Virus-specific factors associated with zoonotic and pandemic potential

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Influenza A is a highly contagious respiratory virus in constant evolution and represents a threat to both veterinary and human public health. IA viruses (IAVs) originate in avian reservoirs but may adapt to humans, either directly or through the spillover to another mammalian species, to the point of becoming pandemic. IAVs must successfully be able to (i) transmit from animal to human, (ii) interact with host cells, and (iii) transmit from human to human. The mechanisms by which viruses evolve, cause zoonotic infections, and adapt to a new host species are indeed complex and appear to be a heterogeneous collection of viral evolutionary events rather than a single phenomenon. Progress has been made in identifying some of the genetic markers mainly associated with virulence and transmission; this achievement has improved our knowledge of how to manage a pandemic event and of how to identify IAVs with pandemic potential. Early evidence of emerging viruses and surveillance of animal IAVs is made possible only by strengthening the collaboration between the public and veterinary health sectors.

Keywords Host range, influenza, interspecies transmission, pandemic, virus adaptation.

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Introduction
IA viruses cause recurrent epidemics and global pandemics. The emergence of a novel H1N1 swine-origin virus (H1N1 S-OIV) in 2009 and the ongoing occurrence of human cases of infection with avian H5N1 IAVs are only recent examples of the zoonotic and pandemic potential of IAVs. Different mechanisms are believed to be able to transform an animal virus to a human pandemic strain, and these include a constellation of viral evolutionary events that are still to be thoroughly investigated. By and large, swine and avian influenza viruses cause the greatest concerns for public health. Understanding the molecular evolution of IAVs in the animal reservoir and the mechanisms associated with interspecies transmission would improve our knowledge and prediction skills on relevant characteristics of zoonotic and pandemic influenza viruses.

Biology of IAVs
IA viruses are members of the Orthomyxoviridae family. On the basis of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), they currently cluster into 17 HA (H1–H17) and nine NA (N1–N9) subtypes. IAVs consist of eight segmented, single-stranded RNA genomes of negative polarity, encoding 11 proteins: polymerase polypeptides PB1, PA, PB2 (polymerase complex), HA and NA, nucleocapsid protein (NP), matrix protein (M1), ionic channel protein (M2), non-structural protein 1 (NS1), nuclear export protein (NEP), and mitochondria-associated protein (PB1-F2). The HA glycoprotein is critical for binding to cellular host receptors and for the fusion of the viral and endosomal membranes. Replication and transcription of viral RNAs are carried out by the three polymerase subunits PB1, PB2, and PA, and by the NP. Newly synthesized viral ribonucleoprotein (RNP) complexes are exported from the nucleus to the cytoplasm by the NEP and M1 and are assembled into virions at the plasma membrane. The NA facilitates the virus release from infected cells by removing sialic acid (SA) from cellular and viral HA and NA proteins. The NS1 protein is an interferon antagonist, and the PB1-F2 protein is an important virulence factor that induces cell apoptosis.

Recently, a novel PA-X fusion protein encoded in part from a + 1 frameshifted X open reading frame (X-ORF) in segment 3 of AI viruses has been described and has been associated with the immune response modulation in the mouse model.
α2-6 linkage.\textsuperscript{13,18} IAVs recognize mainly two species of SAs, NeuAc (N-acetylneuraminic acid), and NeuGc (N-glycolylneuraminic acid), which are attached to galactose in SAα2-3Gal or SAα2-6Gal linkages. For instance, avian viruses preferably recognize SAα2-3Gal linkages, which are mainly found in the intestine and respiratory epithelia of birds,\textsuperscript{11,19} whereas human influenza viruses recognize SAα2-6Gal linkages, which mainly populate the human upper respiratory tract (URT) epithelia.\textsuperscript{20–22} However, toward the lower epithelial tract of humans, there is a relative increase in SAα2-3Gal expression,\textsuperscript{21,23} and this has been associated with severe pulmonary pathology observed in some cases of H5N1 infection.\textsuperscript{24,25}

Pigs are known to exhibit dual expression of both SA linkages in the respiratory tract;\textsuperscript{26–28} however, recent studies indicate that receptor distribution is similar to that found in humans, suggesting that the classical “mixing vessel” hypothesis regarding the unique role played by pigs needs further discussion.\textsuperscript{28,29} Given similar receptor distribution, one might expect humans and pigs to have similar susceptibility to direct infection by IAVs. Additional factors may play a role in species differences however, including relative abundance of the preferred glycan topology (as discussed later), which might influence the viral binding kinetics and/or equilibrium shift.\textsuperscript{30} In addition, the human upper respiratory tract contains many complex types of glycans,\textsuperscript{31} and human bronchial mucus contains a mucin, a heavily glycosylated protein with attached α-2,3-linked oligosaccharides, which may bind avian IAVs and help prevent infection.\textsuperscript{20}

Concerning other species, the presence of SAα2-6Gal in the alveoli of dogs, cats, pigs, and ferrets\textsuperscript{32} and in the trachea of chickens and ducks has been reported,\textsuperscript{33} while both types are present throughout the respiratory tract of pheasant, turkeys, and quails.\textsuperscript{34}

**Viral characteristics of receptor-binding site**

The amino acid residues in the receptor-binding site of HA affect the virus host range.\textsuperscript{35,36} Glutamine (Q) at position 226 and glycin (G) at position 228 of H3 HA confer binding to SAα2-3Gal, while leucine (L) and serine (S) at these positions determine binding to SAα2-6Gal. For H1 strains, glutamic acid (E) and glycine (G) at positions 190 and 225 confer binding to SAα2-3Gal, whereas aspartic acid (D) at the same positions confers binding to SAα2-6Gal.\textsuperscript{11,37,38}

Influenza virus–receptor interactions are more complex than the simple α2-3 versus α2-6 dichotomy on the host range restriction,\textsuperscript{39} suggesting that glycan species (linked to SA) and their topology could also play an important role.\textsuperscript{30} The human respiratory tract expresses only NeuAc, whereas NeuGc is present in other species.\textsuperscript{40} For instance, avian, human, and swine IAVs exhibit preference for NeuAc rather than NeuGc. Interestingly, the abundant presence of NeuAc in swine trachea could render this species as the possible host for adaptation and/or intermediate virus resortment in the creation of novel viruses for humans.\textsuperscript{41} On the other hand, SA glycans are classified as having umbrella-like (long α2-6) and cone-like (α2-3 or short α2-6) structural topology and this may also influence virus–receptor affinity.\textsuperscript{30,31,42}

Recently, it has been demonstrated that human-adapted HAs bind with high affinity to umbrella-like topology SAs, whereas avian and swine HAs preferentially recognize cone-like topology.\textsuperscript{31} These findings indicate that glycan composition and topological changes may also be important determinants in species-specific switch events.\textsuperscript{30,31}

**Viral polymerase complex**

Another determinant of host restriction is the IAV polymerase complex.\textsuperscript{43–45} The amino acid residue 627 in the PB2 subunit regulates polymerase activity in a species-specific fashion.\textsuperscript{46} The PB2 derived from human viruses mainly possesses lysine (K) at position 627 (PB2-K627), whereas glutamic acid (PB2-E627) predominates in avian viruses,\textsuperscript{47–49} with the exception of most of the H5N1 clade 2.2 viruses and their descendants.\textsuperscript{50} PB2-K627 correlates with enhanced polymerase activity, virus replication, transmission, and pathogenicity in mammals,\textsuperscript{51,52} as well as with a possible virus replication at 33°C (human URT temperature).\textsuperscript{53} However, PB2-K627 is not obligatory for efficient infection or disease induction in mammals, as observed in some swine viruses, certain avian H5N1 isolates, and most notably in the H1N1 S-OIV.\textsuperscript{50,54} Indeed, engineering a 627K change into the H1N1 S-OIV did not result in increased virulence.\textsuperscript{54,55} In such cases, other residues within PB2 as T271A, Q591K, D701N, and S714R could contribute to viral adaptation and replication in mammalian cells through the increase in polymerase activity at relatively low temperatures.\textsuperscript{56,57} The amino acid at position 701 of PB2 has emerged as a determinant of virulence, facilitating the binding of PB2 to importin α (a cellular nuclear import factor) in mammalian cells.\textsuperscript{58,59}

**Molecular basis of pathogenicity: role of ha cleavage, NS1, and PB1-F2 proteins**

The HA protein is synthesized as a precursor protein that is cleaved into two subunits (HA\textsubscript{1} and HA\textsubscript{2}) by host cell proteases.\textsuperscript{11,13} This proteolytic cleavage is a prerequisite for fusion of the viral and endosomal membranes to release viral RNP to the cytoplasm.\textsuperscript{60} Low pathogenic avian influenza viruses (LPAI) possess a cleavage site with a monobasic motif recognized by trypsin-like proteases, which confine viral replication to the respiratory and gastrointestinal tracts.\textsuperscript{11,61} In contrast, highly pathogenic avian influenza (HPAI) viruses possess a polybasic HA cleavage site cleavable by the ubiquitous furin, supporting the systemic replication.\textsuperscript{62} This polybasic HA cleavage of HPAI viruses has originated from
LPAI precursors by acquisition of a multibasic cleavage site (MBCS) under both in vitro and in vivo experimental conditions in domestic poultry.

The NS1 protein is an interferon antagonist. The majority of IAV NS1 proteins have a class I PDZ-binding motif at the C-terminus, and its truncation results in attenuation of the virulence in mice, as well as in limited virus replication and enhanced type I IFN induction in human dendritic cells. Additionally, NS1 has been associated with exacerbated pro-inflammatory cytokine production in humans. On the other hand, PB1-F2 is a small protein encoded by the +1 alternate ORF in the PB1 polymerase gene of some IAVs. This protein is thought to play a role as a virulence factor by compromising mitochondrial function and eventually leading to apoptosis.

### Evolutionary pathways and molecular mechanisms of IAVs involved in human adaptation

#### Mutations

IA viruses evolve using different mechanisms. The most prominent is antigenic drift, a result of mutations introduced during replication of the viral genome by viral RNA polymerase, which lacks proofreading activity. The rate of mutation during replication of the influenza genome is about 1 nucleotide change for every copied genome. Antigenic shift occurs through viral reassortment, which can result in the shuffling of entire gene segments. For transmission to humans, animal IAVs need to acquire the ability to recognize SAα2-6Glu as a prerequisite to igniting a pandemic.

Key mutations of HA at positions 138, 190, 194, 225, 226, and 228 (H3 numbering) affect receptor-binding preference of several subtypes including H2, H3, H4, and H9 whereas the HAs from H1 human-adapted viruses bear changes at positions 190 and 225 (Table 1).

Transmission of H5N1 HPAI virus from poultry to humans was first reported in Hong Kong in 1997. From 2003 to 2012, 608 cases of H5N1 virus infections in humans and 359 deaths have been reported in 15 different countries. Even if human-to-human transmission has been limited, H5N1 is believed to be a significant health threat due to “spillover” infections in humans associated with widespread infection in poultry populations. The single mutation HA-Q192H in some H5N1 strains isolated from humans increased viral binding to SAα2-6Glu, correlating as well with an increased virulence in mice.

After the literature search for this review was completed, two works describing potential molecular determinants of airborne transmission were published. Four mutations in the HA of a reassortant virus (N220K, Q222L, N154D, and T314I) possessing the HA from a H5N1 virus and the seven remaining gene segments from a H1N1 S-OIV have been described as important determinants of airborne transmission in ferrets. Similarly, Herfst et al. reported important amino acid substitutions in the HA of a HPAI H5N1 (T156A, N154K, H103Y) that also confer airborne transmission in ferrets.

However, mutations enhancing the binding to SAα2-6Glu are not in themselves sufficient for host switching and transmission, meaning that other virus factors may be involved. In this regard, the adaptation of the IAV polymerase to host factors is an important mechanism underlying interspecies transmission. In addition to the PB2-E627K mutation present in some H5N1 strains, mutations such as PB2-T271A, PB2-Q591K, and PB2-D701N have been associated with elevated avian polymerase activity in human cells, replication, and transmissibility in guinea pigs and with an increased transport of PB2 into the nucleus of mammalian cells.

Prior to 2003, infection with H7 viruses was not considered a serious health threat, although some H7 outbreaks in poultry have been sporadically associated with conjunctivitis in humans. This could be linked to the presence of SAα2-3Glu linkages in corneal and conjunctival epithelial cells of the human eye. During the H7N7 HPAI outbreak in poultry that occurred in the Netherlands in 2003, 86 people involved in a culling operation and three in-contact persons were infected, prompting a reevaluation of the human health risks attributed to this virus, even if the majority of these infections in humans resulted in self-limiting conjunctivitis with occasional mild respiratory illness.

During the 2003 H7N7 Dutch outbreak, different mutations in the polymerase complex, HA, NA, and NS1 were found in viruses isolated from a fatal case when compared with strains isolated from conjunctivitis cases. Among these mutations, PB2-E627K was the main determinant of virus pathogenicity, whereas the HA-A143T mutation correlated with viral attachment to human alveolar macrophages. Additionally, viruses from fatal cases presented the PB2-D701N, PB2-S714R, and PA-K615N mutations, which conferred increased polymerase activity in mammal cells at relatively low temperatures.

H9N2 LPAI viruses have become enzootic in domestic poultry populations of many Eurasian countries, causing sporadic human infections characterized by influenza-like symptoms. H9N2 viruses have been isolated from pigs.
Table 1. Principal amino acid mutations and signatures associated with interspecies transmission of influenza A viruses

| Gene | Mutation | Effect                                                                 | Strain                  | Reference |
|------|----------|------------------------------------------------------------------------|-------------------------|-----------|
| HA   | E190D    | Viral strains with residues D190/D225 are human-specific, D190/G225 swine-specific and E190/G225 avian-specific. Mutations in these residues cause a switch in receptor binding preference from α2-3 to α2-6 SA | H1N1/1918 | 11,37,107 |
|      | G225D    | H1N1 cSIAVs European ‘avian-like’ swine H1N1                           |                         |           |
|      | D225G/E  | Enhances receptor binding to dual hosts (pigs and humans)              | H1N1 S-OIV              | 135,141   |
|      | D222G    | Enhances binding to SAα2-3Glu receptors                               | H1N1 S-OIV              | 142,154   |
|      | Q192R    | Enhance binding to human-type receptors in vitro                      | H5N1                    | 155       |
|      | G139R    |                                                                           |                         |           |
|      | N182K    |                                                                           |                         |           |
|      | Q192H    | Increases binding to SAα2-6Glu and virulence in mice                   | H5N1                    | 77        |
|      | Q226L    |                                                                           |                         |           |
|      | G228S    |                                                                           |                         |           |
|      | 226L     | Signature which exhibits preferential binding to human-like SAα2-6Glu receptors. A key element for the successful infection of humans. | H9N2                    | 82        |
|      | A143T    | Increases viral attachment to human alveolar macrophages              | H7N7                    | 97        |
|      | E391K    | Associated with the fitness of the virus                             | H1N1 S-OIV              | 143,144   |
|      | T160A    | Required to sustain the avian virus transmission in guinea pig model  | H5N1                    | 156       |
|      | K193R    | Decreases binding to SAα2-3Glu or increases binding to SAα2-6Glu       | H5N1                    | 157       |
|      | N220K    | Respiratory droplet transmission in ferrets                           | Reassortant HS/H1N1     | 85        |
|      | Q222L    |                                                                           |                         |           |
|      | N154D    |                                                                           |                         |           |
|      | T314I    |                                                                           |                         |           |
|      | T156A    | Airborne transmission between ferrets                                  | H5N1 genetically modified | 86        |
|      | N154K    |                                                                           |                         |           |
|      | H103Y    |                                                                           |                         |           |
|      | K119N    | Enhancement of virulence in mouse model                               | H1N1 S-OIV              | 129       |
|      | G155E    |                                                                           |                         |           |
|      | S183P    |                                                                           |                         |           |
|      | R221K    |                                                                           |                         |           |
| PB2  | E627K    | Avian strains have 627E and human strains 627K signature. Associated with increased transmission. Important determinant of host range Increases polymerase activity in mammalian cells at relatively low temperatures Determinant of host range. Increases transcription at a low temperature Increases virulence in mammals | H1N1/1918 | 43,51,52,90,91 |
|      | D701N    | Enhances the binding of PB2 to importin α1, increasing the level of PB2 in the nucleus in mammalian cells. Important role in the interspecies transmission of IAVs Increases polymerase activity in mammalian cells at relatively low temperatures Involved in mammalian adaptation Increases transmissibility of Influenza A viruses in guinea pig model Enhances the polymerase activity in mammalian cells | H7N7 isolated from human FC Mouse-adapted H9N2 | 97 |
|      | S714R    | Enhances the polymerase activity in mammalian cells                   | H5N1                    | 51        |
|      | K318R    | Correlates with high pathogenicity in mice in the presence of additional mutations Enhances activity only at higher temperatures (37 and 39°C) Contributes to avian polymerase adaptation to mammalian hosts | H5N1                    | 58        |
|      | T271A    |                                                                           | H5N1                    | 56        |
|      | Q591R/K  | Enhances viral replication in human cells and involved in mammalian adaptation Compensates the lack of PB2-E627K mutation in the S-OIV | H5N1                    | 57        |
|      | G590S    | Associated with mammalian pathogenicity and enhanced replicative ability in mammals | H1N1 S-OIV              | 43        |
|      |          |                                                                           |                         |           |
and humans and are believed to be potential pandemic candidates. Molecular characterization of H9N2 viruses circulating in the Middle East and Asia has revealed that more than 70% of the viruses contained the HA-L226 signature, which modifies receptor preference to SA2-6Glu linkages. Along the same lines, Sorrell et al. demonstrated that the combination of four key amino acid residues at the receptor-binding site of the HA (H183, A189, E190, and L226) in a chimeric virus carrying the surface proteins of avian H9N2 in a human H3N2 backbone is essential for transmission in ferrets. Additionally, the PB2-E627K mutation in mouse-adapted H9N2 viruses was correlated with increased virulence in mammals.

To date, swine influenza viruses (SIAV) H1N1, H3N2, and H1N2 subtypes are circulating in swine all over the world. Classical swine H1N1 viruses (cSIAV) presumably emerged from the 1918 pandemic, circulating, and reassorting with other viruses to give rise to the “triple reassortant” H3N2 SIAV. Independently, an avian-like H1N1 SIAV emerged in Europe. Phylogenetic analysis of different SIAVs showed that cSIAVs analyzed possess the HA-E190D mutation (H3 numbering), which is required to switch the host specificity. In addition, cSIAVs possess the avian signature HA-225G, whereas in the European lineage, this signature is variable (G225E or G225K). Interestingly, the European avian-like H1N1 lineage possesses the PB2-D701N that may play a role in mammalian adaptation.

### Reassortments

Because the genome of IAVs consists of eight separate RNA segments, coinfection of one host cell with two different strains can result in progeny viruses containing gene segments of both parental viruses. Theoretically, there are 256 possible combinations of the eight gene segments between two viruses. Swine are considered as the main candidates for generating reassortant viruses between human and avian IAVs. Available reports have demonstrated the isolation of whole avian IAVs in pigs, meanwhile complete genomic analyses have confirmed the reassortment of swine, avian, and/or human viruses in pigs worldwide, as recently reported in China. Importantly, swine are also capable of transmitting reassortant viruses to humans, as demonstrated during the last 2009 pandemic.

H9N2 IAVs have become established worldwide in poultry and wild birds and have been occasionally transmitted to mammals including humans and pigs. The continuous circulation among different hosts has provided the conditions for the evolution and generation of multiple novel genotypes through reassortment events. Fusaro et al. reported significant inter- and intra-subtype reassortments associated with specific amino acid substitutions that are believed to result in increased transmissibility in mammals. To date, inter-subtype reassortments have been detected between H9N2, H5N1 HPAI, and H7N3 viruses in China and Pakistan. In vivo studies have demonstrated that a

### Table 1. (Continued)

| Gene | Mutation | Effect | Strain | Reference |
|------|----------|--------|--------|-----------|
| A684S | Associated with host shift from avian to swine and the subsequent transfer to humans | Avian IAVs | 79 |
| E158G/A | Associated in the adaptation of PB2 genes to mammals (mouse model) | H1N1 S-OIV | 129 |
| L13P | Enhances the activity of viral polymerase | H7N7 | 58 |
| G375S | Associated with adaptation to a new species (swine to human) | H1N1 | 158 |
| K577E/M | Increase virulence and polymerase activity in mouse model | H3N2 human isolates | 159 |
| K578Q | | | |
| K615N | Enhances activity of viral RNA polymerase and stimulates viral replication and pathogenicity in mouse model | H7N7 | 58 |
| K356R | Associated with host shift from avian to swine and the subsequent transfer to humans | Avian IAVs | 79 |
| T85I | Multiple residues that contribute to the enhancement of avian polymerase activity in mammalian cells which is essential for mammalian host adaptation | H1N1 S-OIV | 160 |
| G186S | | | |
| L336M | | | |
| N319K | Increases binding to mammalian importin α-1 proteins and polymerase activity. Related to host range specificity. | H7N7 | 59 |
| V100I | Increases transmissibility among humans | H1N1 S-OIV | 135 |
| X-ORF Segment 3 | PA-X | Modulates virulence and host immune response in mouse model | IAVs | 17 |

cSIAV, classical swine H1N1 viruses; HA, hemagglutinin; HPAI, highly pathogenic avian influenza; IAVs, IA viruses; SA, sialic acid; SIAV, swine Influenza viruses; S-OIV, swine-origin virus.
reassortant virus containing the surface glycoprotein genes from H9N2 and the six internal genes of a human H3N2 virus, as well as a reassortant virus carrying the HA of H9N2 in the background of a H1N1 S-OIV, were both able to replicate and be transmitted from ferret to ferret.

Among reassortment dynamics of internal IAV gene segments, an avian-origin PB1 segment is present both in the H2N2/57 and in the H3N2/68 pandemic strains. This suggests that the reassortment of polymerase subunit genes between mammalian and avian IAVs might play a role in interspecies transmission. To test this hypothesis, Li et al. studied the compatibility between avian H5N1 and human H1N1 polymerases, observing that recombinant viruses carrying the PB2-H1N1 and PB1-H5N1 had stronger polymerase activity in cell culture. Furthermore, a study demonstrated that in vivo coinfection with avian H5N1 and human H3N2 viruses of ferrets generated reassortant viruses containing genes from both progenitor viruses.

### Genetic markers

Surveillance for genetic markers of adaptation could help in predicting the risk of an epidemic emergence. Previous studies have reported up to 52 species-associated signatures that differentiate between avian and human IAVs. Unfortunately, these methods did not take into account the phylogenetic relationship of the isolates and treated each sequence as an independent observation, resulting in an overestimation of statistical significance. Other studies reported 18 mortality markers in three pandemic strains. To test this hypothesis, Li et al. studied the compatibility between avian H5N1 and human H1N1 polymerases, observing that recombinant viruses carrying the PB2-H1N1 and PB1-H5N1 had stronger polymerase activity in cell culture. Furthermore, a study demonstrated that in vivo coinfection with avian H5N1 and human H3N2 viruses of ferrets generated reassortant viruses containing genes from both progenitor viruses.

### Pandemic overview

To date, only viruses of the H1, H2, and H3 subtypes are capable of replication and be transmitted from ferret to ferret.

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viruses circulating in swine herds, as reported to have occurred in Canada, \(^1\) Hong Kong, \(^2\) and China. \(^3\) A study demonstrated that reassortant viruses containing the HA gene from a seasonal H1N1 on a H1N1 S-OIV background showed enhanced growth in cell culture. \(^4\) However, limited compatibility among polymerase subunits from different IAVs must be considered as a restricting factor for reassortment. \(^5\)

**Conclusion**

Avian IAVs have played an important role in the generation of the well-known H1, H2, and H3 human pandemics, and in all cases, at least one of eight segments was donated by these viruses to the pandemic strains. This proves that the medical and veterinary influenza communities are challenged with a virus that constantly changes through different mechanisms, as it adapts to different species and reassorts with other IAVs of avian and mammalian origin. This indicates the need to strengthen the collaboration in an interdisciplinary manner to identify new emerging viruses with pandemic potential.

Molecular determinants of host specificity and pathogenicity have been identified in most viral genes that encode viral surface glycoproteins, proteins involved in the viral genome replication and those that counteract the host immune response. Recent findings show that not only pigs but also humans and some gallinaceous avian species express both \(\alpha2\)-3- and \(\alpha2\)-6-linked receptors, facilitating possible reassortment events between mammals and avian viruses and probably extending the number of species that need to be considered as “mixing vessels.”

New IAV strains emerge through the accumulation of mutations, natural reassortment, and adaptation to their new host. Recent investigations have demonstrated that mutations in the receptor-binding site of the HA protein of avian IAVs may change the binding preference of these strains toward the human host; however, this factor is not sufficient for host switching and further transmission. In this regard, several studies have shown that adaptation of the IAV polymerase to host factors is one of the most important mechanisms, which highlights interspecies transmission. Therefore, the evolution and adaptation of IAVs are complex and polygenic, involving several viral genes and other unknown host factors.

H5N1 HPAI viruses are still to be considered as a significant threat for public health. In addition, some H9 avian IAVs have the ability to bind to \(\alpha2\)-6 receptors, and the evidence of reassortment with other IAVs emphasizes their potential to emerge as possible pandemic strains. In the same way, H1N1 S-OIV is evolving rapidly and reassorting with other IAVs that are currently circulating. However, further investigations are needed to clarify the rules that govern the reassortment and the successful gene combination in IAVs, as well as the human and host genes involved in modulation of IAV infections.

Several genetic markers in IAV genes have been reported as being associated with certain biological properties, such as receptor binding, host restriction and tropism, virulence and modulation of host immunity, as well as efficiency of replication and transmission. Some studies in animal models have shown the importance of individual virus proteins, such as the HA, NA, polymerase and non-structural proteins, and even point mutations within these molecules. However, they are polygenic, correlation between molecular markers and biological properties is not absolute, and the full understanding of their correlation with biological properties is yet to come.

A valuable means to identify strains of pandemic potential would be to strengthen the use of molecular methods to study IAV evolution, such as (i) large-scale genomic sequencing to improve the surveillance of mutations and gene constellations; (ii) bioinformatics analyses to study the spatio-temporal evolution dynamics to identify mutations under positive selection and protein structural prediction, and (iii) deep sequencing to monitor within-host viral population diversity. In recent times, accessible databases have become available to support the growing pool of genetic data for IAVs, although epidemiological and ecological data should also be included to improve our understanding and establish new research studies regarding IAV emergence. Finally, the development of programs involving interdisciplinary teams to promote a broader collaboration to identify new emerging viruses could be an important approach to improve data collection and integrative analysis in future pandemics.

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**Conflict of interest**

The authors have no potential conflicts to declare.

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