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H5N1 influenza virulence, pathogenicity and transmissibility: what do we know?

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Abstract

Highly pathogenic influenza viruses of the H5N1 subtype have infected more than 600 people since 1997, resulting in the deaths of approximately 60% of those infected. Multiple studies have established the viral hemagglutinin (HA) surface glycoprotein as the major determinant of H5N1 virulence. HA mediates host-specific virus binding to cells, and mutations that allow efficient binding to viral receptors on mammalian cells are critical (although not sufficient) for H5N1 transmissibility among mammals. The viral polymerase PB2 protein is also a critical virulence determinant, and adaptive mutations in this protein are crucial for efficient H5N1 virus replication in mammals. Additionally, viral proteins (such as NS1 and PB1-F2) with roles in innate immune responses also affect the virulence of highly pathogenic H5N1 viruses.

Keywords

H5N1; HA; influenza; NS1; pathogenicity; PB1-F2; PB2; virulence

Influenza A viruses circulating in avian species rarely infect humans. However, since 1997, highly pathogenic avian influenza viruses of the H5N1 subtype have infected more than 600 people. Infection of humans with these viruses typically leads to severe respiratory disease that often progresses to multiorgan failure; approximately 60% of confirmed cases of highly pathogenic H5N1 influenza infection have resulted in death. In addition, seroprevalence studies have suggested that asymptomatic human infections with H5N1 influenza viruses may have occurred [1,2]. To date, human-to-human transmission of these viruses has been limited to a few small clusters. Nonetheless, the circulation and evolution of these viruses should be monitored closely because they continue to infect humans, may acquire the ability to transmit among mammals [3–6] and undergo frequent reassortment, resulting in novel variants that have now been detected in North America [7].

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The first fatal infections of humans with highly pathogenic avian H5N1 influenza viruses were reported in Hong Kong in 1997 [8–10]. Despite several attempts to eradicate these viruses by depopulation of the live poultry markets in Hong Kong, H5N1 viruses re-emerged in 2001 and 2003 and are now enzootic in poultry in parts of Southeast Asia and the Middle East. Cases of infected wild and/or domestic birds have been reported by other Asian countries, as well as several European and African countries. Most human infections with highly pathogenic avian H5N1 influenza viruses have occurred in Indonesia, Vietnam, Egypt, China and Cambodia. Egypt experienced a spike in human infections with these viruses in late 2014 and early 2015, but it is not clear whether this is because of more comprehensive screening and reporting, or because the highly pathogenic avian H5N1 influenza viruses circulating in Egypt have acquired mutations that facilitate human infections [11].

Since their emergence in the late 1990s, highly pathogenic avian H5N1 influenza viruses have undergone multiple reassortment events with avian influenza A viruses of different subtypes, including H6N1, H9N2 and H5N1 [12–16]. Hence, the currently circulating highly pathogenic H5N1 viruses represent a diverse group of viruses. Moreover, the viral surface glycoprotein HA (the major viral antigen), has evolved through point mutations, leading to a number of genetically and antigenically distinct clades and subclades. Currently, ten major clades (i.e., clades 0–9) are recognized, many of which have multiple second, third and fourth tier subclades. The major clades circulating during the past years include clades 2.2.1 (circulating in Egypt, Israel, the Gaza strip and the West Bank), 2.3.2.1 (circulating in China, Bangladesh and India) and 2.1.3.2 (circulating in Indonesia). Although genetically and antigenically diverse, highly pathogenic avian H5N1 viruses share the ability to cause high mortality in poultry and infect humans. Recently, the HA gene of highly pathogenic avian H5N1 influenza viruses of clade 2.3.4.4 has reassorted with the neuraminidase (NA) and other viral genes originating from different avian influenza viruses, giving rise to novel viruses of the H5N2, H5N6 and H5N8 subtypes [7,17–19].

Multiple studies have assessed the virulence and pathogenicity of highly pathogenic avian H5N1 influenza viruses in different cell types and animal models including chickens, ducks, mice, guinea pigs, ferrets, pigs and nonhuman primates (reviewed in [20,21]). Mice are typically used to assess the virulence and immunogenicity of influenza viruses because they are inexpensive and multiple immunological reagents are available. However, mice are not a natural host of influenza viruses and typically do not transmit viruses. Ferrets infected with influenza viruses show signs of respiratory infection similar to those observed in humans, and influenza viruses can transmit among ferrets via respiratory droplets. This animal model is limited by its relatively high cost and the limited number of immunological reagents available. Guinea pigs have recently been established as an additional model for influenza virus transmission studies; however, infected animals typically do not show overt signs of disease. This review focuses on studies of the virulence and pathogenicity of highly pathogenic avian H5N1 influenza viruses in mammals.

The viral HA protein

The viral hemagglutinin (HA) surface glycoprotein mediates virus binding to sialic acids on host cell receptors. Epithelial cells in the intestinal tract of birds (the major target organ of influenza viruses in avian species) express primarily sialic acid linked to the penultimate galactose residue by an α 2,3-linkage, and avian influenza viruses have evolved to preferentially bind to this type of sialic acids. In contrast, epithelial cells in the respiratory tract of humans (the major target organ of influenza viruses in humans) express primarily α 2,6-linked sialic acids. After they infect humans, influenza viruses therefore need to acquire mutations in HA that confer efficient binding to α 2,6-linked sialic acids to enable efficient transmission among humans. The HA proteins of highly pathogenic avian H5N1 influenza viruses preferentially interact with α 2,3-linked sialic acids (reviewed in [22]); human infections with these viruses may, in part, be explained by the finding of α 2,3-linked sialic acids on type II pneumocytes lining the alveolar wall, and on nonciliated bronchiolar cells [23–26]. The lack of α 2,3-linked sialic acids on epithelial cells of the upper respiratory tract of mammals may explain why highly pathogenic avian H5N1 influenza viruses do not transmit efficiently among mammals. Several studies tested highly pathogenic H5N1 influenza viruses with mutations in HA that confer increased binding to α 2,6-sialic acids; none of these viruses transmitted among ferrets (the most widely used animal model for influenza virus transmission studies) via respiratory droplets [3–4,27]. This finding demonstrated that efficient binding to α 2,6-sialic acids alone is not sufficient for avian influenza virus respiratory droplet transmission in mammals.

In 2012/2013, four research groups [3–6] demonstrated that viruses possessing the HA gene of a highly pathogenic H5 influenza virus can acquire the ability to transmit among ferrets or guinea pigs via respiratory droplets. These viruses differed in the origins of their eight viral RNA segments and in the nature of the mutations that were introduced intentionally and/or acquired during virus passages. Nonetheless, common features emerged: first, the HA protein must mediate efficient binding to human-type receptors (however, this function is not sufficient to confer H5 virus transmissibility among mammals). Chen *et al.* [6] identified such an HA variant (possessing the HA-Q196R, Q226L and G228S mutations; all numbers refer to the H3 reference numbering presented by Burke *et al.* [28]) (**Table 1**) by combining *in vitro* selection with mutations previously shown to mediate binding to human-type receptors. We also performed *in vitro* selection starting from a virus library with random mutations in the HA head region (where the receptor-binding pocket is located), resulting in the identification of the HA-N224K and -Q226L mutations, which conferred binding to α 2,6-sialic acids [4]. Herfst *et al.* used a virus possessing HA mutations known to increase binding to α 2,6-sialic acids (i.e., HA-Q226L and -G228S) [3]. Another study found that a virus that transmitted among guinea pigs via respiratory droplets possessed an HA with dual α 2,3/ α 2,6-sialic acid binding properties [5].

Second, the HA proteins of the mammalian-transmissible H5 viruses all lack a glycosylation site in the HA head (amino acid positions 158–160). In two studies, two different mutations resulting in the loss of the same glycosylation site were acquired during virus replication in ferrets (**Table 1**) [3,4]. The other two mammalian-transmissible H5 viruses possess HA

proteins that naturally lack this glycosylation site [5,6]. The presence of the glycosylation site at positions 158–160 of HA may interfere with virus binding to cellular receptors.

Differences in the physical stability of the HA trimer can affect its biological properties [63] and have led to shortened expiration dates for influenza vaccines [64,65]. HA stability can be assessed by incubating viruses at 50–55°C for various periods of time, followed by hemagglutination assays and virus titration in MDCK cells (i.e., plaque assays).

HA stability emerged as a third critical feature of mammalian-transmissible H5 viruses; this trait was not previously known to play a role in influenza virus transmissibility. The mammalian-transmissible viruses isolated by two groups possessed mutations (acquired during virus passage in ferrets) that increased the stability of HA (**Table 1**) [3–4,29]. Further studies revealed that the mutations that conferred efficient binding to α 2,6-sialic acids reduced the thermostability of HA, a defect that had to be compensated for by mutations that increase HA stability.

Fourth, a polymerase complex that enables efficient replication in mammalian cells is critical for respiratory droplet transmission of avian influenza viruses in ferrets (see ‘The viral polymerase PB2 protein’ section for more details).

The viral polymerase PB2 protein

The replication and transcription of influenza viral RNAs is catalyzed by a trimeric polymerase complex that comprises the PB2, PB1 and PA subunits. Two pivotal studies established that PB2 is critical for efficient replication of avian influenza viruses in mammals [34,35]. Specifically, a lysine residue at position 627 is now recognized as a critical host determinant that facilitates the efficient replication of avian influenza viruses in mammalian cells (**Table 1**) [34,35]; this effect is greater at 33°C (i.e., the temperature in the upper respiratory tract of humans) than at 37°C (i.e., the temperature in the lower respiratory tract of humans) [36]. By contrast, the glutamic acid residue encoded by most avian influenza viruses at this position typically limits the replication of avian influenza viruses in mammalian cells.

During an outbreak of highly pathogenic avian H5N1 viruses at Qinghai Lake, China, in 2005, some of the viruses were found to encode the PB2-627K mutation [66–68]. Descendants of the Qinghai Lake-lineage of H5N1 viruses have spread westward into Europe and the Middle East. All contemporary Middle Eastern H5N1 viruses possess the mammalian-adapting PB2-627K mutation. The highly pathogenic avian H5N1 influenza viruses circulating in Indonesia and Vietnam, which continue to infect humans, do not encode PB2-627K, indicating that this residue is not required for infection of humans. However, the PB2-E627K mutation is frequently selected during the replication of highly pathogenic H5N1 influenza viruses in mammals (reviewed in [21]), indicating strong selective pressure. The mechanism through which PB2-627K confers efficient replication in mammalian cells is not yet fully understood.

The amino acid at position 701 of PB2 also affects the virulence and pathogenicity of H5N1 influenza viruses (**Table 1**) [37–39]. This residue interacts with the cellular nuclear import

factor importin α , which mediates the transport of proteins from the cytoplasm to the nucleus. Replacement of aspartic acid at PB2-701 (encoded by most avian influenza viruses) with asparagine increases PB2 binding to importin α in mammalian cells compared with avian cells [38,39], resulting in increased replicative ability and virulence [37–39].

Three of the four mammalian-transmissible H5 viruses identified to date encode PB2 proteins with known mammalian-adaptive residues. The ferret-transmissible H5 virus identified by Herfst *et al.* [3] possesses the intentionally introduced PB2-627K residue. The ferret-transmissible H5 virus described by us [4] carries the PB2 gene of a 2009 H1N1 pandemic virus, which encodes PB2-591R, a residue that can compensate for the lack of PB2-627K (**Table 1**) [32,33]. The guinea pig-transmissible H5 virus encodes PB2-701N [5].

Several other amino acid residues in the polymerase proteins also affect the virulence and pathogenicity of H5N1 influenza viruses [69]. For example, the PB2-591K residue, which compensates for the lack of PB2-627K in 2009 H1N1 pandemic viruses, also increases the replicative ability and virulence in mice of highly pathogenic H5N1 influenza viruses [33]. The alanine residue at position 271 of PB2 (found in most human influenza viruses) confers higher replicative ability in mammalian cells than does the threonine residue typically found in PB2 proteins of avian influenza viruses (**Table 1**) [31]. The PB2-271A residue was detected in a human H5N1 virus isolate and may have contributed to the virulence of this virus in that person. Recently, we demonstrated that the combined effects of the PB2-147T, -339T and -588T residues found in an appreciable number of H5N1 viruses result in a phenotype comparable to that conferred by PB2-627K (**Table 1**) [30]. A virus possessing all four mammalian-adapting residues in PB2 (i.e., PB2-147T, -339T, -588T and -627K, as was found in an H5N1 virus isolated from a fatal human case) was more pathogenic than viruses possessing only PB2-627K or PB2-147T/339T/588T [30].

The viral interferon antagonist NS1 protein

Virus infections stimulate the expression of IFN and the activation of interferon-induced genes (ISGs). Many ISGs encode proteins with antiviral functions, such as PKR, Mx resistance proteins, IFITM proteins, ISG15, OAS, RNase L or Viperin. Most viruses have therefore evolved mechanisms to control the upregulation of IFN and interferon-stimulated genes and/or the actions of proteins with antiviral activities. In 1998, Garcia-Sastre *et al.* reported that the influenza A virus NS1 protein is critical to antagonize innate immune responses, while this protein is dispensable in IFN-deficient systems such as Vero cells [70]. The NS1 protein interferes with the stimulation of innate immune responses through several mechanisms (reviewed in [21,71]): it suppresses the activation of the IFN- β promoter and the upregulation of the IRF-3, NF- κ B and AP-1 transcription factors, all of which regulate IFN- β transcription. NS1 also binds to TRIM25 and the cytoplasmic sensor RIG-I, resulting in suppressed RIG-I signaling and IFN- β synthesis. Binding of NS1 to double-stranded RNA also interferes with the activation of antiviral factors such as OAS/RNaseL and PKR. Moreover, NS1 binds to the 30-kDa subunit of CPSF and to PABII proteins, which prevents the efficient cleavage and polyadenylation of cellular pre-mRNAs; this mechanism may limit the amount of IFN- β produced in response to an influenza virus infection.

Several studies have demonstrated that the NS viral RNA segment of a highly pathogenic H5N1 virus can increase the virulence of a recipient virus, such as an H1N1 or H7N1 virus [43,72]. Moreover, the NS gene has been shown to contribute to the differences in virulence between H5N1 viruses of high or low pathogenicity [52].

Comparisons of viruses that differ in their virulence have led to the identification of several amino acids in NS1 that are responsible for these virulence differences. For example, Seo *et al.* found that the NS gene of an H5N1 virus increased the virulence of A/Puerto Rico/8/34 (H1N1) virus in pigs, and that this effect was dependent on a glutamic acid residue at position 92 of NS1 (**Table 1**) [43]. Another study reported that a five-amino acid deletion (positions 80–84 of the NS1 proteins of most highly pathogenic H5N1 viruses isolated since 2000) increases the virulence of the deletion mutants (**Table 1**) [41]. An artificially generated virus lacking amino acids 80–84 also acquired the NS1-92E mutation [41], and viruses possessing both markers have been detected in nature, suggesting a functional relationship between these mutations. Further studies have shown that the five-amino acid deletion affects TNF- α levels, whereas the amino acid at position 92 regulates the level of IFN induction [42].

NS1's ability to interfere with the splicing, polyadenylation and nuclear export of cellular premRNA requires its interaction with the 30-kDa subunit of CPSF (CPSF30) [44]. The interaction of NS1 with CPSF30 is determined by the amino acids at positions 103 and 106 of NS1 (**Table 1**) [45]: stronger binding by NS1 appears to prevent CPSF30 from processing cellular mRNA (including that encoding IFN- β), resulting in increased virulence [46–50]. In addition, the amino acid residue at position 103 is also important for the activation of the JNK pathway [73], which has pro- and anti-influenza viral functions [74,75].

Most NS1 proteins encode a PDZ domain-binding motif in their carboxy-terminal four amino acids [53], which mediates the interaction with cellular PDZ proteins that play a role in processes such as apoptosis, signaling, trafficking or cell polarity and the maintenance of tight junctions. Most avian influenza viruses encode a PDZ domain-binding motif with the sequence ESEV, whereas most human influenza viruses encode an RSKV motif. The avian-type ESEV motif confers efficient binding to several proapoptotic and scaffolding PDZ proteins, which interact only weakly with the human-type RSKV motif [54–56]. These differences in binding patterns may explain the differences in virulence observed among viruses encoding different PDZ domain-binding motifs. Introduction of the PDZ domain-binding motifs of pandemic 1918 (KSEV) or two H5N1 viruses (ESEV and EPEV, respectively) into the laboratory-adapted A/WSN/33 (H1N1) virus (which possesses an RSEV motif) increased virulence in mice (**Table 1**) [57]. In contrast, H5N1 viruses possessing ESEV, RSKV or no PDZ domain-binding motif showed comparable virulence in chickens and mice [58], suggesting that the effect of the PDZ domain-binding motif on virulence may be strain specific.

Upon influenza virus infection, the cellular PI3K/Akt pathway is activated by NS1 [76] and by viral RNA-mediated upregulation of RIG-I [77]. PI3K has been described as an antiviral factor, but is also required for efficient influenza virus replication. We demonstrated that an unusual PDZ domain-binding motif (ESKV) and an amino acid infrequently found at

position 138 of NS1 (NS-138Y; one of the NS1 residues that may interact with PI3K) cooperatively confined a highly pathogenic H5N1 influenza virus to the respiratory tract of mice, whereas a mutant encoding the consensus amino acids at these positions (i.e., ESEV and NS-138F) caused lethal, systemic infection in mice (**Table 1**) [51]. Interestingly, mutation of the PDZ domain-binding motif also affected Akt activation, and mutation of NS-138 not only affected activation of the PI3K/Akt pathway, but also the interaction of NS1 with cellular PDZ proteins [51], suggesting a functional relationship between these mechanisms.

In addition to the amino acid residues described above, several other positions in the NS1 proteins of highly pathogenic H5N1 influenza viruses affect virulence. For example, replacement of a proline residue at position 42 with serine (commonly found in H5N1 NS1 proteins at this position) increased virulence in mice (**Table 1**) [40]. Further studies showed the NS1-42S is critical to antagonize IRF-3 and IFN induction [40]. Another study demonstrated that the amino acids at positions 200/205 and 205/210 (numbers refer to highly pathogenic H5N1 NS1 proteins that possess or lack the five amino-acid deletion at positions 84–88) affect virulence by modulating the IFN antagonism activity of the protein (**Table 1**) [52].

The viral PB1-F2 protein

The PB1-F2 protein is encoded by the PB1 viral RNA segment in the +1 reading frame relative to that of PB1 [78]. The PB1-F2 open reading frame is accessed through a ribosomal frameshift and yields a protein of 87–90 amino acids in most influenza viruses [78]. Human H1N1 viruses isolated after 1950 encode a truncated version of 57 amino acids, whereas most swine viruses lack this protein due to multiple stop codons. Because 2009 pandemic H1N1 viruses possess a PB1 gene that originated from swine influenza viruses, the current human H1N1 viruses lack PB1-F2. Almost all highly pathogenic H5N1 influenza viruses encode a PB1-F2 protein of 90 amino acids in length.

PB1-F2 has several functions. It was originally described as a proapoptotic factor [78] that interacts with mitochondrial proteins [79] and forms membrane pores [80,81], leading to changes in mitochondrial potential. However, a comparative study of influenza viruses of different subtypes indicated that the proapoptotic function of PB1-F2 is strain specific and may not be the primary function of the protein [82]. For example, the PB1-F2 protein of a highly pathogenic H5N1 influenza virus did not localize to mitochondria and lacked proapoptotic function [83].

Analysis of several highly pathogenic H5N1 influenza viruses isolated in 1997 in Hong Kong revealed a polymorphism at position 66 of PB1-F2 that correlated with pathogenicity in mice: asparagine at this position was associated with low pathogenicity, whereas serine at PB1-F2-66 correlated with high pathogenicity (**Table 1**) [59]. Interestingly, a serine residue at position 66 was also found in the PB1-F2 protein of the 1918 pandemic A/Brevig Mission/1/18 (H1N1) virus. Several studies have demonstrated that highly pathogenic H5N1 and pandemic 1918 viruses encoding PB1-F2-66S are more virulent than control viruses expressing PB1-F2-66N [59–61]. Conversely, 2009 pandemic H1N1 viruses engineered to

encode an PB1-F2 with -66N or -66S do not differ significantly in their virulence in mice [62].

PB1-F2 affects innate immune responses [59–60,84–87], most likely by interacting with the adaptor protein MAVS [84–85,88], which is critical for efficient RIG-I-mediated activation of transcription factors and subsequent IFN expression. In addition, PB1-F2 expression also activates the NLRP3 inflammasome [89]. The effect of PB1-F2 on innate immune responses and inflammasome activation could explain why PB1-F2 expression augments the severity of secondary bacterial co-infections [90,91].

In addition to its immune-modulatory function, PB1-F2 may also affect virulence in a strain-specific manner through its interaction with PB1, which affects the intracellular localization of the polymerase subunit, resulting in altered replicative efficiency [82,92].

Conclusion

Highly pathogenic H5N1 influenza viruses cause severe infections in mammals. Several viral proteins (most notably, HA, PB2, NS1 and PB1-F2) contribute to the virulence of these viruses. The receptor-binding specificity of HA is a critical determinant in the host range of influenza viruses. Most highly pathogenic H5N1 influenza viruses recognize primarily avian-type receptors; however, highly pathogenic H5N1 influenza viruses isolated in Egypt also interact with human-type receptors to some extent and should be monitored closely for additional mutations that may shift their receptor-binding preference to preferentially recognize human-type receptors. It is important to note that the H7N9 viruses that emerged in China in 2013 display a natural affinity for both avian- and human-type receptors (reviewed in [93]), so that their pandemic potential may be greater than that of currently circulating highly pathogenic H5N1 viruses. In addition to HA, the polymerase complex is a critical determinant of host range. Mammalian-adapting residues (such as PB2-E627K or -D701N) have been found repeatedly in avian influenza viruses isolated from infected mammals, suggesting a ‘global’ role for these residues in the adaptation of avian influenza viruses to mammals. Future studies will be needed to identify additional determinants of virulence, to understand potential correlations among the virulence determinants, and to better define the underlying mechanisms.

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Future perspective

Progress in influenza virus research has been made in areas such as evolutionary analysis and prediction, structural resolution and analysis of influenza viral proteins, and OMICS approaches that study multiple host responses to influenza viral infection. Since experimental tools such as large-scale mutagenesis and screening approaches, OMICS studies and next-generation sequencing platforms are becoming more powerful and less cost prohibitive, large datasets will likely be generated over the next decade. The major challenge lies in the analyses and integration of these datasets to better understand (and perhaps even predict) the evolution of influenza viruses, and the determinants of virulence and pathogenicity. Novel bioinformatics approaches and close collaborations between virologists and bioinformaticians could advance the field into areas that could not be explored by either side alone.

EXECUTIVE SUMMARY

The viral hemagglutinin protein

- The receptor-binding specificity of hemagglutinin (HA) is a critical determinant of virulence.
- Mutations that shift the receptor-binding specificity from preferential binding of $\alpha 2,3$ -sialic acids ('avian-type' receptors) to preferential binding of $\alpha 2,6$ -sialic acids ('human-type' receptors) are critical (although not sufficient) for the transmissibility of highly pathogenic H5N1 influenza viruses in mammals.
- The absence or presence of glycosylation sites in the HA head region may be critical for the transmissibility of highly pathogenic H5N1 influenza viruses in mammals.
- HA stability is an important factor in the emergence of highly pathogenic H5N1 influenza viruses that transmit among mammals.

The viral polymerase PB2 protein

- Several amino acid residues in PB2 determine the replicative ability of highly pathogenic H5N1 influenza viruses in mammals.
- PB2-627K is critical for efficient replication of avian influenza viruses in mammals.
- Other amino acids in PB2 (e.g., those at positions 701 or 591) also affect the replicative ability of influenza viruses in mammals.

The viral IFN antagonist NS1 protein

- NS1 is an IFN-antagonist that affects innate immune responses through several mechanisms.
- The NS1 proteins of most highly pathogenic H5N1 influenza viruses isolated after 2000 possess a five-amino acid deletion that increases virulence.
- The amino acids at positions 103 and 106 of NS1 affect virulence through interaction with CPSF30.
- A PDZ-domain-binding motif at the C-terminus of NS1 affects virulence; this activity may be correlated with the activation of the PI3K/Akt pathway.

The viral PB1-F2 protein

- The PB1-F2 protein affects innate immune responses, apoptosis and the interaction with PB1; some of these effects appear to be strain- or host-specific or both.
- The PB1-F2-S66N mutation increases virulence.

Table 1

Summary of the mutations discussed.

Viral protein	Mutation(s)	Biological function(s) affected by mutation	Ref.
HA	H110Y	HA stability; H5 virus respiratory droplet transmissibility in ferrets	[3,29]
	N158D	Loss of glycosylation site; H5 virus respiratory droplet transmissibility in ferrets	[4]
	T160A	Loss of glycosylation site; H5 virus respiratory droplet transmissibility in ferrets	[3]
	Q196R, Q226L, G228S	Receptor-binding specificity; H5 virus respiratory droplet transmissibility in ferrets	[6]
	N224K, Q226L	Receptor-binding specificity; H5 virus respiratory droplet transmissibility in ferrets	[4]
	Q226L, G228S	Receptor-binding specificity; H5 virus respiratory droplet transmissibility in ferrets	[3]
	T318I	HA stability; H5 virus respiratory droplet transmissibility in ferrets	[4]
PB2	I147A, K339T, A588T	Replicative ability of influenza viruses in mammalian cells	[30]
	T271A	Replicative ability of influenza viruses in mammalian cells	[31]
	Q591K/R	Replicative ability of pandemic 2009 H1N1 influenza viruses in mammalian cells	[32,33]
	E627K	Replicative ability of influenza viruses in mammalian cells	[34-36]
	D701N	Interaction with importin α ; replicative ability of influenza viruses in mammalian cells	[37-39]
NS1	P42S	Regulation of IRF3 and IFN levels	[40]
	80-84	Regulation of TNF- α levels	[41,42]
	D92E	Regulation of IFN levels	[42-43]
	F103L, M106I	Binding to CPSF30; virulence in mice	[44-50]
	F138Y	Interaction with cellular PDZ proteins; Akt activation	[51]
	N200S, G205R	Modulation of IFN levels	[52]
	Mutations in PDZ domain	Interaction with cellular PDZ proteins Akt activation	[51,53-58] [51]
PB1-F2	N66S	Virulence in mice	[59-62]