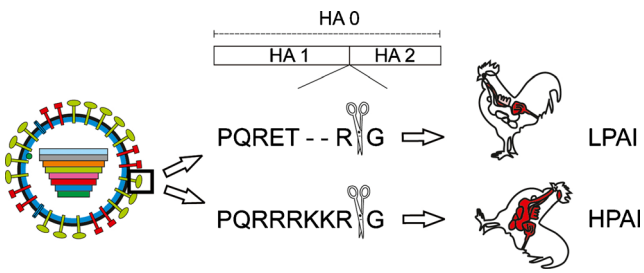

Fig. 3 Cleavage site as an important virulence factor. The HA0 is cleaved into two subunits, HA1 and HA2, by cellular proteases, which recognise either a monobasic or multibasic cleavage site (MBCS). The HA0 of LPAI viruses harbours a monobasic cleavage site and is cleaved by trypsin-like proteases only, thereby limiting replication of these viruses to sites where these enzymes are expressed, i.e. respiratory and intestinal tracts. The HA0 of HPAI viruses of the H5 and H7 subtypes can be cleaved by ubiquitously expressed furin-like proteases, facilitating systemic replication in chickens

Polymerase proteins

The influenza virus polymerase proteins, and in particular PB2, have been shown to be important determinants of virulence. Amino acid substitution lysine (K) to glutamic acid (E) at position 627 in PB2 has been studied extensively in the context of mammalian adaptation [53]. This substitution is suggested to occur in order to adapt to physiological constraints, e.g. differences in body temperature. Most avian influenza A viruses, which preferentially replicate at a relatively high temperature of around 41 °C in the digestive tract of birds, have an E residue at position 627. In contrast, human influenza A viruses replicate at the lower temperature of around 33 °C, which is the temperature in the human URT. These viruses typically have a K residue at this position. The E627K substitution was acquired rapidly when avian viruses were passaged in mice [54]. Moreover, this mutation has been implicated with increased virulence of human HPAI H5N1 virus isolates and was found in a fatal human case of infection with HPAI H7N7 [44, 49]. In the absence of the E627K mutation, an aspartate (D) to asparagine (N) substitution at position 701 of H5N1 PB2 was found to increase virulence and to expand the host range of avian H5N1 virus to mammalian hosts [54–56]. The adaptive mutation D701N caused enhancement of binding of PB2 to importin alpha1 in mammalian cells, resulting in increased transport of PB2 into the nucleus [57]. Noteworthy, unlike other human influenza viruses, pH1N1 does not contain the mammalian adaptation residues 627K and/or 701N. When the mammalian adaptation substitutions E627K or D701N were introduced in pH1N1, no increase in virulence was observed [58]. However, the absence of these mammalian adaptation markers in PB2 have been compensated to some extent by a serine (S) and arginine (R) substitution at positions 590 and 591 that may affect the protein's interaction with viral and/or cellular factors and, hence, its ability to support virus replication in mammals [59]. Recently, numerous other mutations in PB1, PA, NP and NEP have been described that can

overcome the poor polymerase activity of avian influenza viruses in human cells [60].

PB1-F2

PB1-F2 contributes to the virulence of influenza A viruses by inducing apoptosis of infected cells [61]. Moreover, PB1-F2 promotes and increases the severity of secondary pneumonia [62]. The PB1-F2 protein contributed to the virulence of HPAI H5N1 and the 1918 H1N1, 1957 H2N2 and 1968 H3N2 pandemic strains [63, 64]. In particular, an N to S substitution at position 66 (N66S) of HPAI H5N1 and 1918 pandemic influenza PB1-F2 is partly responsible for the high virulence of these viruses [63]. The PB1-F2 N66S variant reduces the production of IFN, which is part of the innate immune response [65]. In contrast to previous pandemic influenza viruses, pH1N1 does not encode a PB1-F2, because of three premature stop codons. Surprisingly, when the pH1N1 PB1-F2's coding capacity was restored, the virulence was only modestly affected in mice and ferrets [66, 67].

PA-X

The PA gene also encodes a newly identified protein, PA-X [4]. This protein modulates the host response by repressing cellular gene expression, i.e. host-cell shutoff. PA-X-deficient influenza viruses cause more severe disease in mice, as a result of an accelerated host response. Moreover, influenza viruses lacking PA-X differ in host-cell shutoff compared to wild-type virus. The truncation of PA-X protein appears to be associated with influenza virus lineages circulating in particular hosts, indicating that there may be some species specificity to the evolution of PA-X [68].

NS1

In response to the presence of pathogens in the host, IFNs are secreted by cells and 'interfere' with viral replication. To establish productive infection, influenza viruses have mechanisms to evade host immune responses, including the type I IFN response. The influenza virus NS1 protein has several ways of acting as an IFN antagonist [69].

NS1 has been studied extensively as a molecular determinant of virulence. Influenza viruses lacking the IFN antagonist NS1 are only able to replicate in cells or mice that have a compromised IFN response [8]. H5N1 viruses, unlike other human, avian and swine influenza viruses, are relatively resistant to the antiviral effects of IFNs, which result in increased levels of pro-inflammatory cytokines [70]. This effect requires an E at amino acid position 92 of the NS1 molecule and allows virus replication in the presence of IFN; this mutation was a determinant of virulence in pigs [71]. Furthermore, an E instead of a D residue at this position increased the virulence of HPAI H5N1 in mice.

Large-scale genome sequence analysis of avian influenza virus isolates indicated that four carboxyterminal residues of the NS1 protein form a PDZ (postsynaptic density protein 95, *Drosophila* disc large tumour suppressor and zonula occludens 1 protein) ligand domain of the X-S/T-X-V type [72]. This PDZ ligand domain of NS1 has also been shown to influence influenza virus virulence. Pandemic 1918 H1N1 and H5N1 HPAI viruses contain a PDZ ligand domain motif which increases virulence when introduced into a mouse-adapted influenza strain [73]. This demonstrates that NS1 can modulate virulence through different mechanisms.

pH1N1 lacks the ability to block host gene expression in both human and swine cell lines [74]. Additionally, pH1N1 has a truncated NS1 protein with an 11 amino acid deletion at its C-terminus and, therefore, lacks the PDZ-binding domain [16]. However, even when these functions are restored for pH1N1, they do not appear to have a significant effect on the replication, virulence or transmission of pH1N1 in various animal models [74].

NA

Considering that HA binds to SA receptors and NA cleaves SA from cellular receptors, the balance between HA and NA activity is critical for virus replication and transmission [75].

Two influenza viruses are known that have developed additional mechanisms which promote the cleavage of HA. The NA of the neurovirulent laboratory H1N1 strain A/WSN/33 recruits plasminogen which, when converted to plasmin, cleaves HA in the absence of trypsin [76]. On the other hand, the 1918 H1N1 NA gene enables the virus to replicate in the absence of trypsin. Additionally, this NA protein was shown to play a critical role in the high virulence of the 1918 pandemic H1N1 in mice [77].

In 2003, an outbreak of HPAI H7N7 virus in poultry in the Netherlands resulted in the death of one person and 89 human cases of conjunctivitis. When the sequence of the virus obtained from the fatal case was compared to the sequence of a virus isolated from a patient with conjunctivitis, four amino acid substitutions in the NA gene were identified [21]. These mutations all contributed to an increased NA activity, resulting in more efficient replication in mammalian cells, most likely by preventing the formation of virus aggregates [44].

When avian influenza viruses are transmitted from wild birds to poultry, genetic changes as a result of adaptation to the new host frequently occur. One example of such a change is a deletion in the stalk region of NA that has been reported in several viruses isolated from unrelated poultry outbreaks [78]. This shortened NA stalk region is frequently detected upon the transmission of avian influenza viruses from waterfowl to domestic poultry, and is associated with increased virulence

[79, 80]. It is not yet clear how this shortened NA stalk region influences virulence; however, deletion in the NA stalk does not enhance the release of progeny viruses, since the active site in the head cannot efficiently access its substrate [81].

Transmission

Human-to-human transmission of influenza viruses can occur through direct contact, indirect contact via fomites (contaminated environmental surfaces) and/or airborne transmission via small aerosols or large respiratory droplets. Efficient and sustained human-to-human transmission is critical for the circulation of seasonal and pandemic influenza viruses in the human population. Transmission has been studied extensively in mammalian models, in particular the ferret and guinea pig [82]. Ferrets are naturally susceptible to both human and avian influenza viruses and, upon infection, develop similar symptoms and pulmonary pathology as humans. In addition, cells of the ferret respiratory tract express predominantly α2,6-linked SA receptors in the URT and α2,3-linked SA receptors in the LRT, similar to humans [83, 84]. Avian influenza viruses do not transmit via the airborne route in the ferret model [85, 86]. Therefore, the ferret model is a valuable tool to study viral traits for influenza virus transmission in mammals. This is highly relevant, as it is currently unclear as to what exactly determines the transmission of influenza viruses in mammals via aerosols or respiratory droplets. For this reason, the ferret model was used extensively to compare the transmissibility of pH1N1 with the contemporary seasonal H1N1 virus, when it first emerged in humans [86]. In addition, transmission of the 1918 H1N1 virus was studied in ferrets. These studies showed that changes in the HA receptor binding domain and PB2 were critical to initiate the transmission of an avian-derived influenza virus [39, 87]. Similar genetic changes were required for the Asian H2N2 virus. An early H2N2 virus (1957) failed to transmit to naïve ferrets. However a Q to L at position 226 in HA was sufficient to change its binding preference from avian to human receptors, subsequently resulting in transmission between ferrets [88]. Overall, amino acid substitutions in HA and polymerase proteins can affect the host range and transmission of influenza viruses [56, 59, 87].

As described above, avian influenza viruses of the H5, H7 and H9 subtypes have infected humans on several occasions and are, therefore, considered a potential pandemic threat. However, the requirements for a virus to become pandemic (i.e. transmissible between humans) are poorly understood. In order to study the determinants that could lead to a pandemic virus, an avian H9N2 virus that harboured the internal genes from a human H3N2 virus was adapted to replication in mammals by serial passaging in ferrets. This ‘adapted’ virus was found to be transmitted efficiently between ferrets via respiratory droplets [89]. This indicates that avian H9N2 viruses may acquire the ability to be transmitted between humans.

The lack of sustained transmission of HPAI H5N1 virus between humans has been confirmed in guinea pigs and ferrets. Early attempts to create airborne-transmissible H5 viruses by generating reassortant viruses between H5N1 and human influenza viruses did not result in H5 viruses that could be transmitted between mammals via the airborne route [90–92]. Based on evidence from previous influenza pandemics, it has been hypothesised that a switch of receptor binding preference from avian α2,3-linked to human α2,6-linked SA receptors is required for an avian virus to become transmissible between humans. Nevertheless, changing the receptor binding preference alone was not sufficient to confer airborne transmission of the H5N1 virus, indicating that additional adaptive changes are required for H5N1 viruses to become transmissible [90, 93]. Herfst et al. recently demonstrated airborne transmissibility of a fully avian, ferret-adapted H5N1 virus. This research proved that avian-origin H5N1 viruses with a human SA receptor binding preference can become airborne-transmissible, but that, indeed, additional mutations were required for this phenotype [93]. Recent transmission studies in guinea pigs demonstrate that reassortants between H5N1 and pH1N1 viruses that harbour the H5N1 HA with a dual SA receptor preference are airborne-transmissible between guinea pigs as well [94]. In addition, phenotypical analysis of airborne H5 virus demonstrated that the stability of HA in an acidic environment is important for airborne transmission [95].

For the H7N9 virus, only one case of non-sustainable transmission between humans has been reported to date [96]. However, transmission experiments in ferrets indicating that this virus has a limited ability to be transmitted via the airborne route are limited [97–100].

The recent H7N9 outbreak again accentuates that increased understanding of the mechanisms and molecular determinants that facilitate avian influenza viruses to cross the species barrier and become airborne-transmissible in humans is urgently needed. It is still impossible to predict when a new influenza virus will emerge in humans to cause the next pandemic, and what the subtype of this virus will be [101]. Therefore, surveillance of bird and swine influenza viruses should specifically target particular mutations that render viruses more virulent or airborne-transmissible in humans, as described above. The detection of such genetic traits should trigger more aggressive control programmes than those employed currently. Improving pandemic preparedness by developing new vaccines that induce broader and stronger immune responses than the current influenza vaccines is another research priority. The ultimate goal of influenza vaccine research should be to design a universal vaccine that would induce protection against all influenza virus subtypes.

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